Australian Native Plants – A Source of Novel Anti-inflammatory Compounds

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full of in part, for a degree at this or any other institution.

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Most A. Akhtar, Ritesh Raju, Karren D. Beattie, Frances Bodkin, and Gerald Münch (2016). Medicinal Plants of the Australian Aboriginal Dharawal People Exhibiting Anti-Inflammatory Activity. *Evidence-Based Complementary and Alternative Medicine*, volume 2016:8.
http://dx.doi.org/10.1155/2016/2935403

Most A. Akhtar, Frances Bodkin, Gerald Münch and Ritesh Raju (2018). A New Anti-inflammatory Chromone from the Leaves of *Eucalyptus viminalis*. *Natural Product Communications,* volume 13 (10):1297-1300.

List of Presentations

1. "The Anti-inflammatory Activity of Australian *Eucalyptus* spp. (Myrtaceae) with Aboriginal Ethnopharmacological Significance" Australian Society for Medical Research 24th Annual Scientific Meeting, 06th June, 2016, Sydney, NSW, Australia.

2. "The Anti-inflammatory Activity of Australian *Eucalyptus* spp. (Myrtaceae) with Aboriginal Ethnopharmacological Significance" Royal Australian Chemical Institute Natural Products Chemistry Group symposium, 30th September, 2016, University of Wollongong, NSW, Australia.

3. The Anti-inflammatory Activity of Australian *Eucalyptus* spp. (Myrtaceae) with Aboriginal Ethnopharmacological Significance" Songline Symposium, 24-25th October, 2016, WSU, NSW, Australia.

4. "The Anti-inflammatory Activity of Australian *Eucalyptus* spp. (Myrtaceae) with Aboriginal Ethnopharmacological Significance" Higher Degree Research Showcase, 27th October, 2016 WSU, NSW, Australia.

5. The Anti-inflammatory Activity of Australian *Eucalyptus* spp. (Myrtaceae) with Aboriginal Ethnopharmacological Significance" ASCEPT-MPGPCR Joint Scientific Meeting 27 -30th November, 2016, Melbourne, Victoria, Australia.

6. "The Anti-inflammatory Activity of *Eucalyptus viminalis* (Myrtaceae) With Aboriginal Ethnopharmacological Significance" 12th Brain Sciences UNSW Symposium, 04th May, 2017, UNSW, NSW, Australia.

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8. "The Anti-inflammatory Activity of Australian Native Plants with Aboriginal Ethnopharmacological Significance" Science in the Wild – Science Festival, 13th August, 2017, The Royal Botanic Garden, Mount Annan, NSW, Australia.

9. "New Compound Identified from *Eucalyptus viminalis* (Myrtaceae) With Aboriginal Ethnopharmacological Significance" Royal Australian Chemical Institute Natural Products Chemistry Group symposium, 22th September, 2017, Macquarie University, NSW, Australia.

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List of Abbreviations

Aβ	β-amyloid
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AIDS	Acquired Immune Deficiency Syndrome
AP-1	Activator Protein 1
APP	Amyloid Precursor Protein
BBB	Blood-Brain Barrier
BSA	Bovin Serum Albumine
CAD	Charged Aerosol Detector
CAM	Complementary and Alternative Medicine
CC	Column Chromatography
CD4	Cluster of Differentiation 4
CE	Capillary Electrophoresis
CLR	C-type Lectin Receptor
CM	Complementary Medicine
CNS	Central Nervous System
COX	Cyclooxygenase
CRP	C-Reactive Protein
CSF	Cerebrospinal Fluid
DCC	Droplet Counter-current Chromatography
DCM	Dichloromethane
DMEM	Dulbeccos's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNP	Dictionary of Natural Products
ELISA	Enzyme-linked Immunosorbent Sandwich Assay
ELSD	Evaporative light Scattering Detector
eNOS	endothelial Nitric Oxide-Synthase
FBS	Foetal Bovine Serum
FC	Flash Chromatography
FDA	Food and Drug Administration
GC	Gas Chromatography
GI	Gastro Intestinal
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HO^-	Hydroxyl radicle
HPLC	High Pressure Liquid Chromatography
HRP	Horseradish peroxidase
IC_{50}	50% Inhibitory Concentration
ICAM-1	Intercellular Adhesion Molecule 1
IL	Interleukin
IFNs	Interferons
I-ƙB	Inhibitor of kappa B
INF-γ	Interferon-γ
INF-β	Interferon-β
iNOS	Inducible Nitric Oxide Synthase
IR	Infra-Red
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase

MS	Multiple Sclerosis
MS	Mass Spectroscopy
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
$NaNO_2$	Sodium nitrite
NED	N-1-napthyl-ethylenediamine
NF-ƙB	Nuclear Factor kappa B
NFT	Neurofibrillary Tangles
NO	Nitric Oxide
NOD	Nucleotide-binding Oligomerization Domain
NOHA	N^{ω}-hydroxy-L-arginine
NLR	NOD Like Receptors
NMR	Nuclear Magnetic Resonance
NSAID	Non-Steroidal Anti-Inflammatory Drugs
PAF	Platelet-Activating Factor
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PD	Parkinson's disease
PDA	Photo Diode Array
PGE_2	Prostaglandin E_2
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
PRR	Pattern Recognition Receptor
RIG	Retinoic acid-Inducible Gene
RLR	RIG-1-Like Receptor
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SAID	Steroidal Anti-inflammatory Drug
SD	Standard Deviation
SEC	Size Exclusion Chromatography
SEM	Standard Error of the Mean
SI	Support Information
SOD	Superoxide Dismutase
SPE	Solid Phase Extraction
STAT	Signal Transducer and Activator of Transcription
TLR	Toll-like Receptor
Th2	T Helper Cells 2
TM	Traditional Medicine
TMB	Tetra Methyl Benzidine
TNF-α	Tumour Necrosis Factor-α
UV	Ultra Violet
uHPLC	Ultra High Performance Liquid Chromatography
VCAM-1	Vascular Cell Adhesion Molecule 1
VLC	Vacuum Liquid Chromatography

Abstract

The aim of this study was to isolate and characterise novel anti-inflammatory compounds from native Australian plants which were important to the D'harawal Aboriginal people for anti-inflammatory and related activities. A total of thirty two plants were screened for their anti-inflammatory and neuroprotective activity.

In chapter 2, ethanolic extracts of seventeen *Eucalyptus* spp. (Myrtaceae) were screened for their nitric oxide (NO) and tumour necrosis factor-α (TNF-α) downregulation activity and cytotoxicity in lipopolysaccharide (LPS) and interferon-γ (IFN-γ) activated RAW 264.7 macrophages. Extracts from seven *Eucalyptus* spp. demonstrated strong activity with IC_{50} values between 7.58 - 19.77 µg/mL for NO inhibition and IC_{50} values for suppression of TNF-α production were between 2.06 – 19.02 µg/mL. These extracts also showed a wide range of cytotoxicity with LC_{50} values between 22.34 – 236.5 µg/mL.

In chapter 3, two of the highly active *Eucalyptus* spp. (Myrtaceae), ***E. viminalis*** and ***E. bosistoana*** were sequentially extracted and screened to find out the most active extracts which were then fractionated to identify bioactive compounds. From *E. viminalis* a new **chromone** (compound **1**) has been identified together with two known compounds **8-β-C-glucopyranosyl-5,7-dihydroxy-2-isobutylchromone** and **globuluside.** The anti-inflammatory and cytotoxic activities of all three compounds were evaluated against RAW 264.7 macrophage and N11 microglial cell line. In RAW 264.7 macrophage, the IC_{50} values for NO down regulation were 44.0, 47.0 and 37.6 µg/mL whereas the IC_{50} of TNF-α suppression were 41.0, 38.3 and 43.2 µg/mL for **compound 1**, **8-β-C-glucopyranosyl-5,7-dihydroxy-2-isobutylchromone** and **globuluside** respectively. In N11 microglia, the IC_{50} values for NO down regulation were 43.4, 34.1 and 21.8 µg/mL whereas the IC_{50} of TNF-α suppression were 20.4, 34.3 and 19.0 µg/mL for **compound 1**, **8-β-C-glucopyranosyl-5,7-dihydroxy-2-isobutylchromone** and **globuluside** respectively. In both cell lines all of the compounds were non-toxic up to the highest concentration (36 µg/mL) tested. **Oleuropeic acid** which was obtained as a hydrolyzed product of **compound 1** was also tested for its anti-inflammatory activity. The NO inhibitory IC_{50} values were 18.7 and 10.2 µg/mL and TNF-α inhibitory IC_{50} values were 21.5 and 17.0 µg/mL for **oleuropeic acid** against RAW 264.7 macrophage and N11 microglial cell line respectively.

From ***E. bosistoana*** (chapter 3a) one known metabolite, **4-coumaroylquinic acid** was identified whose anti-inflammatory and cytotoxic activities were evaluated in RAW 264.7 macrophage and N11 microglial cell line as well. In RAW 264.7 macrophages the compound exhibited IC_{50} values of 95.74 and 52.56 µg/mL for NO and TNF-α inhibition respectively. Whereas in N11 microglia the NO and TNF-α inhibitory IC_{50} values were 44.31 and 35.50 µg/mL. In both cell line the compound was non-toxic up to the highest concentration (36 µg/mL) tested.

In chapter 4, ethanolic extracts of fifteen plant species from 8 different families and 11 different genera were screened for their NO and TNF-α downregulation activity and cytotoxicity in LPS and IFN-γ activated RAW 264.7 macrophages. Extracts from four of the plants, *Syncarpia glomulifera* subsp. *glomulifera*, *Melaleuca linariifolia*, *Baeckea ramosissima* subsp. *ramosissima* and *B. imbricata* exhibited very strong activity with IC_{50} values between 8.25 – 16.78 µg/mL for NO inhibition and IC_{50} values for suppression of TNF-α production were between 8.31 – 23.30 µg/mL. These extracts were found less toxic compared to plants from *Eucalyptus* spp. with LC_{50} values between 52.09 – 130.0 µg/mL.

In chapter 5, *Melaleuca linariifolia* was extracted sequentially with low to high polar solvents and the sequential extracts were screened for their anti-inflammatory activity to identify the most potent one, which was then purified using HPLC. From this plant, two known flavonoids **3, 3', 4', 5, 7-pentahydroxyflavan** and **3, 3', 5, 5', 7- pentahydroxyflavan** were identified along with one known triterpenoid **betulinic acid**. The anti-inflammatory activities as well as cytotoxicity of all three compounds were evaluated in RAW 264.7 macrophage and N11 microglial cell line. In RAW 264.7 macrophage, the IC_{50} values for NO down regulation were 72.81, 39.69 and 2.73 µg/mL whereas the IC_{50} of TNF-α suppression were 58.88, 80.70 and 4.11 µg/mL for **3, 3', 4', 5, 7-pentahydroxyflavan**, **3, 3', 5, 5', 7- pentahydroxyflavan** and **betulinic acid** respectively. In N11 microglia, the IC_{50} values for NO down regulation were 66.27, 58.05 and 2.23 µg/mL whereas the IC_{50} of TNF-α suppression were 17.34, 21.84 and 6.76 µg/mL for **3, 3', 4', 5, 7-pentahydroxyflavan, 3, 3', 5, 5', 7- pentahydroxyflavan and betulinic acid** respectively.

In chapter 6, *Syncarpia glomulifera* subsp. *glomulifera* was extracted sequentially with low to high polar solvents and the sequential extracts were screened for their anti-inflammatory activity to identify the most potent one, which was then purified using HPLC. From this plant, two new compounds compound **6.1** and **6.4** were identified along with three known compounds **tetragocarbone B** (compound **6.2**), **sideroxylin** (compound **6.3**) and **lumaflavanone A** (compound **6.5**). The anti-inflammatory and cytotoxic activities of all five compounds were evaluated in RAW 264.7 macrophage and N11 microglial cell line. In RAW 264.7 macrophage, the IC_{50} values for NO down regulation were 3.91, 35.15, 2.76, 29.42 and 7.84 µg/mL whereas the IC_{50} of TNF-α suppression were 16.90, 32.12, 20.80, 37.57 and 33.35 µg/mL for compound **6.1, 6.2, 6.3, 6.4** and **6.5** respectively. In N11 microglia, the IC_{50} values for NO down regulation were 4.52, 21.17, 3.87, 39.64 and 4.51 µg/mL whereas the IC_{50} of TNF-α suppression were 6.50, 27.01, 13.66, 33.06 and 5.46 µg/mL for compound **6.1, 6.2, 6.3, 6.4** and **6.5** respectively.

In chapter 7, *Baeckea ramosissima* subsp. *ramosissima* and ***B. imbricata*** leaves were extracted sequentially with low to high polar solvents and the sequential extracts were screened for their anti-inflammatory activity to identify the most potent one. Sequential EtOAc extract from both plants showed highest anti-inflammatory activity for NO inhibition (IC_{50} = 9.33 and 8.98 µg/mL for *Baeckea ramosissima* subsp. *ramosissima* and *B. imbricata* respectively) in LPS + IFN-γ activated 264.7 RAW macrophages and subjected to HPLC for fractionation. From ***Baeckea ramosissima* subsp. *ramosissima***, two known flavonoids **quercetin** and **kaemferol** were identified and evaluated for anti-inflammatory and cytotoxic activities in RAW 264.7 macrophage and N11 microglial cell line. In RAW 264.7 macrophage, the IC_{50} values for NO down regulation were 10.95 and 9.18 µg/mL whereas the IC_{50} of TNF-α suppression were 17.35 and 11.26 µg/mL for **quercetin** and **kaemferol** respectively. In N11 microglia, the IC_{50} values for NO down regulation were 19.71 and 16.06 µg/mL whereas the IC_{50} of TNF-α suppression were 8.84 and 8.12 µg/mL for **quercetin** and **kaemferol** respectively. Isolation and characterization of other active constituents are in progress.

Chapter 1
Introduction

1.1 General Introduction

Inflammation is an important biological process for maintaining the body's homeostasis. It is necessary for protecting the body against pathogenic organisms as well as chemical and physical stimuli and initiating wound healing mechanisms to repair damaged tissue [1]. But when uncontrolled, inflammation plays a role in the development and progression of human diseases including asthma, rheumatoid arthritis, inflammatory bowel disease, Crohn's disease and tendonitis. Moreover, chronic inflammatory response is a major driving force for the progression of cancer, atherosclerosis, diabetes, obesity and Alzheimer's disease. In this sense, inflammation can be viewed as a double-edged sword [1,2].

Currently, both steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) are used to treat inflammation. Steroids have an obvious role in the treatment of inflammatory diseases, but can only be used over short periods due to the onset of a number of adverse effects associated with chronic use, except in very serious cases where the benefits outweigh the risks. On the other hand, prolonged use of NSAIDs is also associated with severe side effects, notably gastrointestinal haemorrhage [3-5]. The newer selective cyclooxygenase-2 (COX-2) inhibitors originally designed to overcome such issues also do not seem to be free of risk [4,6]. Consequently, there is some urgency for the discovery of new anti-inflammatory compounds with less or no side effects [1,5].

Plants were the first source of remedies in the history of mankind. Plants or different plant parts were used as drugs against all types of treatment in all cultures and through all ages [1]. The anticancer vinblastine and vincristine from *Catharanthus roseus* and paclitaxel from *Taxus brevifolia*, the antimalarial quinine from *Cinchona* species, the antiglaucoma pilocarpine from *Pilocarpus jaborandi*, the analgesics morphine and its derivative codeine from *Papaver somniferum*, the spasmolytic and cold medicine atropine from *Atropa belladonna* and *Hyoscyamus niger* and the cardiac glycosides (e.g. digoxin) for the treatment of congestive heart failure from *Digitalis* species all share two important characteristics in common: they are the keystones of modern pharmaceutical care and they are all plant-derived natural products [7]. Thus the use of natural substances, especially plants either to prevent, manage or cure diseases is a centuries old practice which has led to discovery of more than half of all modern pharmaceuticals [8,9].

In many developing countries traditional medicine is still the only affordable and thus accessible way to meet the primary healthcare needs of 60-90% of the population [1,10,11]. In the developed world, a growing awareness of the potency and side effects of synthetic drugs, has coincided with a shift towards people seeking natural product remedies [11]. The term, 'complementary and alternative medicine' (CAM) encompasses a broad range of ancient to modern day treatment where complementary interventions are used together with conventional treatment and alternative interventions are used instead of conventional medicine [12]. In Australia, Europe and North America, the usage of CAM is increasing [10]. This does not infer however, that whole plant remedies are devoid of side effects and hence it is important the safety and efficacy of such products are validated using rigorous scientific methodologies.

World Health Organization (WHO) rephrases traditional medicine (TM) and complementary medicine (CM) for T&CM according to 'WHO's Traditional Medicine Strategy 2014-2023' emphasizing the importance of using T&CM in a safe, respectful, cost-efficient and effective manner.

WHO's definition states; "TM: It is the sum total of the knowledge, skill, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness, CM: The terms "complementary medicine" or "alternative medicine" refer to a broad set of health care practices that are not part of that country's own tradition or conventional medicine and are not fully integrated into the dominant health-care system. They are used interchangeably with traditional medicine in some countries, and T&CM: T&CM merges the terms TM and CM, encompassing products, practices and practitioners".

The chemical composition and biological activity of Australian native plants has not been examined in great depth [13,14]. There is large scope to investigate Australian traditional medicinal plants for their bioactivity and chemical constituents. According to literature, a number of plant species used in Australian Aboriginal medicine has been claimed to possess anti-inflammatory activities [15-19]. Traditional medicine is still practised by the Aboriginal people across Australia and the knowledge is recorded in some cases [14]. The 'D'harawal Pharmacopeia' written by Botanist and Aboriginal elder Mrs. Frances Bodkin (known as Aunty Fran) is an example for such compilation of knowledge where thousands of plants are recorded with their taxonomy and uses by the D'harawal people, including medicinal uses [20]. This pharmacopeia has been used to guide the selection and study of plants used by D'harawal Aboriginal people, for treatment of inflammation-related ailments for the present study.

1.2 Inflammation

Inflammation is a pathophysiological process characterized by redness, oedema, fever, pain and loss of function. An inflammatory response may be triggered by biological (e.g. bacteria/virus), chemical (e.g. capsaicin) or physical stimuli (e.g. ultra-violet radiation) leading to disruption of tissue integrity and homeostasis. Under normal conditions, this process usually leads to complete resolution, however inflammation can lead to persistent tissue damage by leukocyte, lymphocyte or collagen, if the targeted destruction and assisted repair are not properly conducted [21].

1.2.1 Inflammatory Response

As a host protective strategy, one of the main aims of inflammation is the maintenance of normal tissue homeostasis. Therefore, inflammation is considered as an 'adaptive response' that operates normal cellular functions and is normally initiated by the host in response to stimuli such as microbial infection and irritants (external or internal).

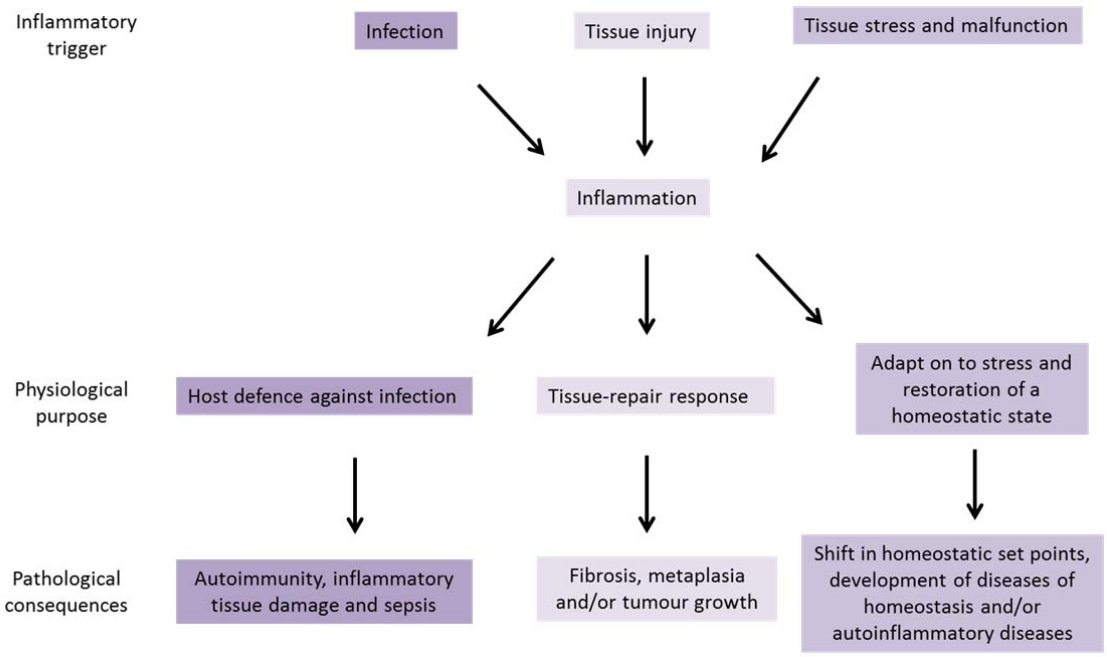


Figure 1-1. Causes and physiological and pathological outcomes of inflammation [22].

Together with immune cells, some non-immune cells including epithelial cells, endothelial cells and fibroblasts respond to inflammatory cues as well as further mediating the process. Different inflammatory pathways are initiated depending on the nature of the stimulant. Bacterial infection causes activation of pathogen specific receptors. Consequently, inflammatory mediators like cytokines (e.g. tumor necrosis factor (TNF), interlukin-1 (IL-1) and interlukin-6 (IL-6)) and chemokines are released and inflammation is progressed through the modification of vascular endothelial permeability and neutrophil recruitment. Neutrophils release highly potent toxic molecules such as reactive oxygen species (ROS), reactive nitrogen species (RNS), proteinase, cathepcin G and elastase which destroy both microbial targets as well as the host tissues at the same time. Viral infection, on the other hand, initiates production of a different class of cytokines such as type-1 interferons (IFNs) and cytotoxic lymphocytes with the same outcome like bacterial infection. Parasitic infections and allergens induce production of IL-4, IL-5, IL-13 and histamine [21-23].

1.2.2 Molecular Mechanism of Inflammation

Pattern recognition receptors (PRRs) (e.g. Toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG-1-like receptors (RLRs) and NOD-like receptors (NLRs)) are the specific transmembrane receptors expressed by both innate and adaptive immune cells. These receptors first recognize the inflammatory stimuli by recognizing structures conserved in microbes, called pathogen-associated molecular patterns (PAMPs) [24]. As these receptors interact with the appropriate stimuli, signal transduction to the nucleus takes place resulting in activation of a selective set of genes through transcriptional or posttranscriptional mechanisms [22,24]. These genes initiate the expression of

precise proinflammatory cytokines such as TNF, IL-1β and IL-6 in response to bacterial infection in a coordinated manner [23].

Signal transduction pathways often converge to the activation of a common set of transcription factors such as nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) which leads to the production of proinflammatory cytokines and chemokines and amplify the inflammatory response [23,25].

1.2.3 Role of Nitric Oxide in Inflammation

Nitric oxide (NO), a toxic atmospheric gas, is also generated by mammalian cells. Its multitude of physiologic and pathophysiologic functions results from both a wide distribution of synthesis and diverse mechanisms of action. Besides its function as a potent vasodilator and neurotransmitter, NO is important in inflammation and immunity [26]. However, there is a large body of evidence that NO is involved in several inflammatory disorders. It can be pro-inflammatory or anti-inflammatory, host-protective or host damaging during infections. Therefore, NO has been described as a "double-edged sword mediator" and this phenomenon is often referred to as the NO paradox. This peculiar aspect of the pathophysiology of NO has hampered the development of new drugs [27]. NO is produced as a metabolic by-product during the conversion of L-arginine to L-citrulline by a class of enzymes called nitric oxide synthases (NOS) [28].

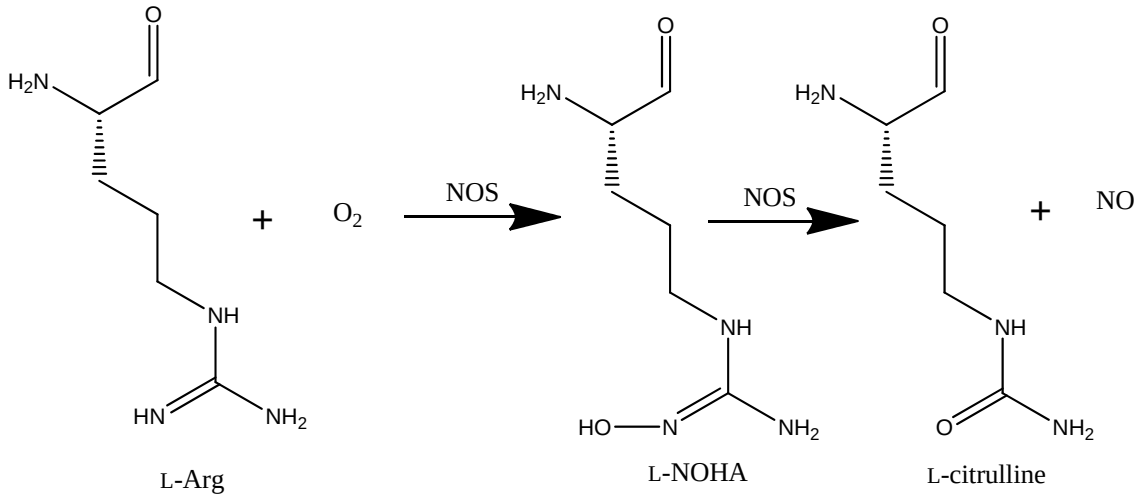


Figure 1-2. Formation of nitric oxide.

There are three isoforms of NOS; neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The nomenclature, nNOS and eNOS, results from the fact that these isoforms were originally isolated from rat neurons and bovine endothelial cells, respectively. Each is constitutively expressed in cells and synthesise NO in response to an increase in calcium or in some cases to calcium independent stimuli such as shear stress. The third isoform iNOS is constitutively expressed only in some tissues such as lung epithelium and is typically rapidly induced and upregulated in response to inflammatory or pro-inflammatory mediators [5,27,28].

Macrophage activation by lipopolysaccharides (LPS), a major component (endotoxin) of gram-negative bacteria cell walls, results in the release of several inflammatory mediators including NO [29]. Upon induction by certain inflammatory stimuli, e.g. bacterial lipopolysaccharide (LPS), the NF-κB signal pathway is activated, regulating the expression of a wide variety of genes involved in inflammatory responses, e.g. the cytokines TNF-α and IL-1β, and the enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). The enzyme iNOS catalyzes the conversion of the amino acid L-arginine to NO, which is cytotoxic and cytostatic. Therefore, inhibition of NO production (through iNOS) is a major target for anti-inflammatory compound development. Many studies have shown plants to be a source of natural products with ability to directly or indirectly modify iNOS activity [5,30].

1.2.4 Role of Cytokines in Inflammation

Cytokines are secreted, highly localized, low molecular weight proteins (molecular weight ranges from 6 to 70 kDa) that regulate the immune response and in large part determine the form of an immune response to be taken place [31,32]. They are produced by many cell types, most importantly by macrophages and mast cells [22]. Through their numerous biological activities either directly on immune cells or alternatively upon adhesion molecules with which immune cells must interact, all of the cytokines may be considered to be pro-inflammatory. Several cytokines, however, are uniquely capable of promoting the cellular infiltrate and damage to resident tissue that are characteristic of inflammation. These cytokines include tumour necrosis factor (TNF), interleukin (IL) -1, IL-6 and members of the small cytokine family [31]. Due to their redundant and pleiotropic actions, cytokines form a network in which one cytokine can induce its own production or even the secondary generation of other cytokines. In addition, it has been widely shown that most cytokine actions involve the activation of transcription factors (e.g., NF-κB and AP-1) and protein kinases (e.g., MAPK and PKC) that, in turn, regulate the expression of many target genes indispensable to the maintenance of the inflammatory state. For instance, cytokines may be responsible for the induction of several enzymes (e.g. iNOS and COX-2), receptors (PAF receptor, IL-2 receptor) and adhesion molecules (E-selectin, α- and β-integrins, ICAM-1, VCAM-1). Besides mediating inflammation they also contribute to a chemical signalling language that regulates development, tissue repair, hemopoiesis and other specific and nonspecific immune responses [33]. It has been observed that several compounds are able to decrease the expression of different pro-inflammatory cytokines/chemokines such as TNF-α, IL-1β, IL-6, IL-8, MCP-1 in LPS-activated mouse primary macrophages [34]. With the knowledge of their interaction and by measuring their expression, cytokines hold considerable promise for providing a range of better treatments for tissue injury and inflammation [35].

1.2.5 Role of Nuclear factor-κB (NF-κB) in Inflammation

Nuclear factor-κB (NF-κB) is a family of dimeric proteins that regulates the expression of multiple immune and inflammatory genes [36-38]. It is present in the cytoplasm in an inactive form complexed to an inhibitory protein, I-κB. Many extracellular stimuli, including viruses, oxidants, inflammatory cytokines and immune stimuli, activate NF-κB. Once activated, it binds to recognition elements in the promoter regions of inflammatory and immune genes, such as proinflammatory cytokines, chemokines, inflammatory enzymes and adhesion molecules. This activation leads to the co-ordinated expression of inflammatory response and plays a key regulatory role in host defence [36]. The dysregulation of NF-κB is associated with many disease states such as AIDS, atherosclerosis, asthma, arthritis, cancer, diabetes, inflammatory bowel disease, muscular dystrophy, stroke and viral infections. Recent evidence also suggests that the dysfunction of NF-κB is a major mediator of some human genetic disorders [38]. NF-κB is therefore an obvious target for new types of anti-inflammatory treatment [37]. Anti-NF-κB therapy provides the means to simultaneously inhibit expression of numerous inflammatory mediators, including TNF-α and IL-1. Recent discoveries of the critical components (e.g. I-κB kinases) of the NF-κB signalling pathway have revolutionized the field and offered the opportunity to start a systematic search for specific inhibitors of the NF-κB pathway. This knowledge should lead to discovery of novel classes of efficacious therapeutic compounds [38,39].

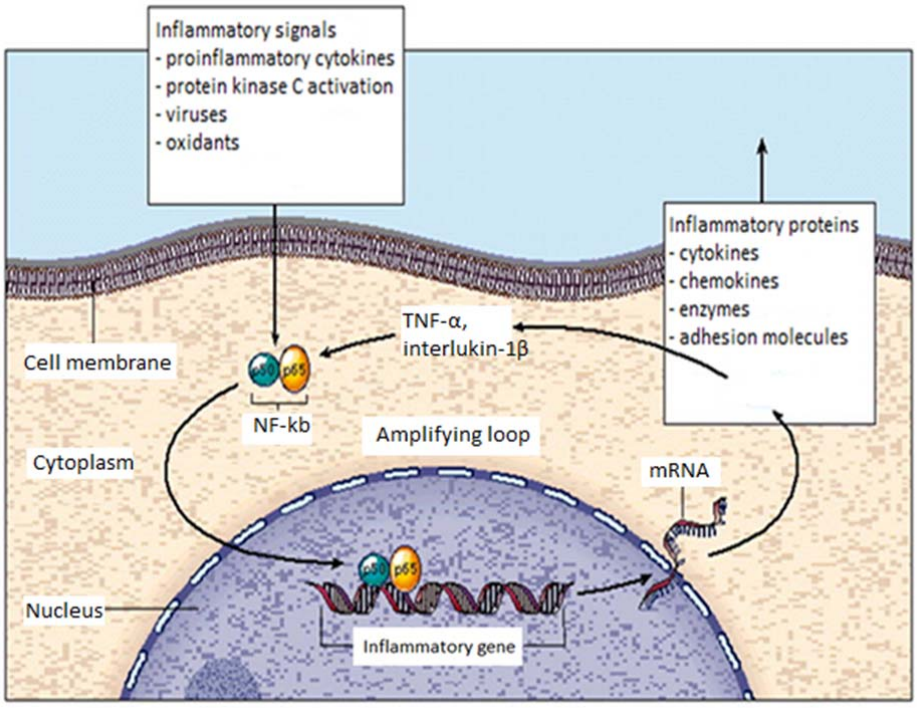


Figure 1-3. Schematic diagram of NF-κB as an inflammatory regulator. (NF-κB may be activated by a variety of inflammatory signals, resulting in the coordinated expression of the genes for several cytokines, chemokines, enzymes, and adhesion molecules. The cytokines interleukin-1β and tumour necrosis factor α (TNF-α) both activate and are amplified by NF-κB. The abbreviation mRNA denotes messenger RNA) [adapted; [37]].

Furthermore, inhibition of NF-κB and subsequent TNF-α production protect animals from the lethal effects of endotoxin. In most cells NF-κB is associated in the cytoplasm with its inhibitory subunit, inhibitory kappa B (I-κB), which prevents NF-κB from translocating into the nucleus. Multiple stimuli, including LPS, induce the phosphorylation and degradation of I-κB , allowing NF-κB to translocate to the nucleus and activate gene expression and thus could be a potential therapeutic target in the inflammatory state [40].

1.3 Chronic Inflammation

Inflammation has evolved as a beneficial strategy for the host in response to any potential danger, since it is normally terminated once the potential danger is eradicated. Usually the inflammatory response reverts to the homeostatic state quite rapidly, a highly regulated process known as the resolution of inflammation [23]. The resolution of inflammation is dominated by a number of anti-inflammatory mediators such as IL-10, TNF-β and glucocorticoids and it also involves the recruitment of monocytes for the clearance of cell debris [41]. If the resolution of inflammation fails for any reason, the acute inflammatory response turns chronic. Chronic inflammation has been a subject of extensive studies over many decades not only for the growing burdens of the associated pathological conditions in the modern societies but also for the underlying mechanisms which remain largely unresolved. Chronic inflammation is generally believed to develop if the host fails to eliminate the inflammatory stimuli, such as persistent infection or chronic cellular injury [23].

1.3.1 Chronic Inflammatory Diseases

Chronic inflammation is a major driver of pathogenesis and is associated with a number of chronic inflammatory diseases, including atherosclerosis, obesity, type 2 diabetes, asthma, inflammatory bowel disease, rheumatoid arthritis, cancer and neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD) and multiple sclerosis (MS) [23,42].

Chronic inflammation can result in obesity as well as obesity associated diabetes partly because of insulin resistance. Rheumatoid arthritis is another chronic inflammatory disorder in which, the synovium, the lining of the joint is chronically inflamed due to the infiltration and persistent activation of macrophages and lymphocytes and the activation of synoviocytes, synovial cells, which produce synovial fluids. In rheumatoid arthritis, the synovial fluid is invaded by billions of neutrophils everyday which are the major contributor of inflammation in synovial tissue [43].

Inflammation in asthma and allergies are caused by dysregulated interactions between mucosal epithelia and innate immune cells [23]. Further studies suggested that apart from innate immunity, cells of adaptive immunity such as CD4 T helper 2 (Th2) cells as well as Th2 associated cytokines were found to be strongly linked to asthma [44]. Even though genetic predisposition to asthma has been reported for years, recent genome-wide studies suggest that the hereditary contribution to asthma is relatively small [45]. Other studies demonstrated that several viral upper respiratory

infections caused by environmental factors, early in the life along with genetic predisposition increases the risks for asthma [46].

Over the last decades it has become evident that inflammation plays a critical role in promoting cancer, in particular tumorigenesis (tumour formation) [23]. In addition to cancer cells, various types of immune cells are commonly found within tumours and an inflammatory microenvironment is also more frequently found as an essential part of all tumours [47,48]. It has also been demonstrated that the inflammatory response triggered by infection is associated with increased cancer risk [49].

The pathological hallmarks of Alzheimer's disease (AD) in the brain include extracellular amyloid plaques comprising aggregated, cleaved products of the amyloid precursor protein (APP) and intracellular neurofibrillary tangles (NFTs) generated by microtubule-binding protein tau phosphorylation [42,50]. Aggregates of Aβ activate microglia and induce production of a number of inflammatory mediators such as NO, ROS, proinflammatory cytokines (e.g. TNF-α, IL-β, IL-6), chemokines (e.g. IL-18) and prostaglandins (e.g. PGE_2) that promote neuronal death. Again an inflammatory environment is thought to be activated by tau kinases to promote formation of NFTs [42].

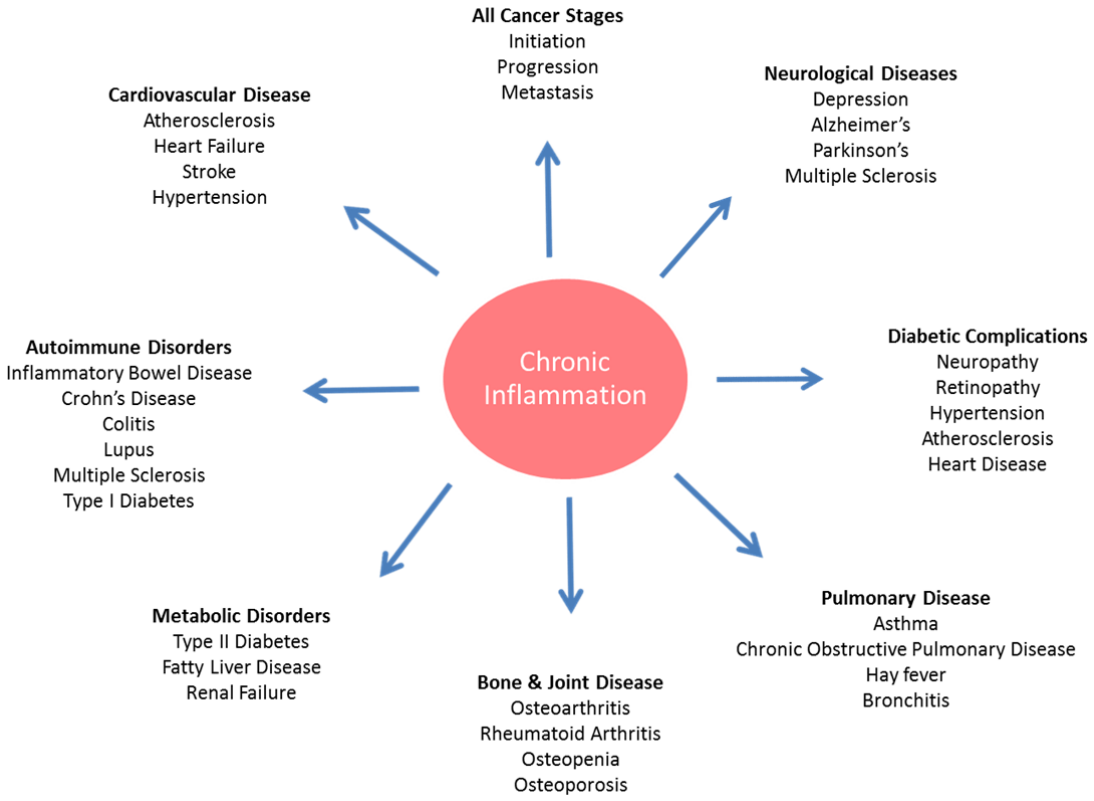


Figure 1-4. Diseases associated with chronic inflammation.

1.4 Anti-inflammatory Drugs

1.4.1 Non-Steroidal Anti-inflammatory Drugs (NSAIDs)

NSAIDs are one of the most commonly prescribed classes of drugs [51]. Approximately 100 million people worldwide use them regularly [52]. Examples of commonly used NSAIDs include aspirin, ibuprofen and naproxen [7].

NSAIDs inhibit cyclooxygenase (COX), the enzyme responsible for conversion of arachidonic acid to prostaglandins. COX exists in 2 isoforms. COX-1 is an ubiquitous constitutive isozyme producing prostaglandins responsible for homeostatic functions such as maintenance of GI mucosal integrity. COX-2 is largely a cytokine-induced isozyme producing prostaglandins that mediate pain and inflammation. NSAIDs inhibit both COX-1 and COX-2 to varying degrees. Thus, the therapeutic effects of conventional NSAIDs are derived from inhibition of COX-2, while the adverse effects of these agents, particularly in the upper GI tract, arise from inhibition of COX-1 activity [53].

NSAIDs use has been associated with colonic bleeding, iron deficiency anaemia, strictures, ulcerations, perforations, diarrhoea and death. In addition, NSAIDS can exacerbate inflammatory bowel disease and ulcerative colitis [54].

1.4.2 Cyclooxygenase 2 Selective Drugs (COX-2 selective drugs)

COX-2 is largely a cytokine-induced isozyme producing prostaglandins that mediate pain and inflammation [53]. Two recently developed COX-2 inhibitors celecoxib and rofecoxib were approved by the US Food and Drug Administration (FDA), as well as some European countries. They were believed to provide therapeutic advantages over conventional currently available non-selective NSAIDs since they do not cause any clinically meaningful inhibition of COX-1 at maximal therapeutic doses [52].

Rofecoxib was approved by FDA in 1999 and was marketed by Merck & Co under the brand name ‘Vioxx’. It gained worldwide acceptance but in 2004, Merck withdrew ‘Vioxx’ from the market because of concerns about increased risk of heart attack and stroke associated with long-term, high-dosage use [55].

1.4.3 Steroidal Anti-inflammatory Drugs (SAIDs)

Steroids are highly potent and effective agents in controlling chronic inflammation [56]. The different types of steroids in tablet form include prednisolone, prednisone and methylprednisolone. Generic inhaled forms include beclomethasone, budesonide, flunisolide, fluticasone propionate and triamcinolone. The high doses of steroids in tablet form (or in smaller doses given for long periods of time) may cause problems including: bruising of the skin, weight gain, osteoporosis, diabetes,

cataracts and swelling of the ankles or feet. On the other hand, the most common side-effects of inhaled steroids are a sore mouth, hoarse voice and infections in the throat and mouth [57].

1.5 Natural Products, Medicinal Plants and Drug Discovery

People have used plants for healing for centuries. Plant products – as part of foods or botanical potions and powders have been used with varying success to cure and prevent diseases throughout history [58]. The strong historic bond between plants and human health began to unveil in 1897, when synthetic aspirin (acetyl salicylic acid) was introduced to the world [58]. Aspirin is a safer and more potent synthetic analogue of salicylic acid, an active ingredient of willow bark and was discovered independently by residents of both the modern and old worlds as a remedy for aches and fevers [59].

Until the last century most medicines were derived directly from plant or animal sources. Despite the increasing use of synthetic drugs, natural organic healing materials have persisted as the "treatment of choice" for a multitude of health problems in populations throughout the world [60].

As stated by the researcher Alan Harvey, "natural products continue to provide greater structural diversity than standard combinatorial chemistry and so they offer major opportunities for finding novel low molecular weight lead structures that are active against a wide range of assay targets. As less than 10% of the world's biodiversity has been tested for biological activity, many more useful natural lead compounds are awaiting discovery" [61].

1.6 Anti-inflammatory Medicinal Plants

Plant-derived natural products represent an important source of new compounds effective against various diseases including inflammatory disorders [62]. In recent years, the search for new anti-inflammatory and anti-allergic agents from a large array of medicinal plant resources is intensifying [63]. In fact, a variety of bioactive components have been shown to modulate inflammatory responses [64].

The anti-inflammatory activity of several plant extracts and isolated compounds has already been scientifically demonstrated. Turmeric (*Curcuma longa*), which has traditionally been used for treatment of rheumatic disorders in Indian traditional medicine, exerts both anti-inflammatory and anti-atherosclerotic effects [65]. Ginger extract (*Zingiber zerumbet*) and its main active compound, 3-*O*-methylkaempferol, inhibited the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2), as well as iNOS expression in a cell culture model and in an *in vivo* model, carrageenan-induced mouse paw oedema was significantly attenuated with this compound [66].

Ethanol extracts of Jordanian plants *Mentha pipirata*, *Jasminum officinale*, *Commiphora molmol* and *Beta vulgaris* possess anti-inflammatory activity against acute (xylene induced ear oedema) and chronic (cotton-pellet granuloma) inflammation [67]. Mediterranean dietary plants borage (*Borago*

officinalis), caper (*Capparis sicula* Veill. subsp. *sicula*), mallow (*Malva sylvestris*), mint (*mentha aquatica*) and radish (*Raphanus raphanistrum*) have shown to inhibit croton oil-induced ear oedema in mice with the ethanolic extracts of each plant [68].

Treatment of LPS-stimulated macrophages with extracts from strawberry (*Fragaria ananassa*), loquat (*Eriobotrya japonica*), mulberry (*Morus alba*) and bitter melon juice (*Momordica charantia*) decreased the secretion of IL-6 and IL-1β pro-inflammatory cytokines and up-regulated the secretion of the anti-inflammatory cytokine IL-10 in a prophylactic cell culture model [69].

Various plant compounds have also been shown to exhibit anti-inflammatory activity: Quercetin inhibits iNOS, COX-2 and C-reactive protein (CRP), and down-regulates NF-κB and TNF-α secretion. Kaempferol inhibits iNOS, COX-2, CRP and NF-κB. Naringenin down-regulates the secretion of pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α and inhibits iNOS expression and NF-κB activation and luteolin inhibits TNF-α, IL-6 and IL-1β secretion [70,71].

1.7 Australian Indigenous Medicinal Plants

For over 40,000 years, Aboriginal Australian peoples have passed down through oral history, traditional knowledge including the preparation and uses of different medicinal plants. A number of studies have revealed biologically active compounds from these plants [72]. The Australian flora offers enormous opportunities for the selection of novel plants that, in addition to being nutritious, possess health enhancing properties. Many of the Australian native fruits have been identified as a novel source of rich polyphenolic complexes with enhanced antioxidant activities [18,73,74]. Kakadu plam (*Terminalia ferdinandiana*) contains an extremely rich mixture of phytochemicals and demonstrated a range of significant biological activities *in vitro*, including antioxidant activity, the inhibition of COX-2 and iNOS in LPS-activated murine macrophages [17]. Illawara plum (*Podocarpus elatus*) extract on the other hand, has been shown to reduce the proliferation of colon cancer cells and could lead to provisions for an alternative chemoprevention strategy to conventional chemotherapy [75]. Mueller and co-workers have identified new antimalarial leads from the Australian tree *Mitrephora diversifolia* [76]. Methanol extracts of traditionally used Australian plants *Eremophila maculata*, *Acacia auriculoformis* and *Acacia bivenosa* exhibited antibiotic effects while another such plant *Eremophila alternifolia* extract induced significant changes in heart activity of spontaneously hypertensive rats [72].

1.8 Anti-inflammatory Australian Indigenous Plants

A number of plant species used in Australian aboriginal medicine have been claimed to possess anti-inflammatory activities [16]. Simpson and co-workers isolated clerodane diterpenoids (Figure 1-5) from the Australian plant *Dodonaea polyandra* and demonstrated anti-inflammatory activity of compound (2) and (4) in a mouse ear oedema model [77]. The same lab later on showed that compound (2) can inhibit IL-1β production and can reduce the secretion on IL-6, both of which are pro-inflammatory cytokines [78].

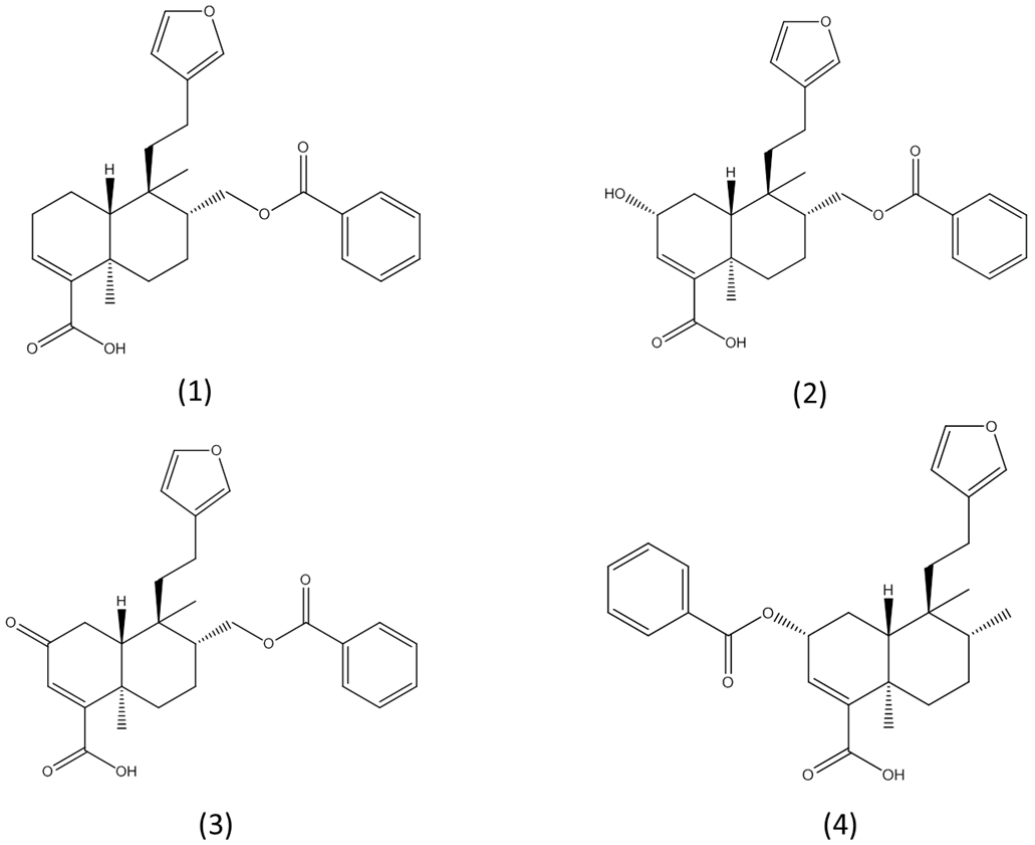


Figure 1-5. Structures of compounds isolated from *Dodonaea polyandra* [78].

An extract with anti-inflammatory activity has been obtained from the seeds of the Australian rainforest vine *Celastrus subspicata* and two agarofurans (Figure 1-6) isolated from the extract shown to possess anti-inflammatory activity [79].

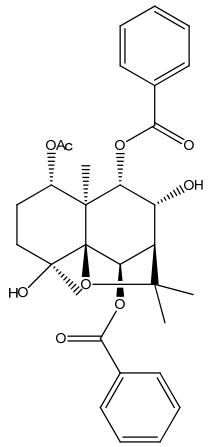


Figure 1-6. Agarofurans (1 and 2) isolated from *Celastrus subspicata* [79].

Triterpenoid saponins from the Australian desert tree *Acacia victoriae* have anti-inflammatory effects by inhibiting activation of NF-κB, through prevention of nuclear localization and binding to DNA [80]. Another Australian medicinal plant *Tinospora smilacina* is claimed to possess long chain unsaturated fatty acids which possess anti-inflammatory properties [81]. Meanwhile, the native Australian fruits Kakadu plum (*Terminalia ferdinandiana*), Illawarra plum (*Podocarpus elatus*) and Native current (*Acrotriche depressa*) all exhibit significant anti-inflammatory activities [17,18].

In an anti-inflammatory study, researchers investigated ethanolic extracts of 13 Australian plants (*Acacia adsurgens, Acacia ancistrocarpa, Abrus precatorius, Acacia catechu, Clematis glycinoides, Clematis pickeringii, Eucalyptus pruinosa, Euphorbia australis, Euphorbia drummondii, Ficus racemosa, Morinda citrifolia, Tinospora smilacina* and *Verbena officinalis*) concluding each as having a COX-1 inhibitory effect to varying degrees [16].

1.9 Anti-inflammatory Plant Product and Cellular Action Mechanisms

Biologically active natural products continue to be useful in the exploration and control of intracellular signalling processes. For example, Kwok and co-researchers demonstrated that a sesquiterpene lactone derived from the herb Feverfew (*Tanacetum parthenicum*) inhibited proinflammatory pathway by inhibiting IκB and thereby preventing NF-κB activation [82]. Polyphenols (e.g., catechins, proanthocyanidin, resveratrol, silymarin) which are a large family of naturally occurring plant products have proved to be anti-inflammatory. For example, catechins can inhibit NO, iNOS; proanthocyanidins can inhibit hydrogen peroxide (H_2O_2), iNOS and NF-κB activation; resveratrol inhibits COX-2 and NF-κB activation [83].

Flavonoids, another class of plant secondary compound show anti-inflammatory activity by inhibiting eicosanoid generating enzymes including phospholipase A_2 (PLA_2), cyclooxygenases, leukotrienes or by modulating proinflammatory gene expression including iNOS and several cytokines. (Figure 1-7) For example, quercetin can inhibit PLA_2. Another flavonoid, apigenin can inhibit COX-2 strongly by inhibiting NF-κB activation and can also inhibit iNOS and cytokines (IL-6 and IL-8). Genistein is also reported to inhibit proinflammatory cytokines (IL-1β, IL-6 and TNF-α) [3].

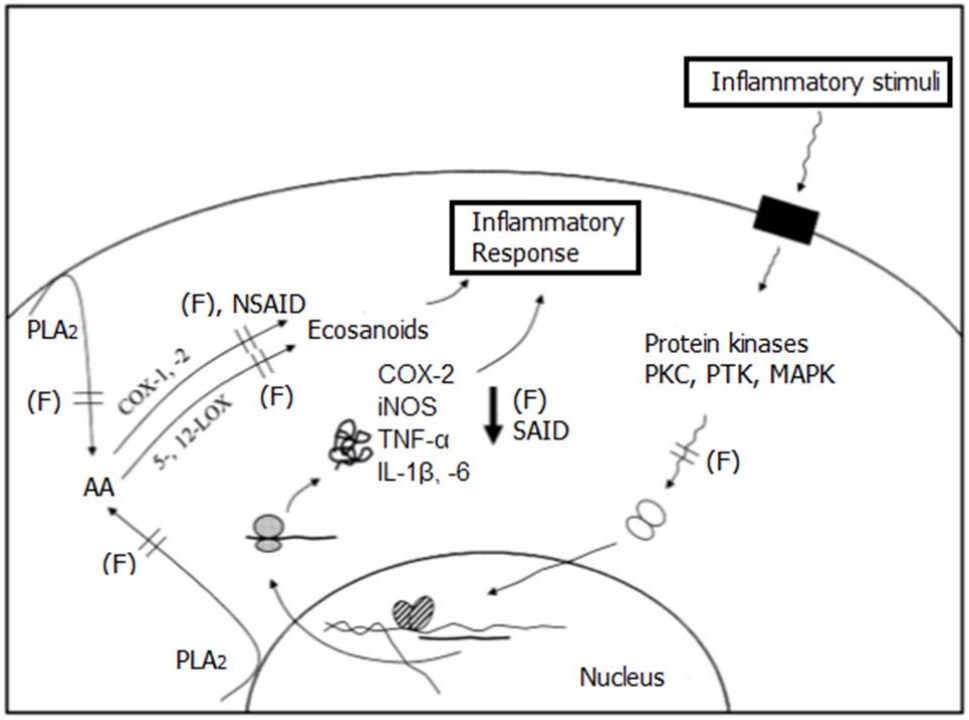


Figure 1-7. The proposed action mechanism of flavonoids. Flavonoid (F), nonsteroidal anti-inflammatory drug (NSAID), steroidal anti-inflammatory drug (SAID), "=" and "↓ " denote enzyme inhibition and down-regulation of the expression, respectively [adapted; [5]].

1.10 Isolation of Bioactive Natural Products

1.10.1 Selection of the Plant

In the present study, the plants were selected studying the D'harawal Pharmacopoeia and under the guidance of D'harawal Aboriginal elder and Botanist Auntie Frances Bodkin. In the first part of the investigation, (Chapter 2) seventeen *Eucalyptus* Spp. (Myrtaceae) were selected for screening, which were used by the Aboriginal D'harawal People to heal inflammation or related disorders. In the later part of the study (Chapter 4) fifteen plant species from eleven different genera [*Adiantum*

(Adiantaceae); *Asplenium* (Aspleniaceae); *Acacia* (Fabaceae); *Mentha* (Lamiaceae); *Baeckea, Melaleuca* and *Syncarpia* (Myrtaceae); *Pittosporum* (Pittosporaceae); *Hakea* and *Telopea* (Proteaceae); *Pimelea*, (Thymelaeaceae)] were screened which were important to the same Aboriginal clan for their anti-inflammatory or related activity. After screening, two plants from *Eucalyptus* Spp. (*E. viminalis* and *E. bosistoana*; Chaper 3) and four other plants [*Melaleuca linarifolia*, *Syncarpia glomulifera* subsp. *glomulifera*, *Baeckea ramosissima* subsp. *ramosissima* and *B. imbricata*); Chapter 5, 6 and 7 respectively] were selected for investigation to isolate anti-inflammatory compounds.

1.10.2 Sequential Solvent Extraction

Sequential solvent extraction is a convenient and commonly used procedure to isolate unknown compounds. A series of solvents from non-polar to more polar were used to comprehensively extract the plant material. The extractant is usually concentrated under vacuum, once the extraction is done ensuring the volatile constituents are not lost [84]. Sequential solvent extraction also facilitates the separation of lipophilic from hydrophilic compounds [85]. Hexane, dichloromethane, ethyl acetate, ethanol, methanol and water were used as sequential solvents and soxhlet extraction has been carried out in this dissertation. Each of the extracts was then screened for bioactivity and the ones with lowest IC_{50} value for NO downregulation was selected for purification to isolate the active molecules.

1.10.3 Bioassay-guided Fractionation

Bioassay-guided fractionation is a modern strategy to isolate and identify active lead compounds from natural sources. This technique facilitates the isolation of bioactive molecules from multi-constituent extracts [86,87]. It is a popular technique in natural product chemistry and is particularly important when the active compound is unknown [84]. According to bioassay-guided fractionation the crude extracts were separated into several fractions using different separation techniques which were then evaluated by the bioassays and fractions with the desired biological activity in the bioassay were selected for further purification to identify the pure compounds with the biological activity [88]. Bioassay-guided fractionation minimizes the chance of isolating inactive compounds to a great extent.

1.10.4 Isolation Techniques

A number of techniques are available to isolate and purify bioactive natural products from plant extracts. Solvent partitioning or liquid-liquid partitioning is a fast and simple technique, which separates fractions based on their relative solubility in different solvents. Chromatographic techniques include vacuum liquid chromatography (VLC), flash chromatography (FC), column chromatography (CC), size exclusion chromatography (SEC), solid-phase extraction (SPE), droplet counter-current chromatography (DCC) or preparative high performance chromatography (prep-HPLC) [89]. The most widely used isolation techniques are probably Liquid Chromatography (LC), including open column chromatography and High Performance Liquid Chromatography (HPLC). Other separation techniques include gas chromatography (GC), capillary electrophoresis (CE) and Ultra High Performance Liquid Chromatography (uHPLC). Relatively non-specific detectors are preferable for HPLC, such as UV, refractive index (RI), evaporative light scattering (ELSD) or charged aerosol detectors (CAD) [88]. In the present study, HPLC with UV detection was used to purify crude extracts from plant.

1.10.5 Anti-inflammatory Bioassays

1.10.5.1 Nitric oxide quantitation via Griess method

Based on the principle of chromophore formation, the Griess method is diazotization of sulphanilamide by nitrite in acidic conditions followed by coupling with bicyclic amines, *i.e. N*-1-(napthyl)ethylenediamine (NED). The concentration of nitrate is calculated as a function of the reduction rate, which is in proportion to the yield of the Griess dye (Figure 1-8) [90,91].

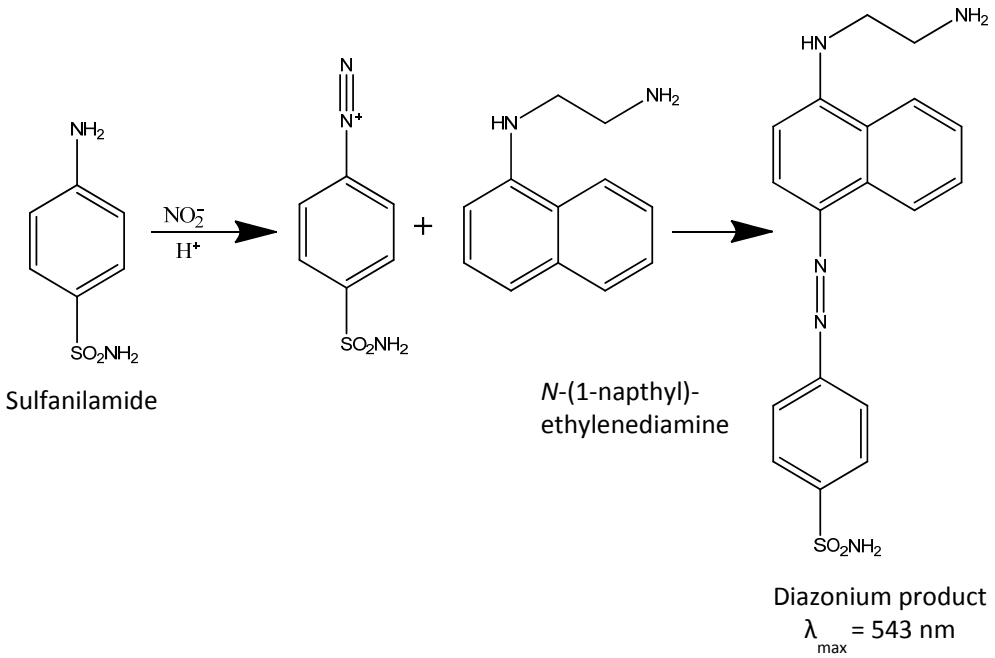


Figure 1-8. Principle of nitrite quantitation using Griess reaction. Formation of azo-dye is detected via absorbance at 540 nm.

1.10.5.2 Measuring TNF-α with enzyme linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA), is a widely used and well validated method to measure cytokines. This method is highly quantitative and generally reproducible. In the traditional double antibody sandwich ELISA (Figure 1-9), an antibody attached to the bottom of a well provides both antigen capture and immune specificity, while another antibody linked to an enzyme provides detection and an amplification factor which enables accurate and sensitive detection of the cytokine (antigen) of interest [92]. To quantify the amount of TNF-α, avidin-horseradish peroxidase (avidin-HRP) conjugate is added to allow colour formation in the presence of tetramethylbenzidine (TMB). Subsequently, brilliant blue colour can be detected with a spectrophotometer at 655 nm. Then addition of stop solution (for example, 5M H_2SO_4) turns the colour from brilliant blue to yellow, which is detectable at 450 nm with a reference wavelength of 655 nm [93].

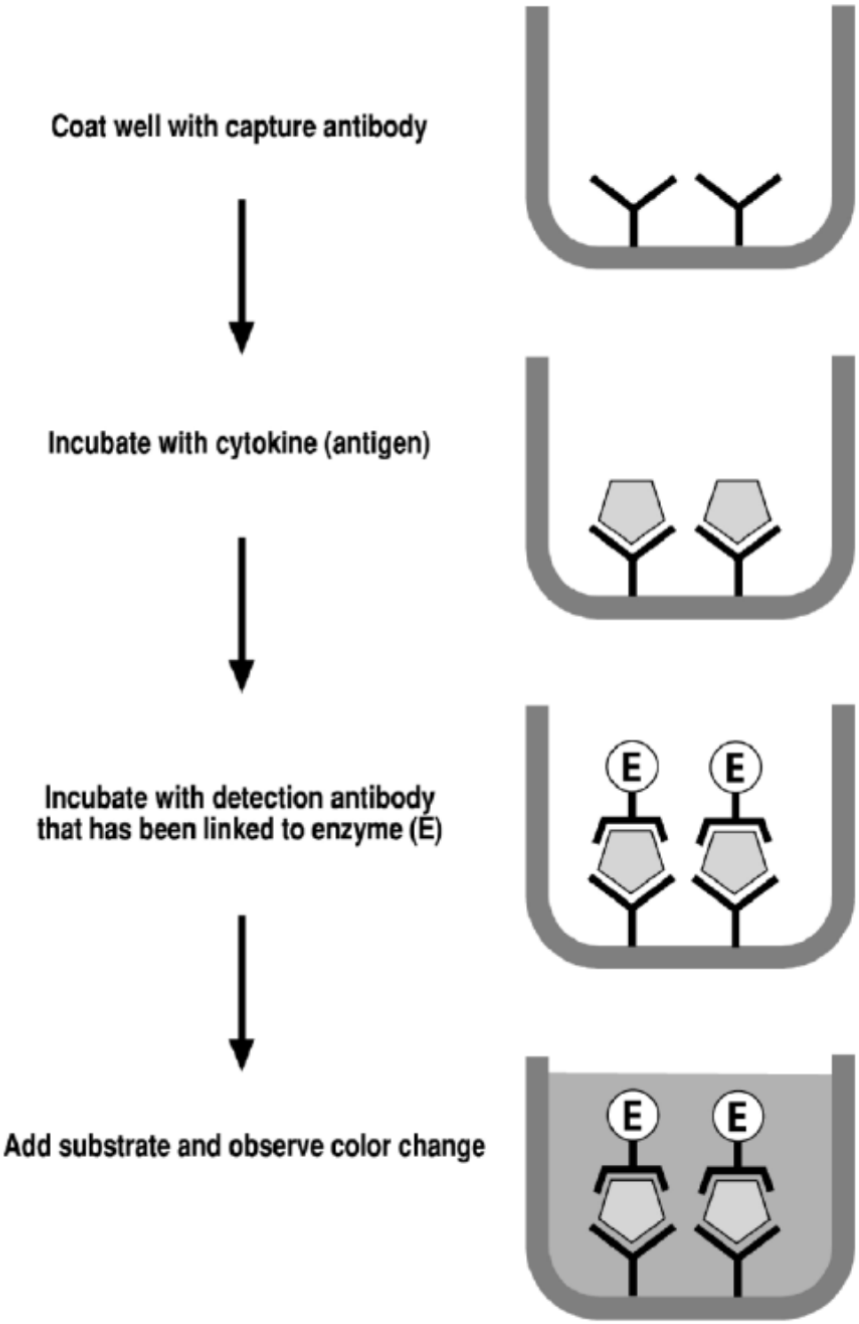
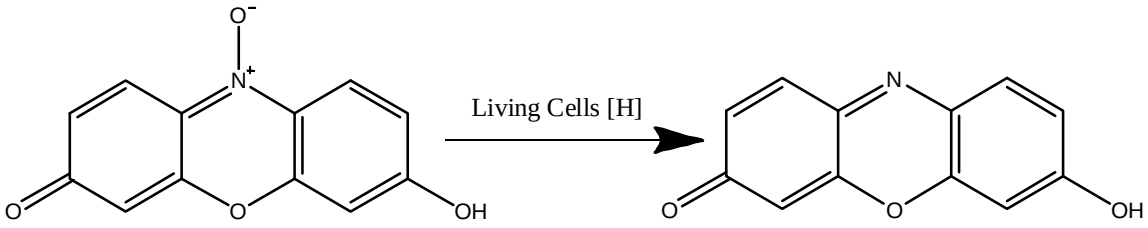


Figure 1-9. Schematic illustration for traditional double-antibody sandwich ELISA [92].

1.10.5.3 Determination of cell viability

Alamar blue (resazurin) is a dye reagent which is blue in colour and virtually nonfluorescent. Upon entering cells, resazurin is reduced to resorufin, which produces very bright red fluorescence. Viable cells continuously convert resazurin to resorufin (Figure 1-10), thereby generating a quantitative measure of cell viability and cytotoxicity [94]. The Alamar Blue assay works on the principle of measuring mitochondrial activity [95].



Resazurin Resorufin

Figure 1-10. Cell viability quantification with resazurin reduction to resorufin

1.10.6 Structure Elucidation of Bioactive Natural Products

The structure elucidation of bioactive natural products traditionally depends on analysing spectroscopic data including mass spectroscopic data, IR, UV and NMR spectroscopy [96,97]. As stated by Halabalaki et al., NMR is an indispensable analytical method in all structure elucidation protocols [98]. Determination of stereochemistry usually depends on interpretation of NMR spectra, including 2D spectra, optical rotation values and circular dicroism spectroscopy. Dereplication of known compounds can be obtained by interpreting mass spectrometry, 1D NMR spectroscopic analysis and database searches. The most used databases in this dissertation are the Dictionary of Natural Products (DNP) and SciFinder. Structure elucidation of new compounds needs more in-depth spectroscopic analysis. If spectroscopic analysis does not allow confident structure elucidation, chemical derivatizations are required.

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Chapter 2

Medicinal Plants of the Australian Aboriginal Dharawal People Exhibit Anti-inflammatory Activity (Publication-1)

Preface to Chapter 2

Author contribution

This chapter is a previously published work (Akhtar M. A.; Raju R.; Beattie K. D.; Bodkin F. and Münch G. Medicinal plants of the Australian Aboriginal Dharawal People exhibit anti-inflammatory activity. *Evidence-Based Complementary and Alternative Medicine.* 2016, 1-8.). Contributions of co-authors of the article are described as follows in the order of names listed. The author of this dissertation (Most. Afia Akhtar) completed extraction of the plant samples and performed anti-inflammatory bioassays as well as preparation of the manuscript. Dr. Ritesh Raju guided the extraction steps and collection of the plant samples. Dr. Karren D. Beattie participated in the manuscript preparation. Mrs. Frances Bodkin guided plant selection. Dr. Gerald Münch was a mentor of this work and is corresponding author for the published article.

Mrs. Frances Bodkin's (Aunty Fran) role for the published work

Mrs. Frances Bodkin is the D'harawal Aboriginal elder and Scientist. She has prepared a Pharmacopoeia and named it D'harawal Pharmacopoeia. In her Pharmacopoeia, Aunty Fran has enlisted approximately 2700 Australian native plant species with their corresponding taxonomical identification. She has described the uses of each plant by the D'harawal Aboriginal people including their medicinal values and thus representing the centuries old knowledge of this Aboriginal clan. This Pharmacopoeia was provided for this project and was studied thoroughly for plant selection. The selected plants were then screened and investigated to identify anti-inflammatory compounds.

Aunty Fran is also an Aboriginal custodian of the botanical garden (The Australian Botanic Garden, Mount Annan), from where we collected our plant samples. She physically attended meetings on this project, guided plant selection, followed up the progress of the work and gave her wise opinions on the findings.

Corrections for the typographical errors in Chapter 2

Chapter 2 has few typographical errors for which the following corrections are provided.

On page 39 (page 3 of the paper), right column, 2nd paragraph (2.5. Activation of RAW 264.7 Macrophages);

Incorrect: is

Correct: was

On page 40 (page 5 of the paper), left column, 4th paragraph (2.9. Data presentation and Analysis), line 6;

Incorrect: In addition, linear relationships and significance tests of these data sets were also conducted.

Correct: Added mistakenly, should be omitted.

Incorrect: growth curve analysis in dose-dependent experiments

Correct: sigmoidal dose-response experiments

On page 40 (page 5 of the paper), right column, 3rd paragraph (3. Results and Discussion), line 5 and 2nd last line;

Incorrect: are

Correct: were

On page 41 (page 6 of the paper), right column, paragraph 2 (4. Conclusion), last sentence;

Incorrect: Purification and structure identification of the most these extracts are currently underway.

Correct: Purification and structure identification of the most active compounds are currently underway.

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Research Article

Medicinal Plants of the Australian Aboriginal Dharawal People Exhibiting Anti-Inflammatory Activity

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Chronic inflammation contributes to multiple ageing-related musculoskeletal and neurodegenerative diseases, cardiovascular diseases, asthma, rheumatoid arthritis, and inflammatory bowel disease. More recently, chronic neuroinflammation has been attributed to Parkinson's and Alzheimer's disease and autism-spectrum and obsessive-compulsive disorders. To date, pharmacotherapy of inflammatory conditions is based mainly on nonsteroidal anti-inflammatory drugs which in contrast to cytokine-suppressive anti-inflammatory drugs do not influence the production of cytokines such as tumour necrosis factor-α or nitric oxide. However, their prolonged use can cause gastrointestinal toxicity and promote adverse events such as high blood pressure, congestive heart failure, and thrombosis. Hence, there is a critical need to develop novel and safer nonsteroidal anti-inflammatory drugs possessing alternate mechanism of action. In this study, plants used by the Dharawal Aboriginal people in Australia for the treatment of inflammatory conditions, for example, asthma, arthritis, rheumatism, fever, oedema, eye inflammation, and inflammation of bladder and related inflammatory diseases, were evaluated for their anti-inflammatory activity in vitro. Ethanolic extracts from 17 *Eucalyptus* spp. (Myrtaceae) were assessed for their capacity to inhibit nitric oxide and tumor necrosis factor-α production in RAW 264.7 macrophages. *Eucalyptus benthamii* showed the most potent nitric oxide inhibitory effect (IC_{50} 5.57 ± 1.4 μg/mL), whilst *E. bosistoana, E. botryoides, E. saligna, E. smithii, E. umbra, and E. viminalis* exhibited nitric oxide inhibition values between 7.58 and 19.77 μg/mL.

1. Introduction

Inflammation is an important biological process and is essential to maintain the body's homeostasis, to fight against pathogens effectively, and to repair the damaged tissue [1]. However when uncontrolled and chronic, inflammation gives rise to a number of (often age related) diseases including asthma, rheumatoid arthritis, inflammatory bowel disease, Crohn's disease, and tendonitis. Furthermore, a chronic inflammatory response with accompanying oxidative stress is a significant force driving the progression of peripheral diseases like atherosclerosis, diabetes, and metabolic syndrome, as well as neurodegenerative diseases such as multiple sclerosis, Parkinson's disease, and Alzheimer's disease [2–5].

While some chronic/remitting neurological diseases, such as multiple sclerosis, have long been recognized as inflammatory, the term "neuroinflammation" is now applied to chronic activation of microglia and astroglia that do not reproduce the classic characteristics of inflammation in the periphery but may cause neurodegeneration [6–8]. Some examples of diseases characterized by neuroinflammation are Alzheimer's disease (AD) and Parkinson's disease and even autism-spectrum and obsessive-compulsive disorders [9–12]. Microglial and astroglial activation, accompanied by increased levels of proinflammatory mediators such as TNF-α, IL-1β and IL-6, prostaglandins, and reactive oxygen and nitrogen species, as well as reactive carbonyl species and advanced glycation end products, is observed in the AD brain at all stages of the disease [13–18]. Genetic and pharmacoepidemiological studies also point to the importance of inflammation in AD. For example, three immune-relevant genes were shown to be associated with an increased risk of

AD; these are CLU (clusterin), CR1 (complement receptor 1), and TREM2 (triggering receptor expressed on myeloid cells 2) [19].

Consequently, targeting chronic neuroinflammation, for example, with plant-derived anti-inflammatory compounds, has been suggested as a promising disease-modifying treatment for many neurodegenerative diseases including AD [12, 20–27].

At present, both steroidal and nonsteroidal anti-inflammatory drugs (NSAIDs) are used to treat inflammation. NSAIDs in particular can cause severe side effects, most importantly gastric ulcers. NSAIDs are specifically designed as inhibitors of cyclooxygenase (COX) enzymes and, in contrast to CSAIDs, do not influence the production of proinflammatory cytokines such as TNF-α or free radicals such as nitric oxide [28]. CSAIDs specifically target p38 MAPK and NF-κB signalling pathways to inhibit cytokine-mediated events with demonstrated efficacy in a range of animal models [29, 30].

Activated inflammatory cells produce a variety of chemokines and cytokines, reactive oxygen species (ROS), reactive nitrogen species (RNS), free radicals, and prostaglandins [31–33] and cease to produce neuroprotective factors such as glutathione [6, 7].

Excessive production of inflammatory cytokines and reactive radical species can damage cellular biomolecules like proteins, lipids, and carbohydrates as well as nucleic acids, leading to cellular and tissue damage, which further perpetuates the inflammatory cascade. Therefore, pharmacological compounds with the ability to attenuate the production of these inflammatory molecules may have potential for the treatment of many inflammatory diseases including AD [21, 22, 28, 34, 35].

The use of natural substances, especially those derived from plants, in order to prevent, manage, or cure diseases is a centuries-old practice which has led to the discovery of many modern pharmaceuticals. In recent years, the search for novel anti-inflammatory drugs from a wide range of medicinal plant resources has been intensified, and a variety of plant secondary metabolites including apigenin, curcumin, cinnamaldehyde, and resveratrol have already been found to suppress inflammatory responses [21, 22, 28].

For example, turmeric (*Curcuma longa*) and its main ingredient curcumin, which has long been used for treatment of rheumatic disorders, exerts both anti-inflammatory and antiatherosclerotic effects [23, 36]. Ginger extract (*Zingiber zerumbet*) and its main active compound, 3-*O*-methyl kaempferol, significantly attenuated carrageenan-induced mouse paw oedema in an in vivo model and were also found to inhibit the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2), as well as iNOS expression in a cell culture model. Aqueous and hydroalcoholic as well as ethanolic extracts from another ginger species (*Zingiber officinale*) demonstrated significant anti-inflammatory activity and its active constituent [6] gingerol again showed anti-inflammatory activity by inhibiting the production of NO and PGE_2 [37] and was also successful in inhibiting carrageenan-induced rat paw oedema [38].

Triterpenoid saponins, from the Australian desert tree *Acacia victoriae,* have shown anti-inflammatory effects *via* inhibiting activation of NF-κB, by preventing its nuclear localization and inhibiting its ability to bind to DNA [39]. Another Australian indigenous plant *Tinospora smilacina* is claimed to possess long chain unsaturated fatty acids which possess anti-inflammatory properties [40]. The fruits of the Australian native Kakadu plum (*Terminalia ferdinandiana*), Illawarra plum (*Podocarpus elatus*), and Native currant (*Acrotriche depressa*) also exhibited significant anti-inflammatory activity [41].

There is large scope to investigate Australian native plants for their bioactivity and chemical constituents [42]. Traditional medicine is still practised by the many tribal Aboriginal people, particularly in Central and Northern Australia and this ethnomedicinal knowledge is recorded in some cases [43]. The "Dharawal Pharmacopeia" written by botanist and Aboriginal Elder Frances Bodkin (known as Aunty Fran) is a compilation of the Aboriginal medicinal and ceremonial uses (and corresponding taxonomic identification) of thousands of native Australian plants. Of interest to our research, a number of plant species described in the Dharawal pharmacopeia have been claimed to possess anti-inflammatory activities (Table 1) [44, 45]. Plants from *Eucalyptus* species have special importance for the Dharawal indigenous people and are used for their anti-inflammatory activity along with other medicinal uses as well as for shelter and weapons. As stated in the Dharawal pharmacopeia, Eucalypts are mostly distributed in Blue Mountains, Southern Highlands, Woronora Plateau, and coastal area of New South Wales, Australia.

The aim of our research is to evaluate the anti-inflammatory activity of Australian native plants with ethnopharmacological importance and subsequently characterise the bioactive components. In this manuscript, dried extracts from 17 *Eucalyptus* spp. were evaluated for anti-inflammatory activity *via* the suppression of NO and TNF-α production induced by lipopolysaccharide (LPS) and interferon gamma (IFN-γ) in RAW 264.7 cells. Cytotoxicity of the crude extracts was also examined using an Alamar blue cell viability assay.

2. Materials and Methods

2.1. Plant Material. Plants known to be used by the Dharawal people (also known as Tharawal) to treat inflammation and related illnesses were selected under the guidance of botanist and Aboriginal Elder Auntie Fran (Frances Bodkin) and the Dharawal pharmacopeia. Leaf material of 17 *Eucalyptus* spp. was collected in the month of August, 2015 from the "Australian Botanic Gardens" at Mount Annan, NSW, Australia (Table 1).

2.2. Chemicals and Reagents. Ethanol was purchased from Chem-Supply (Gillman, SA, Australia); bovine serum albumin, lipopolysaccharide (*E. coli* serotype-0127:B8), EDTA, *N*-(1-napthyl) ethylenediamine dihydrochloride, benzylpenicillin G sodium salt, resazurin sodium salt (10%), streptomycin, sulphanilamide, 3,3′,5,5′-tetramethylbenzidine (TMB), trypan blue, and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma-Aldrich (Castle Hill,

Table 1: Plants collected for the study of anti-inflammatory activity.

Number	Plant	APNI name	Family	Voucher number
(1)	*Eucalyptus acmenoides*	*Eucalyptus acmenoides* Schauer	Myrtaceae	961604
(2)	*Eucalyptus benthamii*	*Eucalyptus benthamii* Maiden & Cambage	Myrtaceae	832452
(3)	*Eucalyptus bosistoana*	*Eucalyptus bosistoana* F. Muell.	Myrtaceae	20070782
(4)	*Eucalyptus botryoides*	*Eucalyptus botryoides* Sm.	Myrtaceae	861776
(5)	*Eucalyptus eximia*	*Eucalyptus eximia* Schauer	Myrtaceae	841857
(6)	*Eucalyptus globoidea*	*Eucalyptus globoidea* Blakely	Myrtaceae	873240
(7)	*Eucalyptus gummifera*	*Eucalyptus gummifera* (Gaertn.) Hochr.	Myrtaceae	892074
(8)	*Eucalyptus maculata*	*Eucalyptus maculata* Hook.	Myrtaceae	20070782
(9)	*Eucalyptus notabilis*	*Eucalyptus notabilis* Maiden	Myrtaceae	20020217
(10)	*Eucalyptus paniculata*	*Eucalyptus paniculata* Sm.	Myrtaceae	840775
(11)	*Eucalyptus pilularis*	*Eucalyptus pilularis* Sm.	Myrtaceae	861796
(12)	*Eucalyptus punctata*	*Eucalyptus punctata* DC.	Myrtaceae	861820
(13)	*Eucalyptus resinifera*	*Eucalyptus resinifera* Sm.	Myrtaceae	911862
(14)	*Eucalyptus saligna*	*Eucalyptus saligna* Sm.	Myrtaceae	872719
(15)	*Eucalyptus smithii*	*Eucalyptus smithii* R. T. Baker	Myrtaceae	361827
(16)	*Eucalyptus umbra*	*Eucalyptus umbra* R. T. Baker	Myrtaceae	900782
(17)	*Eucalyptus viminalis*	*Eucalyptus viminalis* Labill.	Myrtaceae	861830

NSW, Australia). GIBCO, fetal bovine serum (FBS), and glutamine were purchased from Life Technologies (Mulgrave, VIC, Australia). Murine interferon-γ (IFN-γ) and TNF-α ELISA kits were purchased from PeproTech Asia (Rehovot, Israel). Citric acid and monosodium dihydrogen carbonate (NaH_2CO_3) were from AJAX Chemicals (Auburn, NSW, Australia). Tween-20 was from Amresco (Solon, Ohio, USA). Methanol, monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), sodium chloride (NaCl), and sulfuric acid (H_2SO_4) were from Merck (Darmstadt, Germany). Sodium carbonate (Na_2CO_3) was BDH brand supplied by Merck Pty. Ltd. (Kilsyth, VIC, Australia).

2.3. Extraction of Plants Leaves for Biological Assays and HPLC and MS Analysis. Approximately 40 g of fresh leaf material from each plant was extracted using absolute ethanol. The leaves were first cut into small pieces with scissors and then ground to a coarse powder using a hand blender. The coarse powder was filled into the thimbles of an accelerated solvent extraction system (Buchi B-811, Switzerland) and then extracted under standard soxhlet mode (for 2 × 15 minutes cycles). The volume of the extracts was reduced to ca. 2–4 mL using a rotary evaporator and then evaporated to dryness with nitrogen gas for biological assays. Percentage yields (g/g% fresh weight) are recorded in Table 2.

2.4. Maintenance and Preparation of RAW 264.7 Macrophages. RAW 264.7 macrophages were grown in 175 cm^2 culture flasks on DMEM (Dulbecco's Modified Eagle's Medium) containing 5% FBS (fetal bovine serum) that was supplemented with antibiotics (1%) and glutamine (1%). The cell line was maintained in 5% CO_2 at 37°C, with media being replaced every 3-4 days. Once cells had grown to confluence in the culture flask, they were removed using a rubber policeman cell scraper, as opposed to using trypsin, which can remove membrane-bound receptors such as RAGE. The cell suspension was concentrated by centrifugation for 3 min at 900 rpm and resuspended in a small volume of fresh DMEM (with 1% antibiotics and 5% FBS). Cell densities were estimated using a Neubauer counting chamber. Cell concentration was adjusted with DMEM (with 1% antibiotics and 5% FBS) to obtain 60000 cells/100 μL cell suspension. The 100 μL cell suspension was then dispensed into the inner wells of 96-well plates. Plates were incubated at 37°C and 5% CO_2 for 18 h before the activation experiments were carried out.

2.5. Activation of RAW 264.7 Macrophages. From each well, the media were removed and replaced with fresh DMEM containing 0.1% FBS. For assays with extracts, a 90 μL volume of the dilutions in DMEM (with 0.1% FBS) was added an hour prior to addition of the activator. Due to the often inconsistent nature of LPS at activating cells, a combination of LPS (10 μg/mL) and IFN-γ (10 U/mL), both in DMEM (with 0.1% FBS), was used for activation. A maximum dose of the extracts used is 900 μg/mL and diluted serially by 50% up to a minimum of 10 doses (900, 450, 225, 112.5, 56.25, 28.125, 14.062, 7.031, 3.515, 1.7578, and 0.8789 μg/mL in the wells, resp.). After activation, the cells were incubated for 24 h at 37°C and 5% CO_2 and then NO and TNF-α inhibition and cell viability were determined. Cells with media alone were used as negative control and activated cells used as positive control.

2.6. Determination of Nitric Oxide Production by Griess Assay. Nitric oxide was determined by Griess reagent quantification of nitrite, one of its stable reaction products. Griess reagent was freshly made up of equal volumes of 1% sulfanilamide and 0.1% naphthylethylene-diamine in 5% HCl. In the presence of nitrite this reagent forms a violet colour. From each well, 50 μL of supernatant was transferred to a fresh 96-well plate and mixed with 50 μL of Griess reagent, and the colour produced was measured at 540 nm in a microplate

Table 2: Plant common names, ethnomedicine, and yields of ethanolic extracts for the study of anti-inflammatory activity.

Plant species	Common name(s)	Diseases treated using leaves (according to Dharawal Aboriginal medicinal use)	Yield of ethanol extract (%)
Eucalyptus acmenoides	White mahogany/yellow stringybark	Breathing difficulties, chest and muscle pain, fever, and wash for joints	19.2
Eucalyptus benthamii	Camden white gum	Colds, fever, chest and muscle pain, and wash for joints	12.6
Eucalyptus bosistoana	Coastal grey box	Colds, fever, chest and muscle pain, and wash for joints	11.6
Eucalyptus botryoides	Bangalay/southern mahogany	Colds, fever, chest and muscle pain, and wash for joints	41.6
Eucalyptus eximia	Yellow bloodwood	Colds, fever, chest and muscle pain, wash for joints, extreme diarrhea, and syphilitic sores	25.8
Eucalyptus globoidea	White stringybark	Breathing difficulties, chest and muscle pain, fever, and wash for joints	26.2
Eucalyptus gummifera	Red bloodwood/bloodwood	Colds, fever, chest and muscle pain, and wash for joints	15.4
Eucalyptus maculata	Spotted gum	Asthma, colds, fever, chest and muscle pain, and wash for joints	12.6
Eucalyptus notabilis	Mountain mahogany	Colds, fever, chest and muscle pain, wash for joints, and extreme diarrhea	11.0
Eucalyptus paniculata	Grey ironbark	Asthma, morning sickness	16.4
Eucalyptus pilularis	Blackbutt	Colds, fever, chest and muscle pain, and wash for joints	23.2
Eucalyptus punctata	Grey gum	Breathing difficulties, stomach upset, and morning sickness	14.0
Eucalyptus resinifera	Red mahogany	Colds, fever, chest and muscle pain, wash for joints, and extreme diarrhea	12.0
Eucalyptus saligna	Sydney blue gum	Colds, fever, chest and muscle pain, and wash for joints	10.8
Eucalyptus smithii	Gully gum/blackbutt peppermint	Colds, fever, chest and muscle pain, and wash for joints	13.3
Eucalyptus umbra	Broad leafed white mahogany/white mahogany	Colds, fever, chest and muscle pain, wash for joints, and extreme diarrhea	10.5
Eucalyptus viminalis	Manna gum/ribbon gum/white gum	Colds, fever, chest and muscle pain, and wash for joints	19.0

reader (Bio-Rad, Australia). The remaining supernatant from each well was used for a TNF-α assay using commercial sandwich ELISA development kits (catalog number: 900-K54; PeproTech, USA).

2.7. Determination of Cell Viability by Alamar Blue Assay. The Alamar Blue assay is a colorimetric assay involving the cellular reduction of resazurin to resorufin. Alamar Blue solution [100 μL of 10% Alamar Blue (resazurin) in DMEM medium] was added to each well and incubated at 37°C for 1-2 h. After incubation, fluorescence was measured (excitation at 530 nm and emission at 590 nm) using a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia) and expressed as a percentage of that in control wells after background fluorescence was subtracted.

2.8. TNF-α Determination by ELISA. The supernatants obtained from each well (remaining supernatant after 24 hours of activation) were diluted 30 times using diluent (0.1% w/v bovine serum albumin and 0.05% v/v tween-20 in PBS [1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, and 154 mM NaCl; pH 7.4]) and were used for determination of TNF-α using a commercial sandwich ELISA (catalog number: 900-K54; Peprotech, USA) according to the manufacturer's protocol. Capture antibody was used at a concentration of 1.25 μg/mL in PBS. To make a standard curve TNF-α (10 ng/mL standard) was diluted serially by 50% up to a minimum of 10 doses (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019, and 0.0097 ng/mL in the wells, resp.) and was used as the internal standard. TNF-α was detected with a biotinylated second antibody and an avidin peroxidase conjugate with TMB as detection reagent. After ~30 min, the reaction was stopped using 0.5 M sulfuric acid, and the absorbance was measured at 450 nm of measurement filter with a 655 nm of reference filter. The absorbance data was expressed as a percentage of that in control wells after conversion of the concentrations by using a standard curve constructed with defined concentrations of TNF-α. Curve fitting of this standard curve and extrapolation of experimental data were performed using nonlinear regression analysis.

2.9. Data Presentation and Analysis. As the experiments were done in triplicates, the results were expressed as the mean ± SEM. In addition, linear relationships and significance tests of these data sets were also conducted. GraphPad Prism version 6.01 (GraphPad Software Incorporated, USA) was used for growth curve analysis in dose-dependent experiments and to determine the IC_{50} values for NO and TNF-α inhibition as well as LC_{50}.

3. Results and Discussion

In this study, leaves from 17 different *Eucalyptus* spp. were collected in the month of August, 2015. Approximately 40 g of leaves from each of *Eucalyptus acmenoides, E. benthamii, E. bosistoana, E. botryoides, E. eximia, E. globoidea, E. gummifera, E. maculate, E. notabilis, E. paniculata, E. pilularis, E. punctate, E. resinifera, E. saligna, E. smithii, E. umbra, and E. viminalis* were extracted using absolute ethanol (Table 2).

The RAW 264.7 murine macrophages release NO and TNF-α when exposed to bacterial LPS and IFN-γ and on this principle, has become an established experimental model to evaluate in vitro anti-inflammatory activity of extracts [28]. For the purpose of interpretation, the IC_{50} values of NO inhibition are divided into three groups: extracts with IC_{50} < 20 μg/mL are considered as highly potent extracts; a value between 21 and 80 μg/mL is considered as moderately potent, and an IC_{50} < 80 μg/mL is considered as an extract with low potency.

The highest concentration of ethanolic crude extract tested in the anti-inflammatory assay was 900 μg/mL with 0.5-fold serial dilutions. *Eucalyptus benthamii, E. bosistoana, E. botryoides, E. saligna, E. smithii, E. umbra, and E. viminalis* leaf extracts showed the highest activity for NO inhibition with IC_{50} values of 5.57, 7.58, 16.65, 19.77, 17.62, 17.69, and 8.0 μg/mL, respectively (Table 3, Suppl. Figure 1). The extracts from *Eucalyptus acmenoides, E. eximia, E. notabilis,* and *E. pilularis* showed moderate inhibition of NO with IC_{50} values of 56.93, 34.14, 53.84, and 76.17 μg/mL, respectively. Six other species, *E. globoidea, E. gummifera*, *E. maculata*, *E. paniculata, E. punctata, and E. resinifera*, presented low inhibition of NO with IC_{50} values of 82.9, 108.17, 99.94, 130.7, 120.4, and 81.21 μg/mL, respectively (Suppl. Figure 1).

The plant extracts also showed promising TNF-α inhibitory activity (Table 3) with IC_{50} values of 2.06, 8.53, 19.02, 3.41, 2.41, 10.2, and 16.68 μg/mL for *E. benthamii, E. bosistoana, E. botryoides, E. saligna, E. smithii, E. umbra, and E. viminalis*, respectively, which are the same plants in our highly potent NO inhibitor group. On the other hand, the moderately potent extracts from *E. acmenoides, E. eximia, E. notabilis, and E pilularis* showed TNF-α IC_{50} values of 16.53, 4.82, 27.48, and 21.09 μg/mL, respectively (Suppl. Figure 1), whereas extracts from *E. globoidea, E. gummifera, E. maculata, E. paniculata, E. punctata, and E. resinifera* exhibited comparatively lower inhibition of TNF-α production with IC_{50} values of 50.73, 82.73, 136.34, 334.86, 115.73, and 62.11 μg/mL, respectively, which are the plants in our low potency group (Suppl. Figure 1).

The use of Alamar Blue (resazurin) to measure cytotoxicity is an established technique [46]. The results of cytotoxicity (LD_{50}) of our leaf extracts are shown in Table 3. The plants of our highly potent group were also relatively toxic with LC_{50} values of 22.34, 37.17, 108.40, 101.01, 38.96, 236.5, and 31.92 for *E. benthamii, E. bosistoana, E. botryoides, E. saligna, E. smithii, E. umbra*, and *E. viminalis*, respectively, whereas, plants of the lower potency group showed lower toxicity with higher LD_{50} values of 464.74, 313.45, 540.46, 268.59, 522.84, and 268.59 for *E. globoidea, E. gummifera*, *E. maculata*, *E. paniculata, E. punctata*, *and E. resinifera*, respectively. Plants with moderate potency showed a wide range of cytotoxicity with LD_{50} values of 296.22, 64.14, 332.44, and 374.74 for *E. acmenoides, E. eximia, E. notabilis,* and *E. pilularis*, respectively (Suppl. Figure 1).

In future experiments, we will purify the most potent extracts to identify the most active compounds. One major candidate for carrying the anti-inflammatory activity could be 1,8- cineole, the major monoterpene of eucalyptus oil, as it can represent between 60 and 80% of the volatile oils

Table 3: Anti-inflammatory activity and toxicity of extracts determined in RAW 264.7 macrophages.

Plant species	Inhibition of NO production (IC_{50} in μg/mL)	Inhibition of TNF-α production (IC_{50} in μg/mL)	Cytotoxicity (LC_{50} in μg/mL)
Eucalyptus acmenoides	56.93 ± 11.8	16.53 ± 5.9	296.22 ± 189.3
Eucalyptus benthamii	5.57 ± 1.4	2.06 ± 0.7	22.34 ± 9.3
Eucalyptus bosistoana	7.58 ± 1.2	8.53 ± 3.4	37.17 ± 15.6
Eucalyptus botryoides	16.65 ± 2.2	19.02 ± 5.4	108.40 ± 44.9
Eucalyptus eximia	34.14 ± 7.1	4.82 ± 1.6	64.14 ± 23.6
Eucalyptus globoidea	82.9 ± 12.5	50.73 ± 24.0	464.74 ± 199.7
Eucalyptus gummifera	108.17 ± 10.5	82.73 ± 52.3	313.45 ± 125.9
Eucalyptus maculata	99.94 ± 12.1	136.34 ± 78.8	110.22 ± 41.1
Eucalyptus notabilis	53.84 ± 7.7	27.48 ± 14.9	332.44 ± 107.5
Eucalyptus paniculata	130.7 ± 11.6	334.86 ± 192.7	540.46 ± 172.5
Eucalyptus pilularis	76.17 ± 10.3	21.09 ± 9.7	374.74 ± 190.7
Eucalyptus punctata	120.4 ± 15.9	115.73 ± 58.4	522.84 ± 221.4
Eucalyptus resinifera	81.21 ± 13.4	62.11 ± 36.0	268.59 ± 131.6
Eucalyptus saligna	19.77 ± 2.3	3.41 ± 1.3	101.01 ± 36.8
Eucalyptus smithii	17.62 ± 3.5	2.41 ± 1.1	38.96 ± 14.1
Eucalyptus umbra	17.69 ± 2.3	10.2 ± 4.5	236.5 ± 144.3
Eucalyptus viminalis	8.0 ± 1.2	16.68 ± 9.9	31.92 ± 11.9

Note. Results represent the mean ± SEM of 3 experiments in triplicate for NO production and cytotoxicity whereas for TNF-α production it is 1 experiment in triplicate.

derived from eucalyptus leaves depending on the species. Therapeutic concentrations of 1,8-cineol (1.5 μg/mL = 10^{-5}M) inhibited significantly cytokine production in lymphocytes and monocytes [47, 48]. It has to be noted that 1,8-cineol has already gained market acceptance for its anti-inflammatory properties in mouthwashes and cough suppressants or anti-asthmatic medications [48, 49].

The plants studied here were chosen on the basis of their traditional use to treat inflammatory conditions by the Dharawal people of the Campbelltown region (South-west Sydney Australia). All of the plants showed anti-inflammatory activity and demonstrated inhibitory effect on downregulation of NO and TNF-α production with varying potencies, which supports their use in traditional Aboriginal medicine. The content of the anti-inflammatory compounds in the plants, according to traditional knowledge, is also dependent on the plant's environment. In Dharawal country, what is most important when seeking particular medicines from plants is where the plant is growing, that is, not so much the soils, but the other plants that are growing around the particular plant required. For instance, with the Eucalypts, close proximity of an Ironbark (Muggago) and a Ribbon bark (Kai'yeroo) is needed for the anti-inflammatory medicine from the Burringoa (*Eucalyptus tereticornis*) to be most effective. As another example, the Ironbark itself does not need other Eucalypts close by, but it does need the Einadia (one of the saltbushes) to be growing at its base. In addition, if it had been struck by lightning (and this can be confirmed by a line of interrupted bark running from the top of the tree almost to its base), then the anti-inflammatory medicine would be most effective, when using the leaves of the Eucalypts as medicine, the leaves of the trees younger than 7 years were placed on a low fire and the smoke inhaled. However, when the tree is bearing the mature leaves, the leaves were collected and boiled then allowed to cool before being rubbed on the affected part of the body, depending on the species. For the present screening study, the plant material was provided by the Botanical Gardens from random trees in the garden, but for future studies, we will investigate if collection practice based on Dharawal knowledge will improve the inherent activity and/or yield of the anti-inflammatory compounds.

4. Conclusions

The present study suggests that most of the *Eucalyptus* spp. potentially possess interesting anti-inflammatory compounds with low toxicity and the in vitro activity appears to support the traditional use. *Eucalyptus benthamii, E. bosistoana, E. botryoides, E. saligna, E. smithii, E. umbra, and E. viminalis* leaf extracts exhibited strong anti-inflammatory activity by inhibiting NO and TNF-α production in LPS and INF-γ stimulated RAW 264.7 macrophages. Purification and structure identification of the most these extracts are currently underway.

Competing Interests

The authors declare that they have no competing interests.

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Ethical engagement of Aunty Bodkin with Western Sydney University (WSU)

In 2012, Frances Bodkin (Auntie Fran - a D'harawal elder), through the D'harawal Traditional Descendents' and Knowledgeholders' Circle (DTDKC) donated the D'harawal Pharmacopeia to Western Sydney University (WSU), Campbelltown campus, specifically for utilisation in teaching and learning within the Mangamai'bangawarra: Indigenous Science course. This remains a particularly unique circumstance and rarely observed, where cultural traditional knowledge is donated to an institution. A key determinant influencing the decision made by the DTDKC to donate the knowledge to WSU is it was considered the best way to protect the knowledge (in the present state of the law), by entrusting it to the one university situated on the land of their ancestors and knowledge. In doing so, it was requested that if any (economic) benefit is derived from the knowledge, that it be used to allow Aboriginal students born of D'harawal Lands to attend WSU to study science (for example, through scholarships).
A letter provided to the university from the D'harawal Traditional Descendents' and Knowledgeholders' Circle outlines their endorsement of Auntie Fran 'to advise and assist the group in their search for greater knowledge of Aboriginal medicines', entrusting the knowledge to the university as the best means of protecting the knowledge.

Chapter 3

A New Anti-inflammatory Chromone from the Leaves of *Eucalyptus viminalis* (Publication-2)

Preface to Chapter 3

This chapter is a previously published work (Akhtar M. A.; Münch G.; Bodkin F. and. Raju R (A new anti-inflammatory chromone from the leaves of *Eucalyptus viminalis*. *Natural Product Communications*. 2018, 13(10), 1297-1300). Contributions of co-authors of the article are described as follows in the order of names listed. The author of this dissertation (Most. Afia Akhtar), completed extractions and fractionation of the plant samples, performed anti-inflammatory bioassays and structural elucidation as well as prepared the manuscript. Mrs. Frances Bodkin guided plant selection. Dr. Gerald Münch was a mentor of this work. Dr. Ritesh Raju ran NMR of the purified samples, participated in manuscript preparation and is the corresponding author for the published article.

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A New Anti-inflammatory Chromone from the Leaves of *Eucalyptus viminalis*

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A new 5,7-dihydroxychromone compound (**1**) was isolated from the leaf extract of *Eucalyptus viminalis* (Myrtaceae), along with two known compounds (**2** and **3**). Their structures were elucidated on the basis of mass and NMR spectroscopic data analysis. Compound **1** showed moderate anti-inflammatory activity by down-regulating nitric oxide in LPS (Lipopolysaccharide) and IFN-γ (Interferon gamma) treated RAW 264.7 macrophages and N-11 microglial cells with an IC_{50} value for nitric oxide downregulation of 44.0 ± 9.0 µg/mL and 43.4 ± 7.3 µg/mL respectively. Compound **1** was non-toxic to RAW macrophage and N-11 microglial cells (>36 µg/mL).

Keywords: *Eucalyptus*, Anti-inflammatory, Glucopyranoside, Chromone.

Native to Australia, the genus *Eucalyptus* (family Myrtaceae) represents more than 700 species and is distributed throughout the world. This genus is a fast growing tree and has long been used in folk medicine. Eucalypts are rich in variety of essential oils[1-6]. Modern pharmaceutical, food and cosmetic industries also use essential oils from *Eucalyptus* species [7-11].

Plants from *Eucalyptus* species have special importance for the D'harawal indigenous people of Australia and are used for their anti-inflammatory activity along with other medicinal uses as well as for shelter and weapons. *Eucalyptus viminalis* are mostly distributed in Blue Mountains, Southern Highlands, Woronora Plateau, and across the coastal area of New South Wales, Australia [12]. In a recent study sixty six compounds were identified in the oil of *E. viminalis* leaves, the major compounds being 1,8-cineole, α-pinene, limonene and globulol by hydro-distillation and GC/MS [13]. The essential oil from *E. viminalis* has also been investigated for insecticidal and repellent activities [11, 14, 15]. Leaf extracts from *E. viminalis* have also known to demonstrate significant antimicrobial activities [8]. Euvimals derived from *E. viminalis* exhibited protonophoric activity which is considered to be the reason of their cytotoxic and antimicrobial activity [16].

In our previous study, *E. viminalis* leaf extract exhibited strong anti-inflammatory activity by inhibiting NO (nitric oxide) and TNF-α (tumor necrosis factor-α) production in LPS (lipopolysaccharide) and INF-γ (interferon gamma) stimulated RAW 264.7 macrophages indicating the potential of this plant to possess interesting anti-inflammatory compounds with low toxicity [12].

As part of our ongoing investigations into the discovery of new anti-inflammatory metabolites from plants we discovered the new chromone compound (**1**).

Fresh leaves of *E. viminalis* were extracted sequentially using a range of different solvents (dichloromethane, ethyl acetate, ethanol, methanol and finally water). Immediately after the initial stages of sequential fractionation, each corresponding fraction was subjected to anti-inflammatory screening using the inhibition of

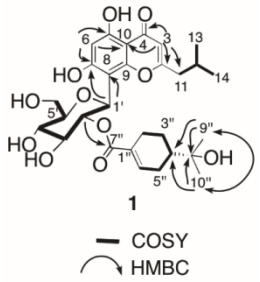

Figure 1: Key 2D NMR correlations for **1**.

NO and TNF-α in LPS and IFN-γ treated RAW 264.7 macrophages following Griess test and TNF-α Elisa. Based on the bioactivity guided fractionation the most potent fraction was determined to be the ethyl acetate fraction which was subsequently further purified by semi-preparative reversed phase HPLC yielding compounds **1** (t_R = 18.6 min, 4.0 mg), **2** (t_R = 15.7 min, 1.1 mg) and **3** (t_R = 19.8 min, 1.0 mg).

HRESI(+)MS analysis of **1** revealed a pseudomolecular ion ($[M+H]^+$) indicative of a molecular formula $C_{29}H_{38}O_{11}$ requiring 11 double bond equivalents. The ^{1}H NMR spectrum (Figure 1, Table 1) exhibited two singlet resonances for H-3 (δ_H 6.07, δ_C 109.1) and H-6 (δ_H 6.14, δ_C 98.8), the first of which showed HMBC correlations to several quaternary carbons C-2 (δ_C 171.5), C-4 (δ_C 184.1), C-10 (δ_C 105.4) and a methylene C-11 (δ_c 44.3). The second showed HMBC correlations to additional quaternary carbons C-5 (δ_C 162.7), C-7 (δ_C 163.8), C-8 (δ_C 103.6) and to C-10. The following correlations suggested the likely existence of a 5,7-dihydroxychromone system. COSY correlations from the methylene H_2-11 extending through to the methine H-12 (δ_H 2.36) and two sets of secondary methyl groups confirmed the attachment of the isobutyl unit at C-2.

A comprehensive presence of oxymethines from H-1′ to H-5′ terminating of at the hydroxy methylene H-6′ prompted the presence of a sugar residue. Interestingly, the lack of an anomeric

Table 1: NMR (400 MHz, MeOH-d_4) data for **1**.

Position	δ_H, (m, *J* in Hz)	δ_C*	COSY	HMBC
2		171.5		
3	6.07, s	109.1		2, 4, 5, 10, 11
4		184.1		
5		162.7		
6	6.14, s	98.8		5, 7, 8, 10
7		163.8		
8		103.6		
9		158.8		
10		105.4		
11	2.63, 2.57, m	44.3	12	2, 3, 12, 13, 14
12	2.36, m	28.1	11, 13, 14	11, 13, 14
13	1.09, d (*6.7*)	22.8	12	11, 12, 14
14	1.04, d (*6.7*)	22.9	12	11, 12, 13
1′	5.05, d (*9.7*)	72.5	2′	2′, 3′, 7, 8, 9
2′	5.61, dd (*10.0, 9.7*)	73.7	1′, 3′	8, 1′, 3′, 7″
3′	3.69, dd (*10.0*, *9.9*)	77.8	2′, 4′	1′, 2′, 4′
4′	3.46[a], m	72.6	3′, 5′	
5′	3.45[a], m	82.7	4′, 6′	
6′	3.92, 3.65, m	63.3	5’	
1″		NO		
2″	6.82, s	140.9	3″	
3″	2.24, 1.92, m	28.3	2″, 4″	
4″	1.43, m	45.4	3″, 5″	
5″	2.29, 1.96, m	26.3	4″, 6″	
6″	1.91, m	24.3	5″	
7″		168.0		
8″		72.7		
9″	1.13[b], s	26.7		4”, 8”, 9”
10″	1.13[b], s	26.8		4”, 8”, 9”

[a,b]overlapping signals, *assignments supported by HSQC and HMBC experiments. NO-Not observed.

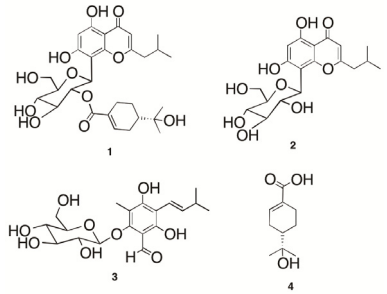

Figure 2: Chemical structures of **1 – 4**.

proton, but the presence of a downfield resonance for H-1′ (δ_H 5.05, δ_C 72.5) coupled with its HMBC correlations to C-7, C-8 and C-9 established the carbon linkage of the sugar residue to the chromone system (Figure 1). The final subunit was identified by a series of COSY and HMBC correlations to confirm the presence of oleuropeic acid (Figure 1. Table 1). A characteristic HMBC correlation from the oxymethine H-2′ to the ester carbonyl C-7″ confirmed the likely attachment of the oleuropeic ester carbonyl to C-2′ of the sugar residue. Further confirmation of the oleuropeic acid moiety presence was determined by performing an acid hydrolysis on **1** to yield oleuropeic acid (**4**), confirmed by HRMS analysis (Figure S11) and the 8-β-glucopyranosyl residue (**2**). The furnished oleuropeic acid exhibited an optical rotation of $[\alpha]_D^{20}$ +68 ($CHCl_3$) the closest comparison was with the reported literature value of oleuropeic acid methyl ester $[\alpha]_D^{20}$ +42.5 ($CHCl_3$) [17], confirming the absolute stereochemistry at C-4″ to be *R.* A relatively low yield of oleuropeic acid (**4**) after acid hydrolysis prevented any further NMR analysis. The relative stereochemistry of H-1′ to H-2′ was determined to be *trans*-1,2-diaxial to each other based on a large coupling of $J_{1',2'}$ (9.7 Hz), while also the large coupling of $J_{2',3'}$ (10.0 Hz) confirmed the *trans*-1,2-diaxial relationship between H-2′ and H-3′. Also observed was a large coupling of $J_{3',4'}$ (9.9 Hz), the coupling between H-4′ and H-5′ was not resolved, however based on the similarity of the glucosyl residue in the known metabolite **2** [18], we propose a *trans*-diaxial orientation between these protons and the identity of the sugar as glucose.

Table 2: NO and TNF-α down regulation and cytotoxicity of compounds (**1 – 4**) in LPS and IFN-γ induced RAW 264.7 macrophages. [Results represent mean ± SEM; 2 experiments as quadruplicate (n=8)].

Compound	Inhibition of NO production (IC_{50} in µg/mL)	Inhibition of TNF-α production (IC_{50} in µg/mL)	Cytotoxicity (LC_{50} in µg/mL)
1	44.0 ± 9.0	41.0 ± 23.8	> 36
2	47.0 ± 7.8	38.3 ± 14.8	> 36
3	37.6 ± 4.9	43.2 ± 18.4	> 36
4	18.7 ± 0.5	21.5 ± 4.4	54.4 ± 21.4
Positive control			
Diclofenac [19]	64.2 ± 12.7	> 99	> 99
Ibuprophen [19]	237.9 ± 27.4	148.9 ± 53.8	> 309
***E*-Cinnamaldehyde[20]**	7.3 ± 1.2	8.3 ± 1.2	157.2 ± 29.8

Compounds **1** – **4** were evaluated for their anti-inflammatory activity by determining the inhibition of NO and TNF-α in both RAW 264.7 macrophage (Table 2) and N-11 microglial cells (Table 3) [18, 21]. Although compounds (**1** – **3**) exhibited moderate anti-inflammatory activities, it was oleuropeic acid [22] (**4**) that displayed the highest potency with an IC_{50} value from 10 – 20 µg/mL (Tables 2 and 3). Our current focus on Australian native plants, and a detailed anti-inflammatory chemical profiling has still to date lead to the discovery of new derivatives, and in the process has led us to evaluate for the first time a comprehensive anti-inflammatory evaluation of a well known molecule, oleuropeic acid.

Table 3: NO and TNF-α down regulation and cytoxicity of compounds (**1 - 4**) in LPS and IFN-γ induced N11 microglia. [Results represent mean ± SEM; 2 experiments as quadruplicate (n=8)].

Compound	Inhibition of NO production (IC_{50} in µg/mL)	Inhibition of TNF-α production (IC_{50} in µg/mL)	Cytotoxicity (LC_{50} in µg/mL)
1	43.4 ± 7.3	20.4 ± 10.5	> 36
2	34.1 ± 4.2	34.3 ± 18.1	>36
3	21.8 ± 3.7	19.0 ± 7.1	> 36
4	10.2 ± 1.0	17.0 ± 5.0	> 22.5

Experimental

General: Acetonitrile, dichloromethane and methanol were supplied by Merck. Ethyl acetate from Fisher Chemical, ethanol from Chem-Supply and water used was Milli-Q water. Bovine serum albumin, lipopolysaccharide (*E. coli* serotype-0127:B8), EDTA, N-(1-napthyl) ethylenediamine dihydrochloride, benzylpenicillin G sodium salt, resazurin sodium salt (10%), streptomycin, sulphanilamide, 3,3′,5,5′-tetramethylbenzidine (TMB), trypan blue and Dulbecco’s Modified Eagle’s Medium (DMEM) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). GIBCO Foetal bovine serum (FBS) and glutamine were purchased from Life Technologies (Mulgrave, VIC, Australia). Murine interferon-γ (IFN-γ) and TNF-α ELISA kits were purchased from PeproTech Asia (Rehovot, Israel). Citric acid and monosodium dihydrogen carbonate (NaH_2CO_3) were from AJAX Chemicals (Auburn, NSW, Australia). Tween-20 was from Amresco (Solon, Ohio, USA). Methanol, monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), sodium chloride (NaCl) and sulfuric acid (H_2SO_4) were from Merck (Darmstadt, Germany). Sodium carbonate (Na_2CO_3) was BDH brand supplied by Merck Pty. Ltd. (Kilsyth, VIC, Australia).

Semi-preparative HPLC was performed using an Agilent 1260 Infinity Series (Santa Clara, CA, USA). NMR spectra were recorded on a Bruker Ascend 400 MHz spectrometer (Bruker Biospin GmbH, Bremen, Germany), in the solvents indicated and referenced to residual ^{1}H signals in deuterated solvents. HRMS was carried out using a Waters Xevo Q-TOF mass spectrometer operating in the positive ESI mode. UV-Vis was measured using Shimadzu model UV-2550, and optical rotation measured on Polax-D, ATAGO system at 22°C.

Fresh leaves of *E. viminalis* (voucher no. 861830) were collected in July, 2016 from the 'Australian Botanic Gardens' at Mount Annan, NSW, Australia. The voucher specimen is deposited at the National Herbarium of NSW, Australia.

Approximately 120 g of fresh leaf material from *E. viminalis* was first cut into small pieces and later ground to a coarse powder using a hand blender. The coarse powder was then extracted using an accelerated solvent extraction system (Buchi B-811, Switzerland) under standard soxhlet mode (for 2 × 15 min cycles) using five different solvents (dichloromethane, ethyl acetate, ethanol, methanol and water) sequentially from low to high polarity. The sequential extracts were then screened for their anti-inflammatory activity. The ethyl acetate extract was found to be most potent and so was subjected directly through reversed phase semi-prep HPLC (phenyl-hexyl column, 250 x 10 mm, Phenomenex) eluting with a gradient starting from 10% acetonitrile/water to a 100% acetonitrile (with a constant 0.01% formic acid modifier) over 30 min and held at 100% acetonitrile for 10 min to yield compounds **1** (t_R = 18.6 min, 4.0 mg), **2** (t_R = 15.7 min, 1.1 mg) and **3** (t_R = 19.8 min, 1.0 mg).

Acid Hydrolysis of 1: A solution of **1** (2.0 mg) in 6 M HCl (2.0 mL) was heated for 3 hours at 70°C. After cooling, sodium hydroxide (2M) was added to the reaction mixture and the products purified using a Kinetex phenyl-hexyl column (250 x 4.6mm, 5µm) eluting with a gradient starting from 10% methanol/water to a 100% methanol (with a constant 0.01% formic acid as modifier) over 5 min and held at 100% methanol for 5 min to yield compound **2** (1.0 mg) and **4** (0.4 mg)

Maintenance and Preparation of RAW 264.7 Macrophages and N11 Microglia: RAW 264.7 macrophages were grown in 175 cm^2 culture flasks on DMEM (Dulbecco's modified eagle's medium) containing 5% FBS (fetal bovine serum) that was supplemented with antibiotics (1%) and glutamine (1%). The cell line was maintained in 5% CO_2 at 37°C, with media being replaced every 3-4 days. Once cells had grown to confluence in the culture flask, they were removed using a rubber policeman cell scraper, as opposed to using trypsin, which can remove membrane-bound receptors such as RAGE. The cell suspension was concentrated by centrifugation for 3 min at 900 rpm and re-suspended in a small volume of fresh DMEM (with 1% antibiotics and 5% FBS). Cell densities were estimated using a Neubauer counting chamber. Cell concentration was adjusted with DMEM (with 1% antibiotic and 5% FBS) to obtain 60000 cells/100 µL cell suspension. The 100 µL cell suspension was then dispensed into the wells of 96-well plates and incubated at 37°C; 5% CO_2 for 18 h before the activation experiments were carried out.

Activation of RAW 264.7 Macrophages and N11 Microglia: From each well, the media was removed and replaced with fresh DMEM containing 0.1% FBS. For assays with extracts (conducted only with RAW 264.7 macrophages), a 90 µL volume of the dilutions in DMEM (with 0.1% FBS) was added an hour prior to addition of the activator. Due to the often inconsistent nature of LPS at activating cells, a combination of LPS (10 µg/mL) and IFN-γ (10 U/mL), both in DMEM (with 0.1% FBS), were used for activation. A maximum dose (36 µg/mL) of the compounds (**1** - **3**) was used, and diluted serially by 50% up to a minimum of 7 doses (36, 18, 9, 4.5, 2.25, 1.12 and 0.56 µg/mL in the wells respectively), whereas a maximum dose of 90 µg/mL was used for compound **4**, and diluted serially by 50% up to a minimum of 7 doses (90, 45, 22.5, 11.25, 5.62, 2.81 and 1.41 µg/mL in the wells respectively). After activation, the cells were incubated for 24 h at 37°C and 5% CO_2 and then NO and TNF-α inhibition, and cell viability was determined. Cells with media alone were used as negative control and activated cells used as positive control.

Determination of Nitric Oxide Production by Griess Assay: Nitric oxide was determined by quantification of nitrite one of its stable reaction products with the Griess reagent (GR). GR was freshly made up of equal volumes of 1% sulfanilamide and 0.1% naphthylethylene-diamine in 5% HCl. In the presence of nitrite this reagent forms a violet colour. From each well, 50 µL of supernatant was transferred to a fresh 96-well plate and mixed with 50 µL of GR, and the colour produced was measured at 540 nm in a microplate reader (Bio-Rad, Australia). The remaining supernatant from each well was used for a TNF-α assay using commercial sandwich ELISA development kits (catalog number: 900-K54; PeproTech, USA).

Determination of Cell Viability of RAW 264.7 Macrophages and N11 Microglia by Alamar Blue Assay: The Alamar Blue assay is a colorimetric assay involving the cellular reduction of resazurin to resorufin. Alamar Blue solution [100 µL of 10% Alamar Blue (Resazurin) in DMEM medium] was added to each well and incubated at 37°C for 1-2 h. After incubation, fluorescence was measured (excitation at 544 nm and emission at 590 nm) using a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia) and expressed as a percentage of that in control wells after background fluorescence was subtracted.

TNF-α Determination by ELISA: The supernatants obtained from each well (remaining supernatant after 24 h of activation) were diluted 30 times using diluent (0.1% w/v bovine serum albumin and 0.05% v/v tween-20 in PBS [1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl; pH7.4]) and were used for determination of TNF-α using a commercial sandwich ELISA (Catalog number: 900-K54; Peprotech, USA) according to the manufacturer's protocol. Capture antibody was used at a concentration of 1.25 µg/mL in PBS. To make a standard curve TNF-α (10 ng/ml standard) was diluted serially by 50% up to a minimum of 10 doses (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019 and 0.0097 ng/mL in the wells respectively) and was used as the internal standard. TNF-α was detected with a biotinylated second antibody and an avidin peroxidase conjugate with TMB as detection reagent. After ~30 min, the reaction was stopped using 0.5 M sulfuric acid, and the absorbance was measured at 450 nm of measurement filter. The absorbance data was expressed as a percentage of that in control wells after conversion of the concentrations by using a standard curve constructed with defined concentrations of TNF-α. Curve fitting of this standard curve and extrapolation of experimental data were performed using non-linear regression analysis.

Data Presentation and Analysis: Two experiments as quadruplicates (n=8) were combined to determine the IC_{50} (for NO and TNF-α inhibition) and LC_{50} (for cell viability) values using the four parameter sigmoidal dose-response function in GraphPad Prism version 6.1 (GraphPad Software, La Jolla, CA, USA).

Compound 1

Yellow solid.

$[\alpha]_D$: + 5.5 (c 0.01, MeOH).

UV/Vis λmax (MeOH) nm (log ε): 228 (4.19), 249 (4.01), 295 (4.08)

^{1}H NMR (400 MHz, DMSO-d_6): Table 1

^{13}C NMR (100 MHz, DMSO-d_6): Table 1

HRMS-TOF: m/z [M + H^+] calcd for $C_{29}H_{39}O_{11}$: 563.2492; found: 563.2498

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Chapter 3a

4-Coumaroyl quinic acid Isolated from *Eucalyptus bosistoana* (Myrtaceae)

3a.1 Introduction

Eucalyptus bosistoana belongs to the family Myrtaceae. As described in the D'harawal pharmacopoeia (unpublished), the plant is commonly known as coastal grey box and is an evergreen tree that grows to a height of 50 m.

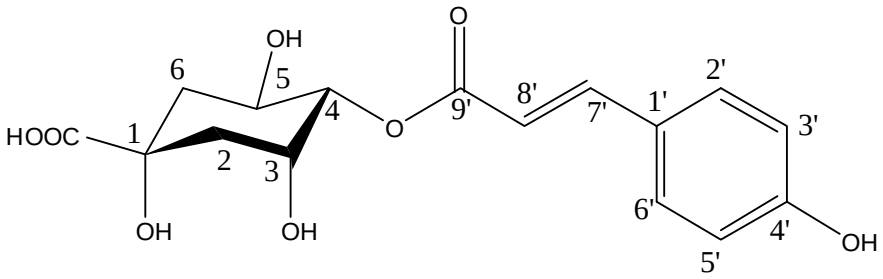
The essential oil samples of *Eucalyptus bosistoana* from Algerian Sahara have been previously investigated and the main constituents shown to be *p*-cymene, cryptone, 1,8-cineole, spathulenol and α-pinene [1].

The plant has a number of medicinal uses according to the D'harawal pharmacopoeia including; i) the nectar was collected by washing the flowers in water until it had become sweet to taste, it was then taken to treat colds and breathing difficulties in the very young and very old, ii) the bark exudate was used to relieve the pain of toothache, iii) the gum was soaked in hot water until it had completely dissolved and the liquid was then allowed to cool and carefully strained before being used to bathe sore eyes or applied to burns, iv) during smoky times, the inner bark was soaked in water, then placed over the eyelids whilst sleeping to relieve pain and inflammation, v) the bark was pounded, then boiled in water, allowed to cool, strained and used to bathe running sores and ulcers, vi) the young leaves were boiled until the water had turned green, then the liquid was allowed to cool, and strained and used as a wash for joint, chest or muscle pain and to relieve the symptoms of colds [2].

Figure 3a-1. *Eucalyptus bosistoana*

The crude EtOH extract of the leaf of *E. bosistoana* exhibited strong anti-inflammatory activity (IC_{50} = 7.58 µg/mL for NO inhibition) in LPS + IFN-γ activated 264.7 RAW macrophages and was selected for investigation to identify bioactive compounds. Fresh leaves of the plant were then sequentially extracted with DCM, DCM:MeOH (50:50) and MeOH. The MeOH extract showed highest anti-inflammatory activity (IC_{50} = 2.75 µg/mL for NO inhibition) in LPS + IFN-γ activated 264.7 RAW macrophages and was subjected to HPLC for fractionation.

The bioassay-guided fractionation of the extract led to the isolation of one known compound, **3a.1.** The structure was confirmed by comparison of its mass spectrometry and NMR data with data in the literature [3].



3a.1

4-*O*-*p*-Coumaroylquinic acid

Figure 3a-2. Structure of compound **3a.1**

3a.2 Results

3a.2.1 Extraction and yield

Two hundred grams of fresh *E. bosistoana* (20070782) leaves were extracted sequentially with three solvents from low to high polarity which were DCM, DCM:MeOH (50:50) and MeOH in order to separate lipophilic from hydrophilic compounds. The mass yields obtained from extraction are presented in Table 3a.1.

Table 3a-1. Yield of the extracts after sequential extraction of fresh leaves from *E. bosistoana*

Extract	Yield of the extract (g)
DCM	9.15
DCM:MeOH (50:50)	14.64
MeOH	18.67

3a.2.2 Anti-inflammatory activity of extracts

The DCM, DCM:MeOH (50:50) and MeOH extracts were tested in LPS + IFN-γ induced RAW 264.7 macrophages to obtain IC_{50} values for NO and TNF-α down regulation and also LC_{50} for cytotoxicity. Due to low solubility of the extracts in the cell culture media, they were diluted in DMSO, leading to a final concentration of up to 0.5% DMSO in cell culture medium. This concentration of DMSO showed a cell viability of less than 10% (data not shown). All three sequential extracts demonstrated variable anti-inflammatory activity to down regulate NO production which was less than 10 µg/mL. The IC_{50} values obtained for the inhibition of TNF-α production had a wide range of 6 to 55 µg/mL whereas LC_{50} for cytotoxicity also exhibited values between 14 to 62 µg/mL (Figure 5-1 and Table 5-2). The sequential MeOH extract showed highest activity with IC_{50} of 2.75 and 6.22 µg/mL for suppression of NO and TNF-α production respectively and LC_{50} of 14.61 µg/mL.

Table 3a-2. Anti-inflammatory activity of *E. bosistoana* sequential extracts

Sequential Extract	Inhibition of NO production (IC_{50} in µg/mL)	*Inhibition of TNF-α production (IC_{50} in µg/mL)	Cytotoxicity (LC_{50} in µg/mL)
DCM	9.07 ± 1.0	34.47 ± 14.7	42.49 ± 3.5
DCM:MeOH (50:50)	9.63 ± 0.9	55.55 ± 29.6	62.49 ± 21.1
MeOH	2.75 ± 0.2	6.22 ± 1.5	14.61 ± 0.9

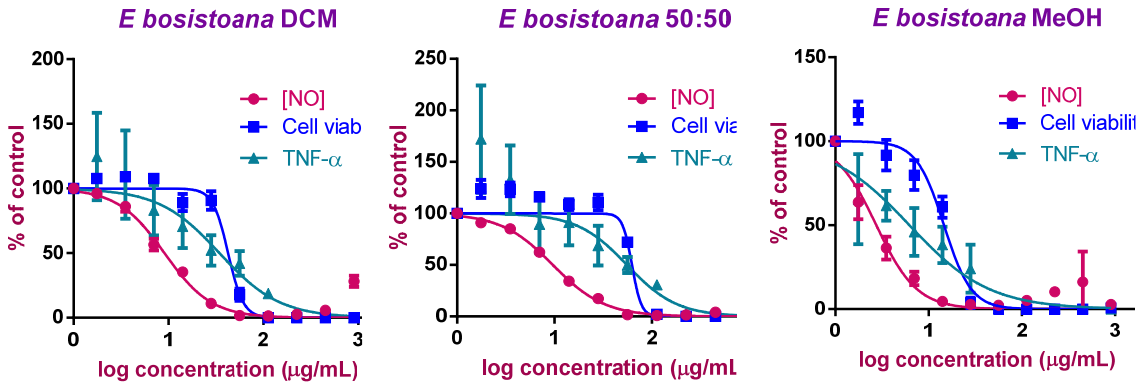
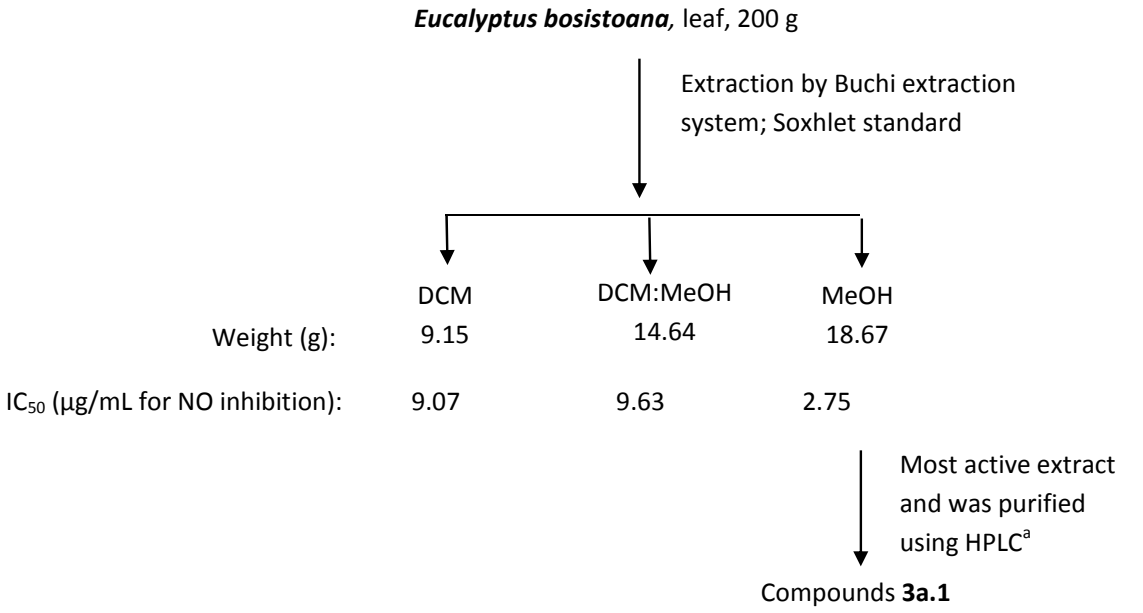


Figure 3a-3. Down regulation of LPS and IFN-γ induced NO and TNF-α production and cell viability of *E. bosistoana* sequential extracts in RAW 264.7 macrophages

Data represented as three experiments assayed in quadruplicates (n=12, mean ± SEM). * One experiment in triplicate (n=3, mean ± SEM).

3a.2.3 Isolation of active compound

In order to identify bioactive compounds, the sequential MeOH extract (IC_{50} = 2.75 µg/mL for NO inhibition) was subjected to HPLC on a phenyl-hexyl column. This process led to the isolation of one known compound (**3a.1**) (Scheme 3a-1).



(a) Semi-preparative HPLC: Phenyl (250x10 mm; 5 µm), 10-100% MeOH/H_2O (0.01% HCO_2H modifier) over 20 mins, held for 20 min then to 10% MeOH/H_2O on 45 min and held for last 5 minutes.

Scheme 3a-1 Bioassay-guided separation of *E. bosistoana* leaf extract

3a.2.3.1 Identification of compound **3a.1**

Compound **3a.1** was obtained as white amorphous powder. Its structure was elucidated as the known compound 4-*O*-*p*-coumaroyl quinic acid (Figure 3a-2) by comparison of its mass spectrometry and NMR data[SI] with literature data [3].

3a.2.4 Anti-inflammatory activity of identified compound

Compound **3a.1** was evaluated for anti-inflammatory activity in LPS + IFN-γ induced RAW 264.7 macrophages and N11 microglia (Figure 3a-4 and Table 3a-3).

In RAW 264.7 macrophages, compound **3a.1** showed moderate activity. The IC_{50} values were 95.74 and 51.56 µg/mL for NO and TNF-α inhibition respectively. The compound did not show any toxicity up to the highest concentration (36 µg/mL) tested. In the N11 microglial cell line, compound **3a.1**

also showed moderate activity. The IC_{50} value was 44.31 and 35.50 µg/mL for NO and TNF-α inhibition respectively. The compound did not show any toxicity up to the highest concentration (36 µg/mL) tested.

Table 3a-3. Anti-inflammatory activity of compound isolated from *E. bosistoana* in LPS + IFN-γ induced RAW 264.7 macrophage and N11 microglia

Cell line	Inhibition of NO production (IC_{50} in µg/mL)	Inhibition of TNF- α production (IC_{50} in µg/mL)	Cytotoxicity (LC_{50} in µg/mL)	% Cytotoxicity
RAW 264.7 macrophage	95.74 ± 14.4	51.56 ± 4.6	> 36	144.5
N11 microglia	44.31 ± 6.1	35.50 ± 11.3	> 36	97.7

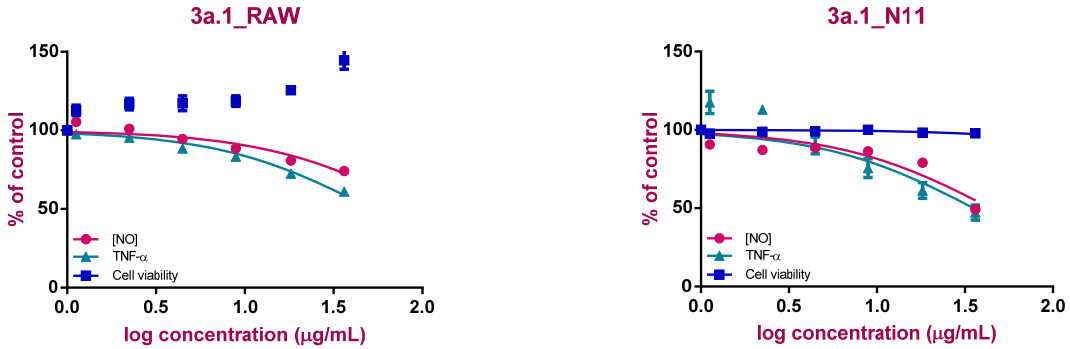


Figure 3a-4. Down regulation of LPS and IFN-γ induced NO and TNF-α production and cell viability of isolated compounds from *E. bosistoana* in RAW 264.7 macrophage and in N11 microglia

3a.3 Discussion

Compound **3a.1** was the major constituent of the sequential MeOH extract from *E. bosistoana;* however, its activity was moderate and was found non-toxic in our investigation. Compound **3a.1** or 4-*O*-*p*-coumaroyl quinic acid is a well-known compound but its complete NMR information was first published by Will et al., in 2007 where they isolated the compound from apple juice [3]. There is no report on its identification from *Eucalyptus* spp.

*Purity of compound **3a.1** and its effect on the biological results*

Little impurities were present in compound **3a.1** as evident from its proton NMR spectrum[SI]. Though we were able to characterize this compound by analyzing its NMR and Mass spectroscopic data, the anti-inflammatory activities remained questionable due to the presence of impurities. Impurities have impact on bioactivity as demonstrated in many studies [4,5]. However, compound **3a.1** or 4-*O*-*p*-coumaroyl quinic acid was a well-known compound [Will, 2007] and it was only moderately active

to suppress NO or TNF-α production with IC_{50} values 95.74 and 51.56 µg/mL respectively in LPS + IFN-γ induced RAW 264.7 macrophages. Therefore, we did not give much effort to re-purify the compound and moved to our next potent extract.

Reason for missing mass of the materials

We found only one major peak (fraction 3 or compound **3a.1,** Figure 3a-5) after optimizing the reverse-phase HPLC method for sequential MeOH extract obtained from *E. bosistoana*. We tried to identify one other minor peak (fraction 4) isolated from this extract but was not successful to identify the structure due to low yield and impurities. We were unable to isolate other compounds present in the crude extract due to inseparable nature of the extract though we tried different HPLC methods including change of HPLC columns, solvents and gradient system. The HPLC chromatogram of the extract in question has been added below for reference.

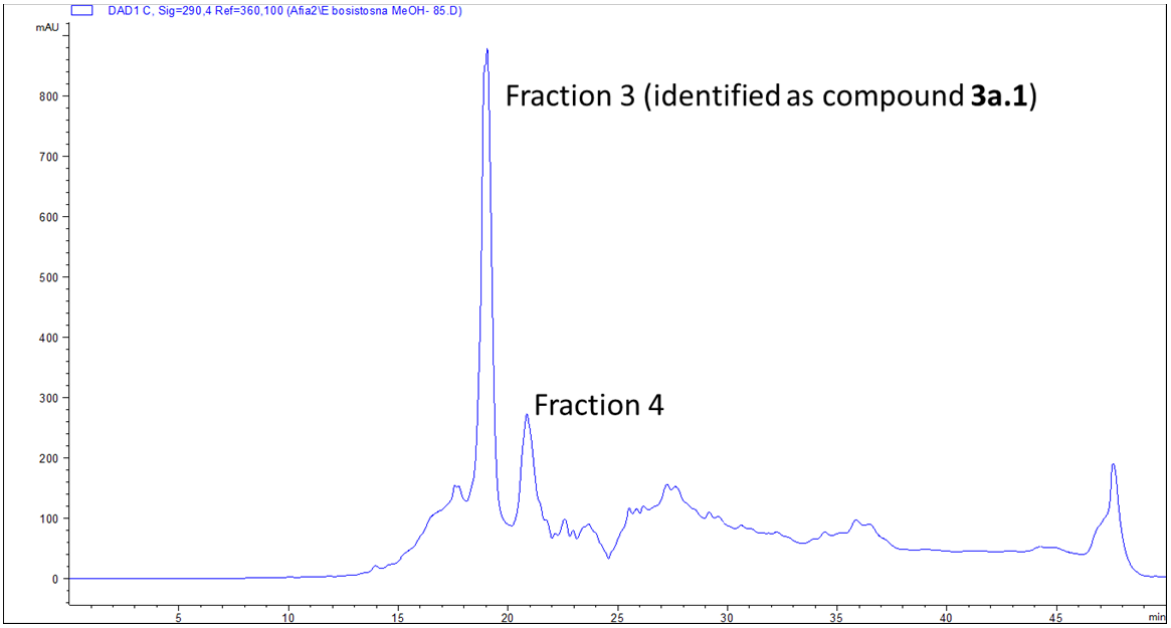


Figure 3a-5. HPLC chromatogram obtained from *E. bosistoana* MeOH extract. [Semi-preparative HPLC: Phenyl (250x10 mm; 5µm), 10-100 % H_2O/MeOH (0.01% HCO_2H modifier) over 20 mins, held for 20 mins then to 10% H_2O/MeOH on 45 min and held for last 5 minutes].

3a.4 Experimental section

3a.4.1 General experimental procedures

Dichloromethane and methanol were supplied by Merck. Ethyl acetate from Fisher Chemical, ethanol from Chem-Supply and water used was Milli-Q water. Bovine serum albumin, lipopolysaccharide (*E. coli* serotype-0127:B8), EDTA, N-(1-napthyl) ethylenediamine dihydrochloride, benzylpenicillin G sodium salt, resazurin sodium salt (10%), streptomycin, sulphanilamide, 3,3',5,5'-tetramethylbenzidine (TMB), trypan blue and Dulbecco's Modified Eagle's Medium (DMEM) were

purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Foetal bovine serum (FBS) and glutamine were purchased from Life Technologies (Mulgrave, VIC, Australia). Murine interferon-γ (IFN-γ) and TNF-α ELISA kits were purchased from PeproTech Asia (Rehovot, Israel). Citric acid and monosodium dihydrogen carbonate (NaH_2CO_3) were from AJAX Chemicals (Auburn, NSW, Australia). Tween-20 was from Amresco (Solon, Ohio, USA). Methanol, monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), sodium chloride (NaCl) and sulfuric acid (H_2SO_4) were from Merck (Darmstadt, Germany). Sodium carbonate (Na_2CO_3) was BDH brand supplied by Merck Pty. Ltd. (Kilsyth, VIC, Australia).

3a.4.2 Experimental procedures for MS, HPLC and NMR

Semi-preparative HPLC was performed using an Agilent 1260 Infinity Series (Santa Clara, CA, USA). NMR spectra were obtained on a Bruker Ascend 400 MHz spectrometer (Bruker Biospin GmbH, Bremen, Germany). TopSpin software was used to analyse the spectral data. ^{1}H NMR spectrum was recorded at 400 MHz. The chemical shifts (δ) are expressed in parts per million (ppm) as δ values and coupling constant *J* in Hertz (Hz). COSY, HSQC and HMBC were acquired using the standard Bruker pulse program. The experiments were performed in dimethyl sulfoxide-d_6 (DMSO-d_6) and chemical shifts were calibrated relative to the DMSO solvent peak (^{1}H δ at 2.50 and ^{13}C δ 39.51 ppm). High resolution mass spectrometry (HRMS) was carried out using a Waters Xevo Q-TOF mass spectrometer operating in positive electrospray ionization (ESI) mode.

3a.4.3 Plant material

Fresh leaves of *E. bosistoana* (voucher no. 20070782) were collected in the month of August, 2015 from the 'Australian Botanic Gardens' at Mount Annan, NSW, Australia. A voucher specimen was deposited at the National Herbarium of NSW, Australia.

3a.4.4 Extraction and isolation

Approximately 200 g of fresh leaf material from *E. bosistoana* was first cut into small pieces with scissors and then ground to a coarse powder using a hand blender. The coarse powder was then filled into the thimbles of an accelerated solvent extraction system (Buchi B-811, Switzerland) and extracted under standard Soxhlet mode (for 2 × 15 minutes cycles) using three solvents (dichloromethane, dichloromethane and methanol in 50:50 ratio and methanol) sequentially from low to high polarity. The volume of the extracts were reduced to a volume of 2—4 mL using a rotary evaporator and then evaporated to dryness with nitrogen. The sequential methanol extract was found most potent when screened for anti-inflammatory activity. This crude extract was then resuspended in methanol and subjected directly through reversed phase semi-prep HPLC (Phenyl column, 250 x 10 mm, 5µm; Merck). Peak detection was performed with a photodiode array detector (PDA) using three detection wavelengths (210 nm, 254 nm and 290 nm). A gradient starting from 10% methanol/water to a 100% methanol (with a constant 0.01% formic acid modifier) over 20 min and held at 100% methanol for 20 min to yield compound **3a.1** (t_R = 18.2 min, 2.5 mg). The isolated compound was purified to 95% purity or more as judged by HPLC (UV detection) and ^{1}H NMR spectroscopy before determining the bioactivity.

In the present study, plant materials were extracted using standard Sohxlet mode where boiling temperature of the solvents were set for the extraction. We were concerned that high temperature

may cause degradation of our compounds of interest. Therefore, we compared HPLC chromatograms of two types of samples obtained from Sohxlet extraction and extraction at room temperature keeping other parameters fixed. In this experiment, we found the HPLC chromatograms were overlapping considering the number of peaks in both modes but the yield of extraction was increasing with increasing temperature (data not shown). From this finding we chose Sohxlet extraction for our plant samples which was faster and more convenient compared to extraction at room temperature.

3a.4.5 Maintenance and preparation of RAW 264.7 macrophages and N11 microglia

RAW 264.7 macrophages and N11 microglial cells were grown in 175 cm^2 culture flasks on DMEM (Dulbecco's Modified Eagle's Medium) containing 5% FBS (foetal bovine serum) that was supplemented with penicillin (100 U/mL), streptomycin (100 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. Once cells had grown to confluence in the culture flask, they were removed using a rubber policeman cell scraper, as opposed to using trypsin, which can remove membrane-bound receptors. The cell suspension was concentrated by centrifugation for 3 min at 900 rpm and resuspended in a small volume of fresh DMEM (with 1% antibiotics and 5% FBS). Cell densities were estimated using a Neubauer counting chamber. The cell concentration was adjusted with DMEM (with 1% antibiotics and 5% FBS) to obtain 60,000 cells/100 µL cell suspension. 100 µL of cell suspension was then dispensed into the wells of 96-well plates (Eppendorf, Hamburg, Germany). Plates were incubated at 37 °C; 5% CO_2 for 24 h before the activation experiments were carried out.

3a.4.6 Activation of RAW 264.7 macrophages and N11 microglia

From each well, the media was removed and replaced with fresh DMEM containing 0.1% FBS. For assays with extracts, a 90 µL volume of the dilutions in DMEM (with 0.1% FBS) was added an hour prior to addition of the activator. Due to the often inconsistent nature of LPS at activating cells, a combination of LPS (10 µg/mL) and IFN-γ (10 U/mL), both in DMEM (with 0.1% FBS), were used for activation. For the sequential extracts a maximum dose of 900 µg/mL was used, and diluted serially by 50% up to a minimum of 10 doses (900, 450, 225, 112.5, 56.25, 28.125, 14.062, 7.031, 3.515, 1.7578 and 0.8789 µg/mL in the wells respectively). A maximum dose of the compound (**3a.1**) used was 36 µg/mL and diluted serially by 50% up to a minimum of 7 doses (36, 18, 9, 4.5, 2.25, 1.12 and 0.56 µg/mL in the wells respectively). After activation, the cells were incubated for 24 h at 37 °C and 5% CO2 and then NO and TNF-α inhibition and cell viability were determined. Cells with media alone were used as negative control and activated cells were used as positive control. The effects of solvents on readouts were initially determined, but as the anti-inflammatory or cytotoxic effects of the solvents were <10% even at the highest concentration used, parameters were compared to the "no solvent" controls.

3a.4.7 Determination of nitric oxide production by Griess assay

Nitric oxide was determined by Griess reagent quantification of nitrite, one of its stable reaction products. Griess reagent was freshly made up of equal volumes of 1% sulphanilamide and 0.1% naphthylethylene-diamine in 5% HCl. In the presence of nitrite this reagent forms a violet colour. From each well, 50 µL of supernatant was transferred to a fresh 96-well plate and mixed with 50 µL of Griess reagent, and the colour produced was measured at 540 nm in a microplate reader

(POLARstar Omega, BMG Labtech, Mornington, Australia). The remaining supernatant from each well was used for a TNF-α assay. The concentration of nitrite was calculated using a standard curve with sodium nitrite (0 to 250 μM) and linear regression analysis.

3a.4.8 Determination of cell viability by Alamar blue assay

The Alamar blue assay is a colorimetric assay involving the cellular reduction of resazurin to resorufin. Alamar blue solution [100 μL of 10% Alamar blue (Resazurin) in DMEM medium] was added to each well and incubated at 37 °C for 1-2 h. After incubation, fluorescence was measured (excitation at 544 nm and emission at 590 nm) using a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia). The results were expressed as a percentage of the intensity of that in control cells after background fluorescence was subtracted.

3a.4.9 TNF-α determination by ELISA

The supernatants obtained from each well (remaining supernatant after 24 hours of activation) and experiment that gave better readout for NO and Alamar blue assay were used for determination of TNF-α using a commercial sandwich ELISA (Catalog number: 900-K54; Peprotech, USA) according to the manufacturer's protocol. The supernatants were diluted 30 times using diluent (0.1% w/v bovine serum albumin and 0.05% v/v tween-20 in PBS [1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl; pH 7.4]). Capture antibody was used at a concentration of 1.25 μg/mL in PBS. To make a standard curve TNF-α (10 ng/mL standard) was diluted serially by 50% up to a minimum of 10 doses (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, and 0.019 ng/mL in the wells respectively) and was used as the internal standard. TNF-α was detected with a biotinylated second antibody and an avidin peroxidase conjugate with TMB as detection reagent. The colour development was monitored at 655 nm, taking readings every 5 min. After about 30 min, the reaction was stopped using 0.5 M sulfuric acid, and the absorbance was measured at 450 nm of measurement filter using a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia). The absorbance data was expressed as a percentage of that of control wells after conversion of the concentrations by using a standard curve constructed with defined concentrations of TNF-α. Curve fitting of this standard curve and extrapolation of experimental data were performed using non-linear regression analysis.

3a.4.10 Data presentation and analysis

For sequential extracts, three experiments in quadruplicates (n=12) were combined to determine the IC_{50} (for NO inhibition) and LC_{50} (for cell viability) whereas one experiment in triplicate (n=3) was conducted to obtain IC_{50} for TNF-α inhibition using the dose-response inhibition function in GraphPad Prism version 6.01 (GraphPad Software Incorporated, USA). For purified compounds, two experiments in quadruplicates (n=8) were combined to determine the IC_{50} (for NO and TNF-α inhibition) and LC_{50} (for cell viability) using the dose-response inhibition function in same version of GraphPad.

3a.5 References

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Chapter 4

Screening of Plants used in Australian Folk Medicine for Anti-inflammatory Activity

4.1 Introduction

The process of inflammation is defined as a sequence of events that takes place in response to foreign stimuli, trauma or infection and is characterized by redness, heat, swelling, loss of function and pain [1,2]. It is an important biological process for maintaining homeostasis, responding to foreign agents (i.e. chemicals and microorganisms) and repairing damaged tissue [3,4]. Diseases like asthma, rheumatoid arthritis, tendonitis, Crohn's disease, inflammatory bowel disease and psoriasis may develop in part from the body's inability to resolve chronic inflammation. A key driver of this is oxidative stress, which contributes to the progression of atherosclerosis, diabetes and metabolic syndrome, multiple sclerosis, Parkinson's and Alzheimer's disease [5-7].

Drugs can cause potential harms beside their beneficial activities [8]. Non-Steroidal Anti-inflammatory Drugs (NSAIDs) are most commonly prescribed anti-inflammatory medication and many individuals may be susceptible to a number of side effects, gastrointestinal complication being the most frequent. Corticosteroids on the other hand are highly effective at controlling chronic inflammation by suppressing the immune response but can give rise to a number of side effects including weight gain, osteoporosis, thinning and bruising of skin amongst others [9,10].

Plants having medicinal values are recognised as the oldest and most widespread form of medication throughout the world. Plant derived natural products have proven to be an important source of active ingredients in pharmaceutical preparations, setting the foundation of the pharmaceutical industry, while in more recent times synthetic compounds are used in modern formulations [11]. Salicylic acid is an active ingredient of willow bark, while the introduction of its synthetic analogue (aspirin) in 1897 was a breakthrough for the treatment of various inflammatory disorders [12].

The diverse Australian flora in association with their more than 40,000 years old Indigenous medicinal knowledge provides great scope to select novel plants for health enhancing properties. It is already demonstrated that many of the plants used by native Australians contain important biologically active molecules [13,14]. Clerodane diterpenoid compounds isolated from *Dodonaea polyandra,* which is a native Australian plant, were found to inhibit pro-inflammatory cytokines like IL-1β and IL-6 [15,16]. In another investigation, anti-inflammatory agarofurans have been isolated from the seeds of the Australian rainforest vine *Celastrus subspicata* [17]. Six steroids isolated from other Australian rainforest tree *Alphitonia petriei* showed strong anti-inflammatory in RAW 264.7 murine macrophages [18].

D'harawal Aboriginal elder and botanist Mrs Frances Bodkin (commonly called Aunty Fran) reported more than 2700 native Australian plants alphabetically in her pharmacopoeia named as 'D'harawal Pharmacopoeia' (unpublished). In this Pharmacopoeia plants are described with their corresponding taxonomic identification and diverse uses, most importantly their medicinal uses by the D'harawal Aboriginal people for centuries. In our previous study we investigated crude ethanolic extract of 17 species of *Eucalyptus* (Myrtaceae), most of which showed strong (IC_{50} < 20 µg/mL for NO inhibition) to moderate (IC_{50} < 85 µg/mL for NO inhibition) anti-inflammatory activity [19]. Furthermore, a number of compounds isolated from *Eucalyptus* have been shown to have anti-inflammatory activity [20,21].

In our present study, we screened ethanolic extracts of leaves of 15 plant species from 11 different genera and 8 different families to evaluate their anti-inflammatory activity. LC-MS profiles of the extracts were also been obtained. These plants were important for their medicinal value to the D'harawal Aboriginal people to heal cuts and wounds, infection, arthritis, cramp, congested chest, fever and other disorders indicative of inflammation.

4.2 Results

In this study, leaves from 15 different plants were collected in the month of July, 2016. Approximately 20g of leaves from each of *Acacia falcata*, *A. leprosa*, *A. melanoxylon*, *Adiantum formosum*, *Asplenium australasicum*, *A. flavelifolium*, *Baeckea imbricata*, *B. ramosissima* subsp. *ramosissima*, *Hakea salicifolia*, *Melaleuca linariifolia*, *Mentha satureioides*, *Pimelea linifolia*, *Pittosporum undulatum*, *Syncarpia glomulifera* subsp. *glomulifera* and *Telopea speciossissima* were extracted using absolute ethanol (Table 4-2).

The RAW 264.7 murine macrophages release NO and TNF-α when exposed to bacterial LPS and IFN-γ and this principle has become an established experimental model to evaluate in vitro anti-inflammatory activity of extracts [14,22-24]. For the purpose of interpretation, the IC_{50} values of NO inhibition are divided into three groups: extracts with IC_{50} < 20 µg/mL are considered as highly potent extracts; a value between 21 and 70 µg/mL as moderately potent and an IC_{50} < 70 µg/mL is considered as an extract with low potency.

The highest concentration of ethanolic crude extract tested in the anti-inflammatory assay was 900 µg/mL with serial ½ dilutions. *Syncarpia glomulifera* subsp. *glomulifera*, *Melaleuca linariifolia*, *Baeckea ramosissima* subsp. *ramosissima* and *B. imbricata* leaf extracts showed the strongest inhibition of NO production with IC_{50} values of 8.25, 9.74, 15.68 and 16.78 µg/mL respectively (Figure 4-1, Table 4-4). The extracts from *Acacia melanoxylon*, *Pimelea linifolia*, *Acacia leprosa* and *Pittosporum undulatum* showed moderate inhibition of NO with IC_{50} values of 28.94, 30.36, 35.49 and 66.36 µg/mL respectively. The remaining seven plants, *Telopea speciossissima*, *Hakea salicifolia*, *Acacia falcata*, *Mentha satureioides*, *Adiantum formosum*, *Asplenium flavelifolium* and *A. australasicum* presented low inhibition of NO with IC_{50} values of 116.5, 195.9, 215.5, 218.7, 238.0, 280.3 and 503.3 µg/mL respectively.

Four of the plant extracts also showed promising TNF-α inhibitory activity (Figure 4-1, Table 4-4) with IC_{50} values of 23.30, 16.40, 8.31 and 22.77 µg/mL for *Syncarpia glomulifera* subsp. *glomulifera*, *Melaleuca linariifolia*, *Baeckea ramosissima* subsp. *ramosissima* and *B. imbricata* respectively, which are the same plants in our highly potent NO inhibitor group. On the other hand, the moderately active extracts from *Acacia melanoxylon*, *Pimelea linifolia*, *Acacia leprosa* and *Pittosporum undulatum* showed TNF-α IC_{50} value of 68.20, 49.70, 63.05 and 66.14 µg/mL respectively. Whereas extracts from *Telopea speciossissima*, *Hakea salicifolia*, *Acacia falcata*, *Mentha satureioides*, *Adiantum formosum*, *Asplenium flavelifolium* and *A. australasicum* exhibited lower to moderate inhibition of TNF-α production with IC_{50} values of 555.1, 697.7, 133.6, 62.75, 86.65, 62.07, 917.0 µg/mL respectively which are the plants in our least potent group.

The use of Alamar Blue (rezazurin) to measure cytotoxicity is an established technique [25]. The results of cytotoxicity (LC_{50}) of our leaf extracts are shown in Table 4-4 and Figure 4-1. The plants of our highly potent group showed moderate toxicity with LC_{50} values of 52.09, 101.6, 85.68, and 130.0 µg/mL for *Syncarpia glomulifera* subsp. *glomulifera*, *Melaleuca linariifolia*, *Baeckea ramosissima* subsp. *ramosissima* and *B. imbricata* respectively. Plants with moderate potency showed a wide range of toxicity with LC_{50} values of 36.26, 226.3, 103.9, and 108.1 µg/mL for *Acacia melanoxylon*, *Pimelea linifolia*, *Acacia leprosa* and *Pittosporum undulatum* respectively. The plants of the least potent group showed no toxicity (> 900 µg/mL) for *Telopea speciossissima*, *Hakea salicifolia*, *Acacia falcata*, *Adiantum formosum*, *Asplenium flavelifolium* and *A. australasicum* with the highest concentration of 900 µg/mL except *Mentha satureioides* which gave an LC_{50} of 242.4 µg/mL.

The LC-MS profile of the 15 extracts were also obtained.

In our LC-MS study of the leaf extracts, polar compounds were detected in the early phase of the separation and non-polar compounds were detected in the later phase. Interestingly, extracts rich in nonpolar constituents (Figure 4-2) with relatively higher abundance exhibited stronger anti-inflammatory activity when screened in LPS and INF-γ induced RAW macrophages.

4.3 Discussion

In a continuing search for anti-inflammatory natural products, fifteen different plants were extracted and screened. The plants studied here were chosen on the basis of their traditional use to treat inflammatory and related conditions by the D'harawal people of the Campbelltown region (Southwest Sydney Australia). Most of the plants showed anti-inflammatory activity, demonstrated inhibitory effect on NO and TNF-α production with varying potencies, which provides some evidence supporting their use in traditional Aboriginal medicine. The amount of the anti-inflammatory compounds in the plants - according to traditional knowledge - is also dependent on the plant's environment. In D'harawal country, what is most important when seeking particular medicines from plants is where the plant is growing, that is, not so much the soils, but the other plants that are growing around the particular plant required [26]. For the present screening study, the plant material was provided by the Botanical Gardens from random trees in the garden, but for future studies, we will investigate if traditional collection practices based on D'harawal knowledge will improve the inherent activity and/or yield of the anti-inflammatory compounds.

We also obtained LC-MS profile of the 15 extracts. Since, chemical profiling of plant secondary metabolite has the potential to generate structural information [27] and liquid chromatography coupled to mass spectrometry has emerged as a key technology for broad screening of biomolecular components across multiple samples (corresponding to different condition, interventions or time points [28-30].

In our LC-MS study of the leaf extracts, polar compounds were detected in the early phase of the separation and non-polar compounds were detected in the later phase. Interestingly, extracts rich in nonpolar constituents (Figure 4-2) with relatively higher abundance exhibited stronger anti-inflammatory activity when we tested them in LPS and INF-γ induced RAW macrophages.

4.4 Experimental Section

4.4.1 Plant material

Plants known to be used by the D'harawal people (also known as Tharawal) to treat inflammation and related illnesses were selected under the guidance of botanist and Aboriginal Elder Auntie Fran (Mrs. Frances Bodkin) and following the D'harawal Pharmacopeia. Leaf materials of 15 selected plants were collected in the month of July, 2016 from the 'Australian Botanic Gardens' at Mount Annan, NSW, Australia (Table-1). Voucher specimens were deposited at the National Herbarium of NSW, Australia.

4.4.2 Chemicals and reagents

Ethanol was purchased from Chem-Supply (Gillman, SA, Australia), Bovine serum albumin, lipopolysaccharide (*E. coli* serotype-0127:B8), EDTA, N-(1-napthyl) [2] ethylenediamine dihydrochloride, benzylpenicillin G sodium salt, resazurin sodium salt (10%), streptomycin, sulphanilamide, 3,3',5,5'-tetramethylbenzidine (TMB), trypan blue and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Foetal bovine serum (FBS) and glutamine were purchased from Life Technologies (Mulgrave, VIC, Australia). Murine interferon-γ (IFN-γ) and TNF-α ELISA kits were purchased from PeproTech Asia (Rehovot, Israel). Citric acid and monosodium dihydrogen carbonate (NaH_2CO_3) were from AJAX Chemicals (Auburn, NSW, Australia). Tween-20 was from Amresco (Solon, Ohio, USA). Methanol, monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), sodium chloride (NaCl) and sulfuric acid (H_2SO_4) were from Merck (Darmstadt, Germany). Sodium carbonate (Na_2CO_3) was BDH brand supplied by Merck Pty. Ltd. (Kilsyth, VIC, Australia).

4.4.3 Extraction of plants leaves for biological assays, HPLC and MS analysis

Approximately 20 g of fresh leaf material from each plant was extracted using absolute ethanol. The leaves were first cut into small pieces with scissors and then ground to a coarse powder using a hand blender. The coarse powder was filled into the thimbles of an accelerated solvent extraction system (Buchi B-811, Switzerland) and extracted under standard Soxhlet mode (for 2 × 15 minutes cycles). The volume of the extracts was reduced to ca. 2—4 mL using a rotary evaporator and then evaporated to dryness with nitrogen gas for biological assays. Percentage yields (% g extract/g fresh weight) are recorded in Table-2.

4.4.4. Chemical profiling of the extracts by LC-MS

LC-MS profiles of the ethanolic leaf extracts of each plant were obtained using a Waters Acquity UPLC instrument. A Waters Acquity UPLC BEH C18 column (2.1 mm x 150 mm, 1.7 µm) was used and a column temperature of 35 °C was maintained. Injections of 10 µL were full loop mode (loop overfill factor x 4) using a method run time of 35 min and solvent flow rate of 0.2 mL/min. Solvent A consisted of 0.1% (v/v) formic acid in ultrapure water and solvent B was 0.1% (v/v) formic acid in LC-MS grade acetonitrile. The chromatographic method consisted initially of 10% solvent B ramped linearly to 95% solvent B over 20 min. At 25 min, the solvent composition returned to 10% B and maintained this composition until completion of the run. Detection of analytes was accomplished with photodiode array detector (PDA) and mass spectrometry. 3D spectra were obtained over the

wavelength range 190-450 nm with resolution of 1.2 nm and sampling rate of 20 points/sec. 2D spectra were measured at 210 nm with resolution of 4.8 nm.

Mass spectrometry was performed using a Waters Xevo TQ-MS triple quadruple mass spectrometer with the following settings: desolvation gas temperature 300 °C; desolvation gas flow 500 L/h; cone gas 0 L/h; and source temperature of 150 °C. In positive mode the capillary voltage was 3.0 kV and the cone voltage was 30 kV. In negative mode the capillary voltage was 3.0 kV and the cone voltage was 30 kV. Positive and negative spectra were recorded simultaneously over the *m/z* range 100-1500 using a scan time of 1 second. Samples were prepared at a concentration of 10 mg/mL of methanol from each of the extract. The operation of the LC-MS system was controlled by MassLynx V4.1 software.

4.4.5 Maintenance and preparation of RAW 264.7 macrophages

RAW 264.7 macrophages were grown in 175 cm^2 culture flasks on DMEM (Dulbecco's Modified Eagle's Eedium) containing 5% FBS (foetal bovine serum) that was supplemented with penicillin (100 U/mL), streptomycin (100 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. Once cells had grown to confluence in the culture flask, they were removed using a rubber policeman cell scraper, as opposed to using trypsin, which can remove membrane-bound receptors. The cell suspension was concentrated by centrifugation for 3 min at 900 rpm and resuspended in a small volume of fresh DMEM (with 1% antibiotics and 5% FBS). Cell densities were estimated using a Neubauer counting chamber. The cell concentration was adjusted with DMEM (with 1% antibiotics and 5% FBS) to obtain 60,000 cells/100 µL cell suspension. 100 µL of cell suspension was then dispensed into the wells of 96-well plates (Eppendorf, Hamburg, Germany). Plates were incubated at 37 °C; 5% CO_2 for 24 h before the activation experiments were carried out.

4.4.6. Activation of RAW 264.7 macrophages

From each well, the media was removed and replaced with fresh DMEM containing 0.1% FBS. For assays with extracts, a 90 µL volume of the dilutions in DMEM (with 0.1% FBS) was added an hour prior to addition of the activator. Due to the often inconsistent nature of LPS at activating cells, a combination of LPS (10 µg/mL) and IFN-γ (10 U/mL), both in DMEM (with 0.1% FBS), were used for activation. A maximum dose of the extracts used was 900 µg/mL, and diluted serially 1 in 2 up to a minimum of 10 doses (900, 450, 225, 112.5, 56.25, 28.125, 14.062, 7.031, 3.515, 1.7578 and 0.8789 µg/mL in the wells respectively). After activation, the cells were incubated for 24 h at 37 °C and 5% CO_2 and then NO and TNF-α inhibition, and cell viability was determined. Cells with media alone were used as negative control and activated cells used as positive control. The effects of solvents on readouts were initially determined, but as the anti-inflammatory or cytotoxic effects of the solvents were < 10% even at the highest concentration used, parameters were compared to the "no solvent" controls.

4.4.7. Determination of nitric oxide production by Griess assay

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

From each well, 50 µL of supernatant was transferred to a fresh 96-well plate and mixed with 50 µL of Griess reagent, and the colour produced was measured at 540 nm in a microplate reader (POLARstar Omega, BMG Labtech, Mornington, Australia). The remaining supernatant from each well was used for a TNF-α assay. The concentration of nitrite was calculated using a standard curve with sodium nitrite (0 to 250 µM), and linear regression analysis.

4.4.8. Determination of cell viability by Alamar blue assay

The Alamar blue assay is a colorimetric assay involving the cellular reduction of resazurin to resorufin. Alamar blue solution [100 µL of 10% Alamar blue (Resazurin) in DMEM medium] was added to each well and incubated at 37 °C for 1-2 h. After incubation, fluorescence was measured (excitation at 544 nm and emission at 590 nm) using a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia). The results were expressed as a percentage of the intensity of that in control cells after background fluorescence was subtracted.

4.4.9. TNF-α determination by ELISA

The supernatants obtained from each well (remaining supernatant after 24 hours of activation) and experiment that gave better readout for NO and Alamar blue assay were used for determination of TNF-α using a commercial sandwich ELISA (Catalog number: 900-K54; Peprotech, USA) according to the manufacturer's protocol. The supernatants were diluted 30 times using diluent (0.1% w/v bovine serum albumin and 0.05% v/v tween-20 in PBS [1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl; pH 7.4]). Capture antibody was used at a concentration of 1.25 µg/mL in PBS. To make a standard curve TNF-α (10 ng/mL standard) was diluted serially by 50% up to a minimum of 10 doses (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019 and 0.0097 ng/mL in the wells respectively) and was used as the internal standard. TNF-α was detected with a biotinylated second antibody and an avidin peroxidase conjugate with TMB as detection reagent. The colour development was monitored at 655 nm, taking readings every 5 min. After about 30 min, the reaction was stopped using 0.5 M sulfuric acid, and the absorbance was measured at 450 nm of measurement filter using a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia). The absorbance data was expressed as a percentage of that of control wells after conversion of the concentrations by using a standard curve constructed with defined concentrations of TNF-α. Curve fitting of this standard curve and extrapolation of experimental data were performed using non-linear regression analysis.

4.4.10. Data presentation and analysis

Three experiments in quadruplicates (n=12) were combined to determine the IC_{50} (for NO inhibition) and LC_{50} (for cell viability) whereas one experiment in triplicate (n=3) was conducted to obtain IC_{50} for TNF-α inhibition using the dose-response inhibition function in GraphPad Prism version 6.01 (GraphPad Software Incorporated, USA).

Table 4-1. Plants collected for the study of anti-inflammatory activity

No.	**Plant**	**APNI name**	**Family**	**Voucher No.**
1	*Acacia falcata*	*Acacia falcata* Willd.	Fabaceae/Mimosaceae	20041358
2	*Acacia leprosa*	*Acacia leprosa* Sieber ex DC.	Fabaceae/Mimosaceae	AA 20150001
3	*Acacia melanoxylon*	*Acacia melanoxylon* R.Br.	Fabaceae/Mimosaceae	20040377
4	*Adiantum formosum*	*Adiantum formosum* R.Br.	Adiantaceae	882136
5	*Asplenium australasicum*	*Asplenium australasicum* (J.Sm.) Hook.	Aspleniaceae	20120611
6	*Asplenium flavelifolium*	*Asplenium flavelifolium* Cav.	Aspleniaceae	20120621
7	*Baeckea imbricata*	*Baeckea imbricata* (Gaertn.) Druce	Myrtaceae	AC 942545
8	*Baeckea ramosissima* subsp. *ramosissima*	*Baeckea ramosissima* A.Cunn. subsp. *ramosissima*.	Myrtaceae	AD 823136
9	*Hakea salicifolia*	*Hakea salicifolia* (Vent.) B.L.Burtt.	Proteaceae	AA 873438
10	*Melaleuca linariifolia*	*Melaleuca linariifolia* Sm.	Myrtaceae	AA 922868
11	*Mentha satureioides*	*Mentha satureioides* R.Br.	Lamiaceae	20021036
12	*Pimelea linifolia*	*Pimelea linifolia* Sm..	Thymelaeaceae	AF 933363
13	*Pittosporum undulatum*	*Pittosporum undulatum* Vent.	Pittosporaceae	20000309
14	*Syncarpia glomulifera* subsp. *glomulifera*	*Syncarpia glomulifera* (Sm.) Nied. subsp. *glomulifera*.	Myrtaceae	891962
15	*Telopea speciossissima*	*Telopea speciosissima* (Sm.) R.Br.	Proteaceae	AB 20040680

Table 4-2. Plant common names and yields of ethanolic extracts for the study of anti-inflammatory activity

Plant species	Common name (s)	Yield of ethanol extract (%)[a]
Acacia falcata	Sickle Wattle	15.45
Acacia leprosa	Cinnamon Wattle/ Leper Wattle	13.35
Acacia melanoxylon	Blackwood	8.7
Adiantum formosum	Black Stem/ Giant Maidenhair/ Maidenhair/ Black Stem Maidenhair	9.4
Asplenium australasicum	Bird's Nest Fern	15.75
Asplenium flavelifolium	Necklace Fern	7.3
Baeckea imbricata	Heath Myrtle/ Ridged Heath Myrtle	9.45
Baeckea ramosissima subsp. *ramosissima*	Rosy Baeckea	22.4
Hakea salicifolia	Willow Leafed Hakea	27.95
Melaleuca linariifolia	Flax Leafed Paperbark/ Snow in Summer/ Snowstorm	29.45
Mentha satureioides	Creeping Mint/ Native Pennyroyal	4.0
Pimelea linifolia	Slender Rice Flower/ Queen of the Bush	7.95
Pittosporum undulatum	Native Daphne/ Sweet Pittosporum/ Snowdrop Tree/ Mock Orange	29.2
Syncarpia glomulifera subsp. *glomulifera*	Turpentine	13.75
Telopea speciossissima	Waratah	18.8

[a]Gram of solid extract/100 g of dried plant material

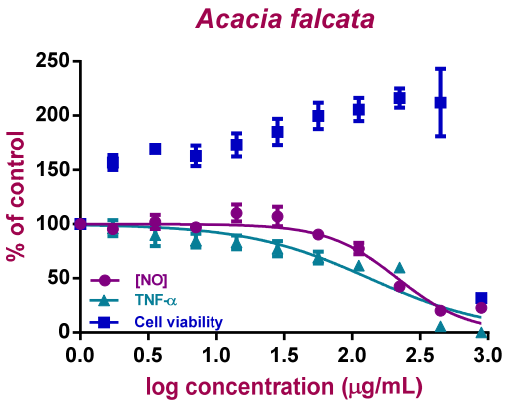
Table 4-3. D'harawal medicinal use of the plants according to D'harawal Pharmacopoiea

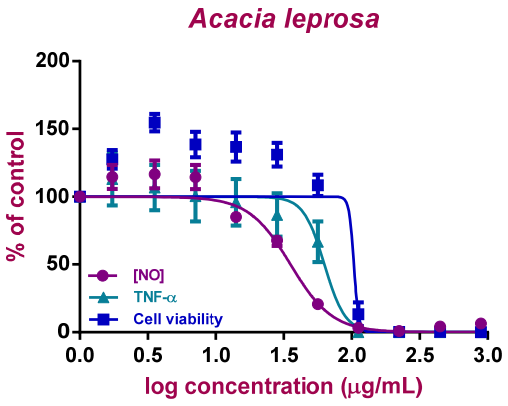
Plant species	D'harawal Medicinal Use (according to D'harawal Pharmacopoiea)
Acacia falcata	The bark was soaked overnight in warm water, then the liquid applied to skin rashes.
Acacia leprosa	The bark was dried, then soaked in hot water for several days. It was then applied to skin complaints.
Acacia melanoxylon	The bark was heated over hot coals, then placed over the areas of the body suffering rheumatic pain. The inner bark was roasted, then soaked overnight in warm water and the liquid used to bathe rheumatic and arthritic joints.
Adiantum formosum	The fronds were sundried, then, when required, soaked in hot water and the liquid sweetened with honey and taken to relieve fevers. The fronds were smoked to relieve the congestion of colds. The fronds were bruised, then soaked in warm water and the liquid was used as a mouth wash to relieve mouth ulcers.
Asplenium australasicum	The young fronds were mashed to a pulp and applied to sores and wounds.
Asplenium flavelifolium	The segments were chewed and the pulp applied to wounds.
Baeckea imbricata	The leaves were bruised, soaked in hot water, and the liquid used as a wash for wound and open cuts. The leaves and branches were placed on a low fire, and the vapour inhaled to ease breathing difficulties.
Baeckea ramosissima subsp. *ramosissima*	The entire plant was placed on a low fire and the vapour inhaled to ease breathing difficulties. The leaves were bruised, then soaked in hot water and the liquid used, when cool enough, as a wash for wounds and open cuts.
Hakea salicifolia	The bark was burned to charcoal, ground to powder, and applied daily to wounds and to lip and mouth sores.
Melaleuca linariifolia	The very fine inner bark layers were used to cover burns.
Mentha satureioides	The leaves were soaked in boiling water and the liquid taken to relieve stomach cramps. The leaves were bruised then soaked in hot water and taken to relieve the discomfort of colds and coughs. The whole plant was bruised, then boiled and the liquid given to women who were suffering menstrual disorders.
Pimelea linifolia	The bark was stripped from the roots, bruised and soaked in hot water until cool. The liquid was then strained and taken to relieve sore throats or relieve chest complaints. The bark was removed in strips, then plaited and tied around the head to ease headaches. At certain times of the year and under certain conditions, parts of this plant were collected, then treated and used as a treatment for cancerous growths.
Pittosporum undulatum	The seeds were crushed, the fruit pulp and the leaves bruised, then placed in boiling water or a few minutes. This was then allowed to stand until it was cool enough to drink, and taken to relieve pain and cramps. The fruit and leaves were bruised and soaked in hot water until cool. The liquid was strained and taken to relieve colds. The leaves were warmed then placed against the breasts of nursing mothers to promote lactation The seeds were ground to a paste, wrapped in paperbark and heated in the fire. This poultice was then placed over sore and swollen joints.
Syncarpia glomulifera subsp. *glomulifera*	The resin was applied to sores and ulcers.
Telopea speciossissima	The nectar was gathered and given to children who were sickly. The sap was used to heal burns.

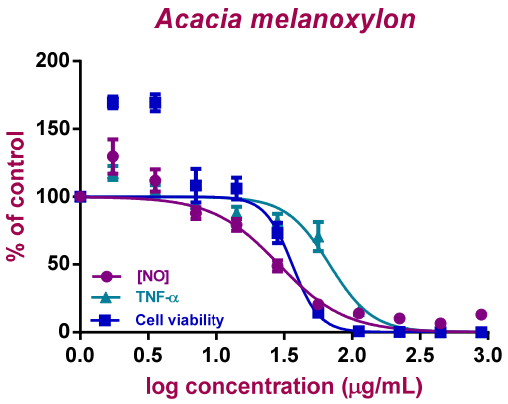
Table 4-4. Anti-inflammatory activity and toxicity of extracts determined in RAW 264.7 macrophages*

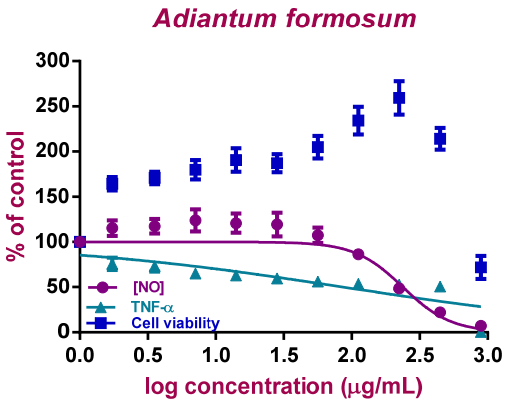
Potency scale	Plant Species	Inhibition of NO production (IC_{50} in µg/mL)	Inhibition of TNF-α production (IC_{50} in µg/mL)	Cytotoxicity (LC_{50} in µg/mL)	% Cytotoxocity
Highly potent	*Syncarpia glomulifera* subsp. *glomulifera*	8.25 ± 1.1	23.30 ± 3.4	52.09 ± 20.9	
	Melaleuca linariifolia	9.74 ± 2.0	16.40 ± 2.9	101.6 ± 13.5	
	Baeckea ramosissima subsp. *ramosissima*	15.68 ± 1.6	8.31 ± 3.3	85.68 ± 19.8	
	Baeckea imbricata	16.78 ± 4.0	22.77 ± 5.9	130.0 ± 47.5	
Moderately potent	*Acacia melanoxylon*	28.94 ± 5.0	68.20 ± 9.5	36.26 ± 7.2	
	Pimelea linifolia	30.36 ± 2.9	49.70 ± 9.9	226.3 ± 46.9	
	Acacia leprosa	35.49 ± 4.4	63.05 ± 13.4	103.9 ± 16.4	
	Pittosporum undulatum	66.36 ± 3.5	66.14 ± 15.0	108.1 ± 24.5	
Least potent	*Telopea speciossissima*	116.5 ± 20.1	555.1 ± 87.5	> 900	63.15
	Hakea salicifolia	195.9 ± 30.7	697.7 ± 185.3	> 900	149.93
	Acacia falcata	215.5 ± 31.1	133.60 ± 38.6	> 900	212.00
	Mentha satureioides	218.7 ± 20.8	62.75 ± 11.4	242.4 ± 88.7	
	Adiantum formosum	238.0 ± 46.8	86.65 ± 39.9	> 900	71.92
	Asplenium flavelifolium	280.7 ± 57.5	62.07 ± 28.0	> 900	190.01
	Asplenium australasicum	503.2 ± 60.5	917.0 ± 219.3	> 900	182.33

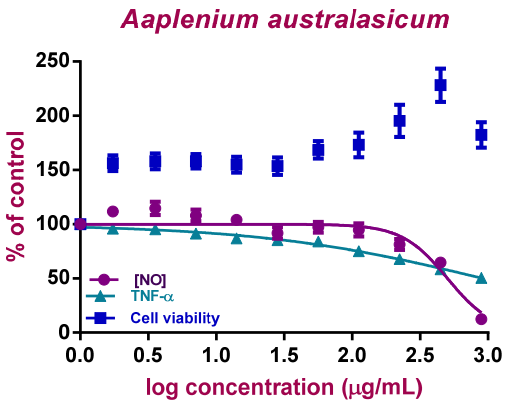
*Results represent the mean ± SEM of 3 experiments in quadruplicate for NO production and cytotoxicity whereas for TNF-α production it is 1 experiment in triplicate.

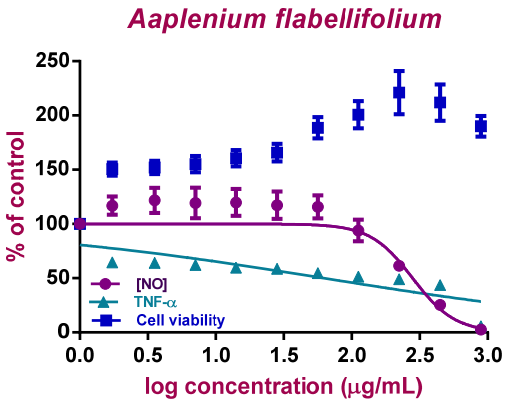

Acacia falcata
% of control
log concentration (μg/mL)
[NO]
TNF-α
Cell viability


Acacia leprosa
% of control
log concentration (μg/mL)
[NO]
TNF-α
Cell viability

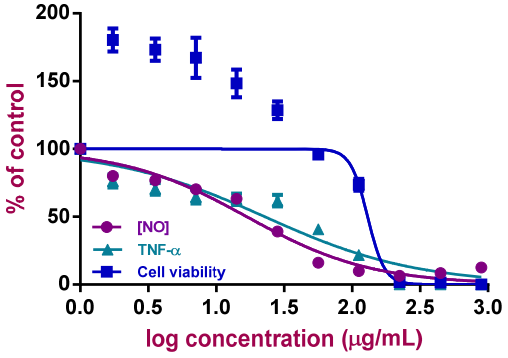

Acacia melanoxylon
% of control
log concentration (μg/mL)
[NO]
TNF-α
Cell viability


Adiantum formosum
% of control
log concentration (μg/mL)
[NO]
TNF-α
Cell viability


Aaplenium australasicum
% of control
log concentration (μg/mL)
[NO]
TNF-α
Cell viability


Aaplenium flabellifolium
% of control
log concentration (μg/mL)
[NO]
TNF-α
Cell viability

Baeckea imbricata



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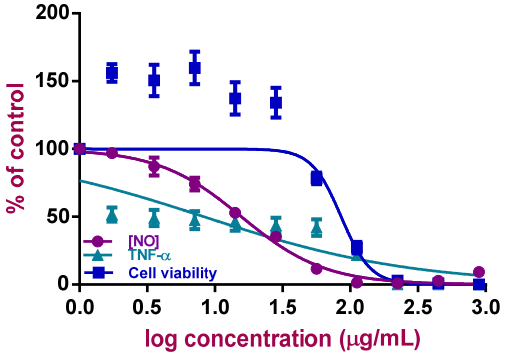

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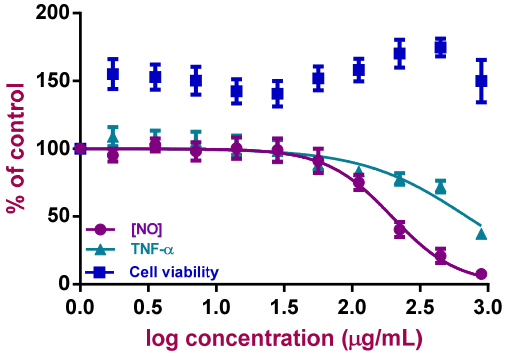
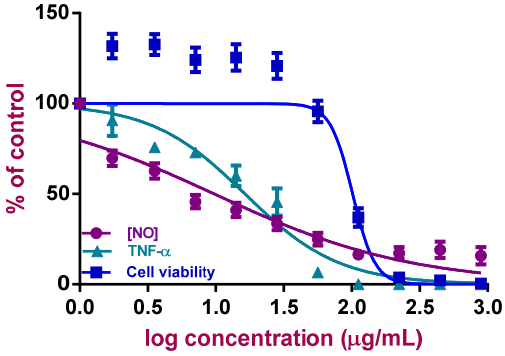


Melaleuca linariifolia



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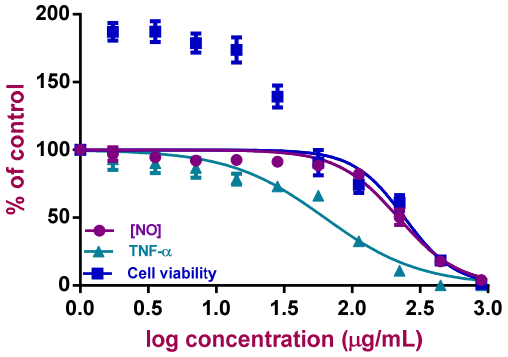

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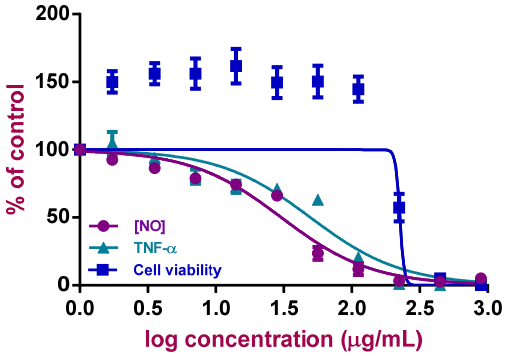


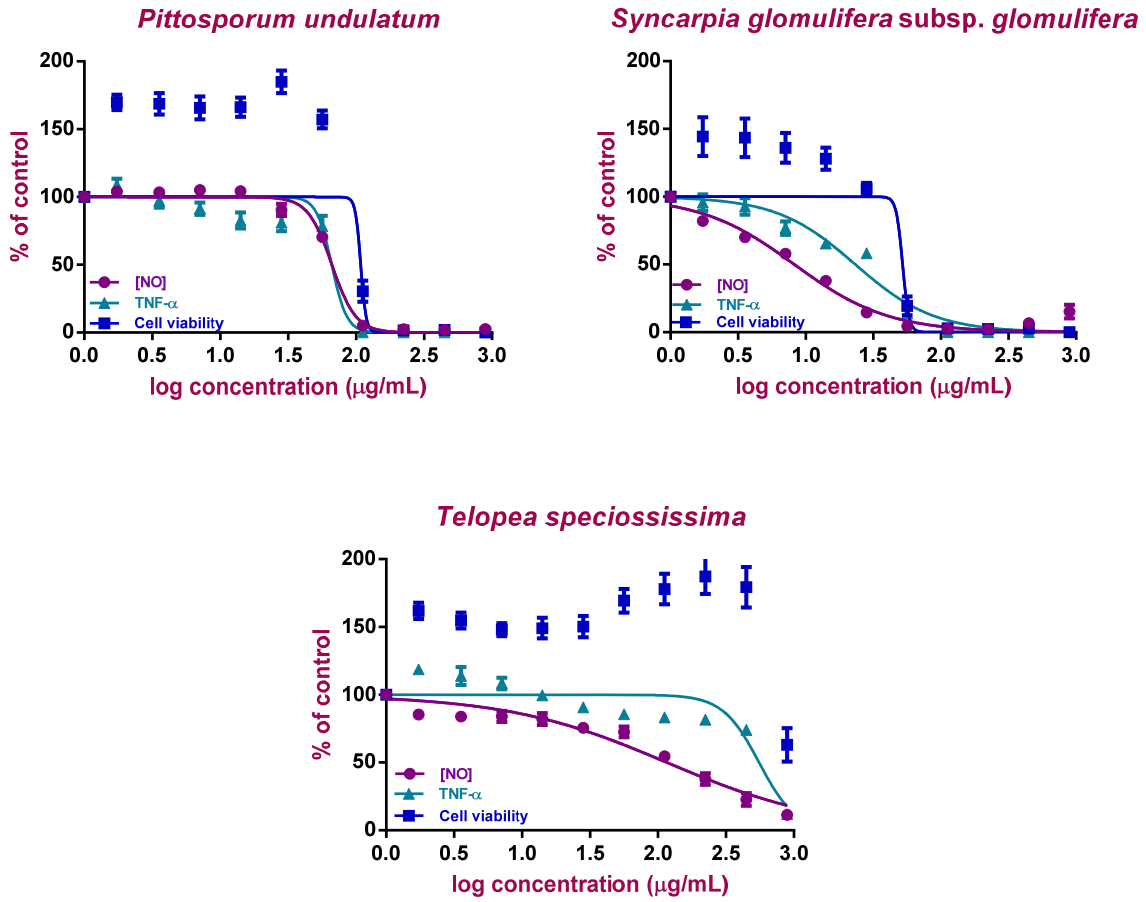
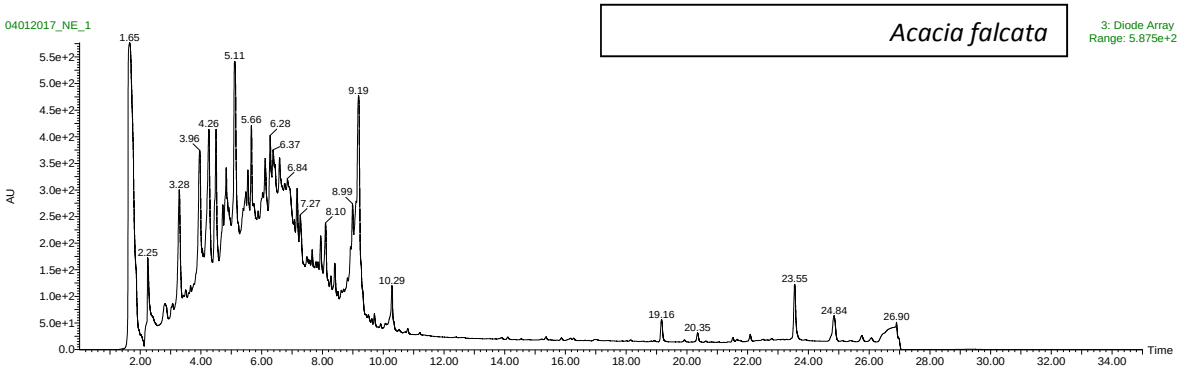


Figure 4-1. NO & TNF-α down regulation and cell viability of 15 extracts in LPS & IFN-γ induced RAW 264.7 macrophages

[Results represent mean ± SEM; 3 experiments in quadruplicate (n=12) for NO downregulation and cell viability and 1 experiment in triplicate (n=3) TNF-α inhibition]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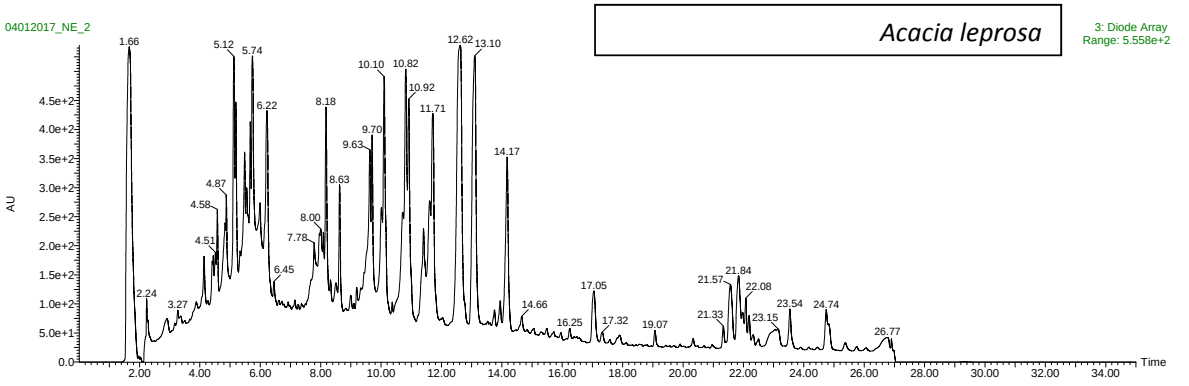
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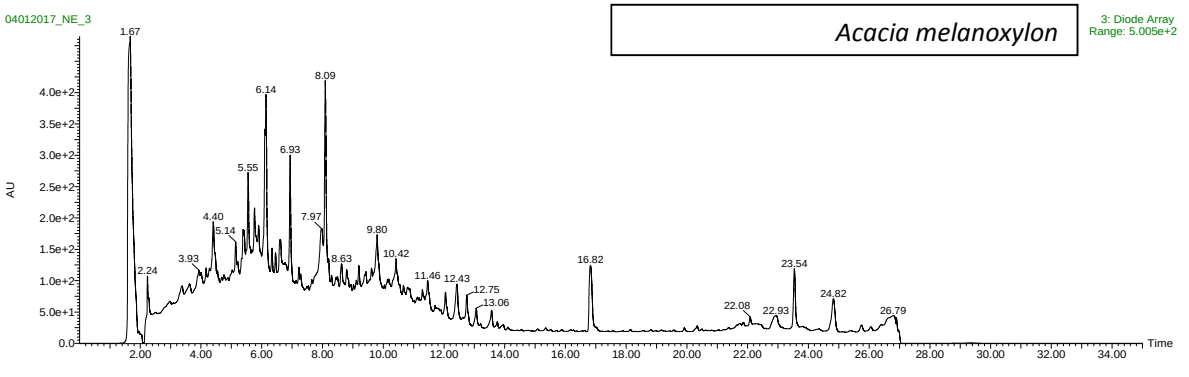
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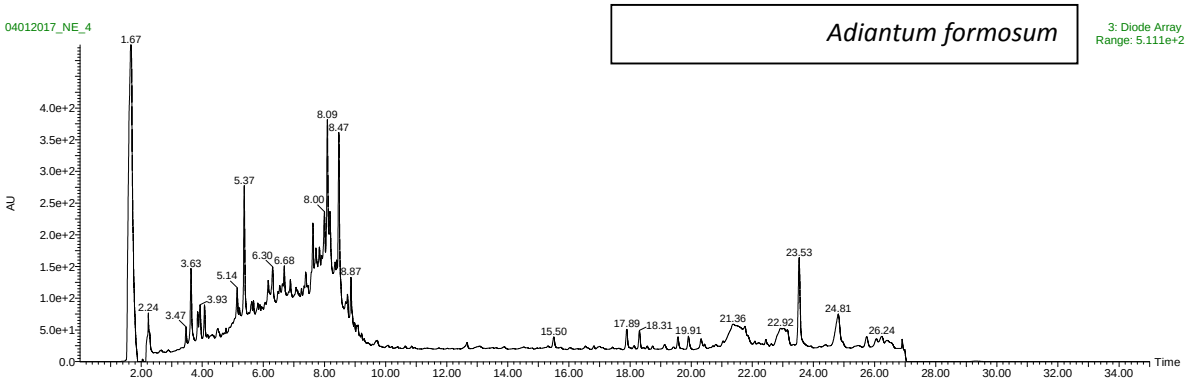
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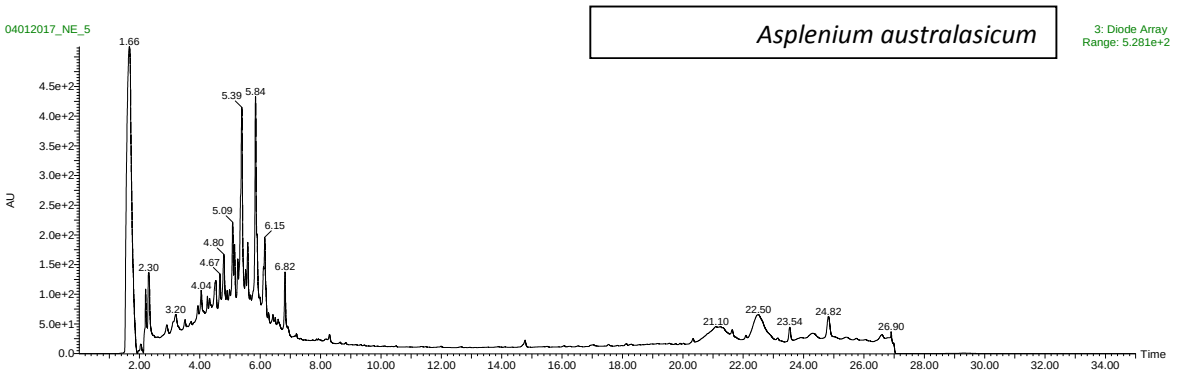
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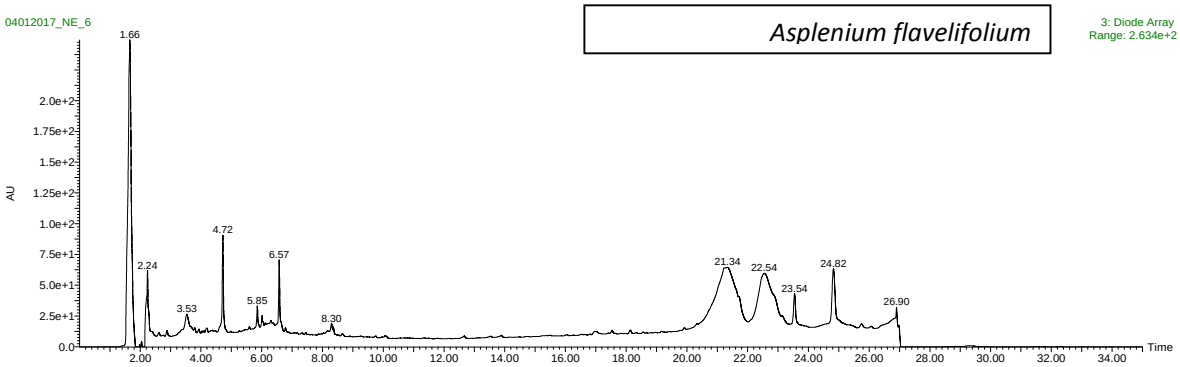
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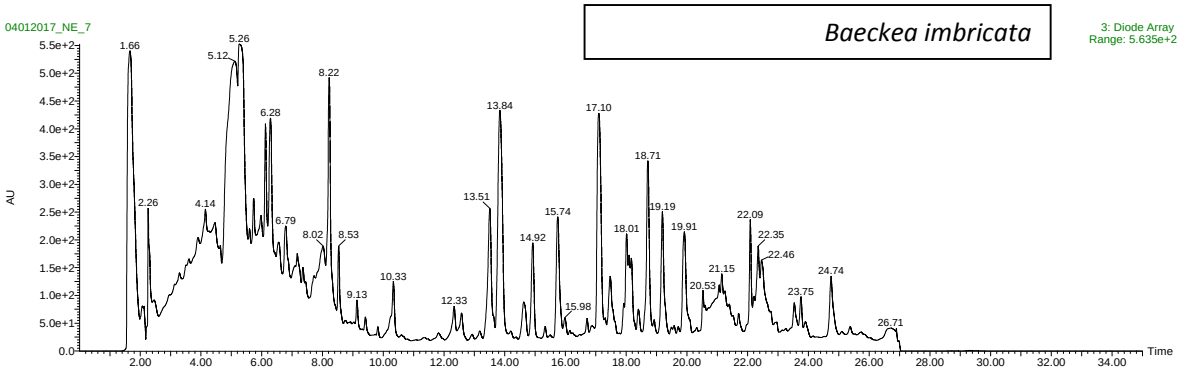
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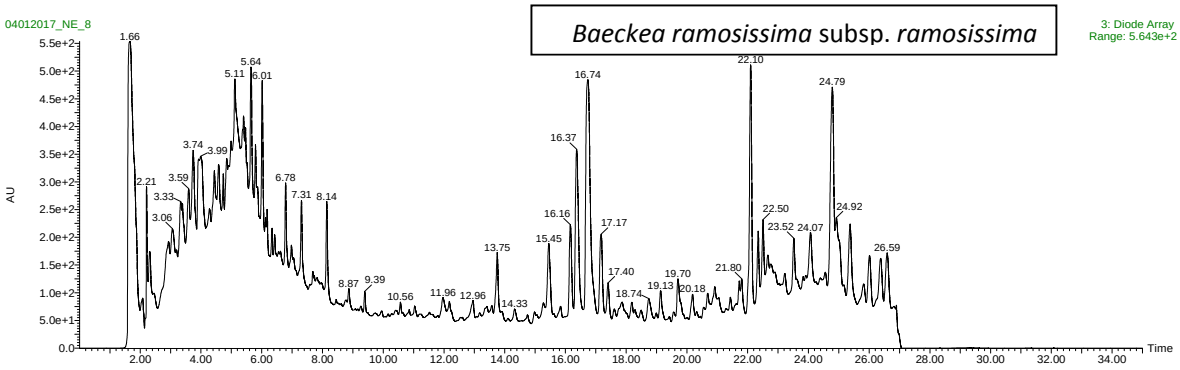
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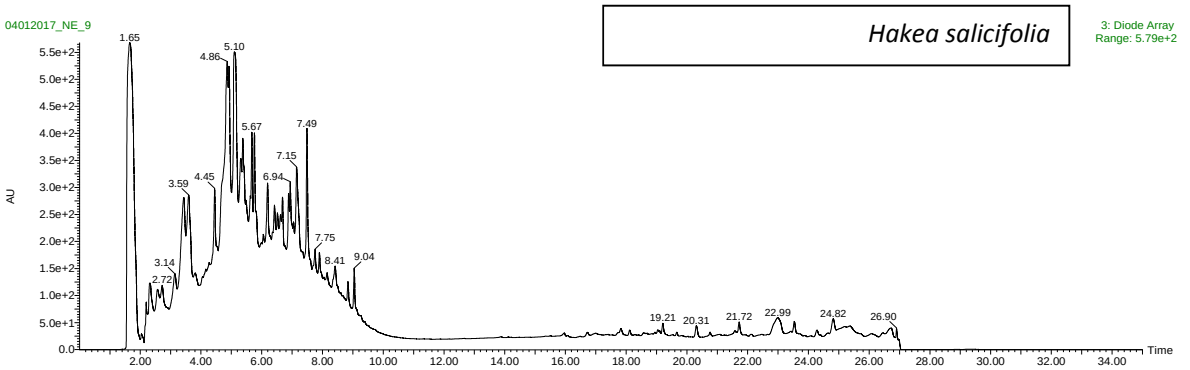
Time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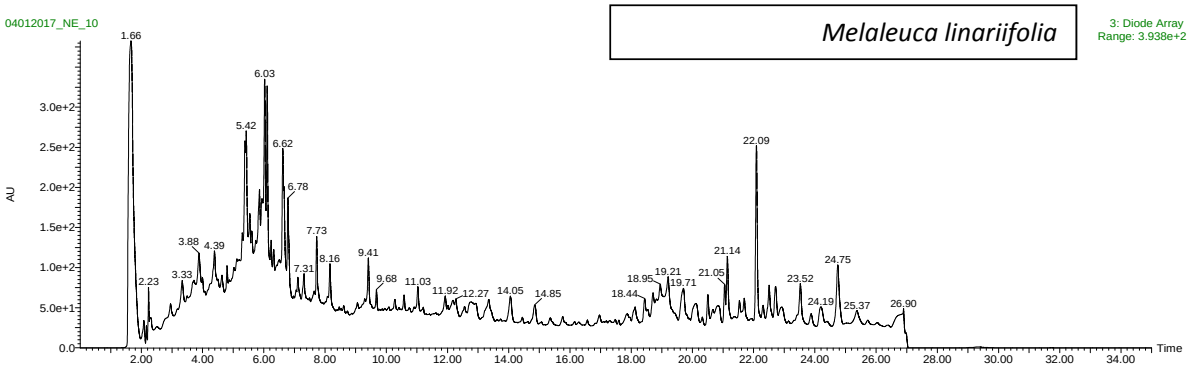
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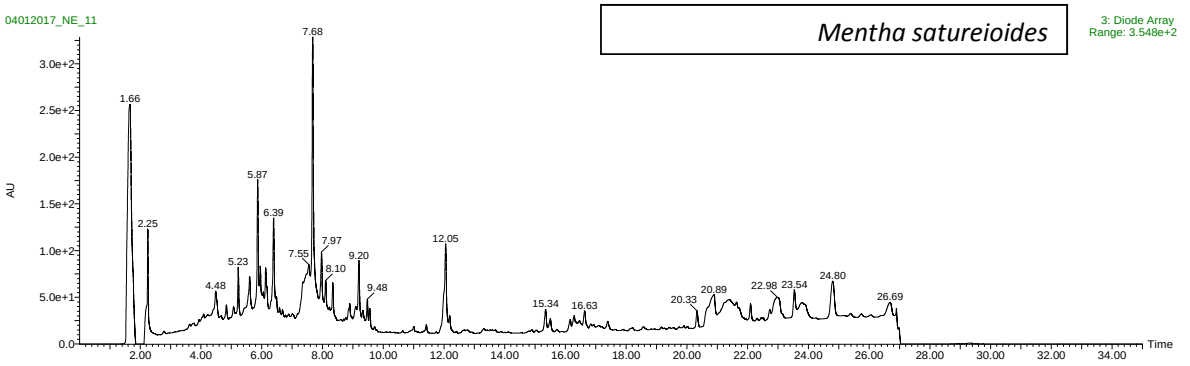
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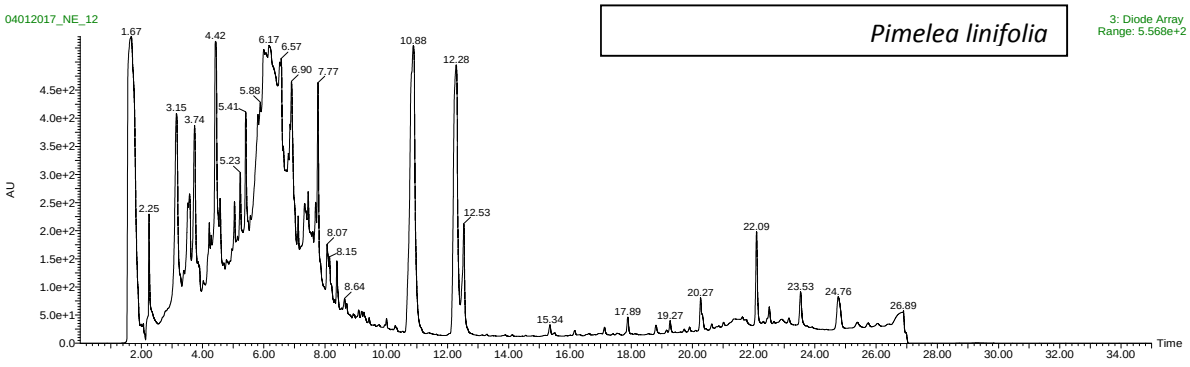
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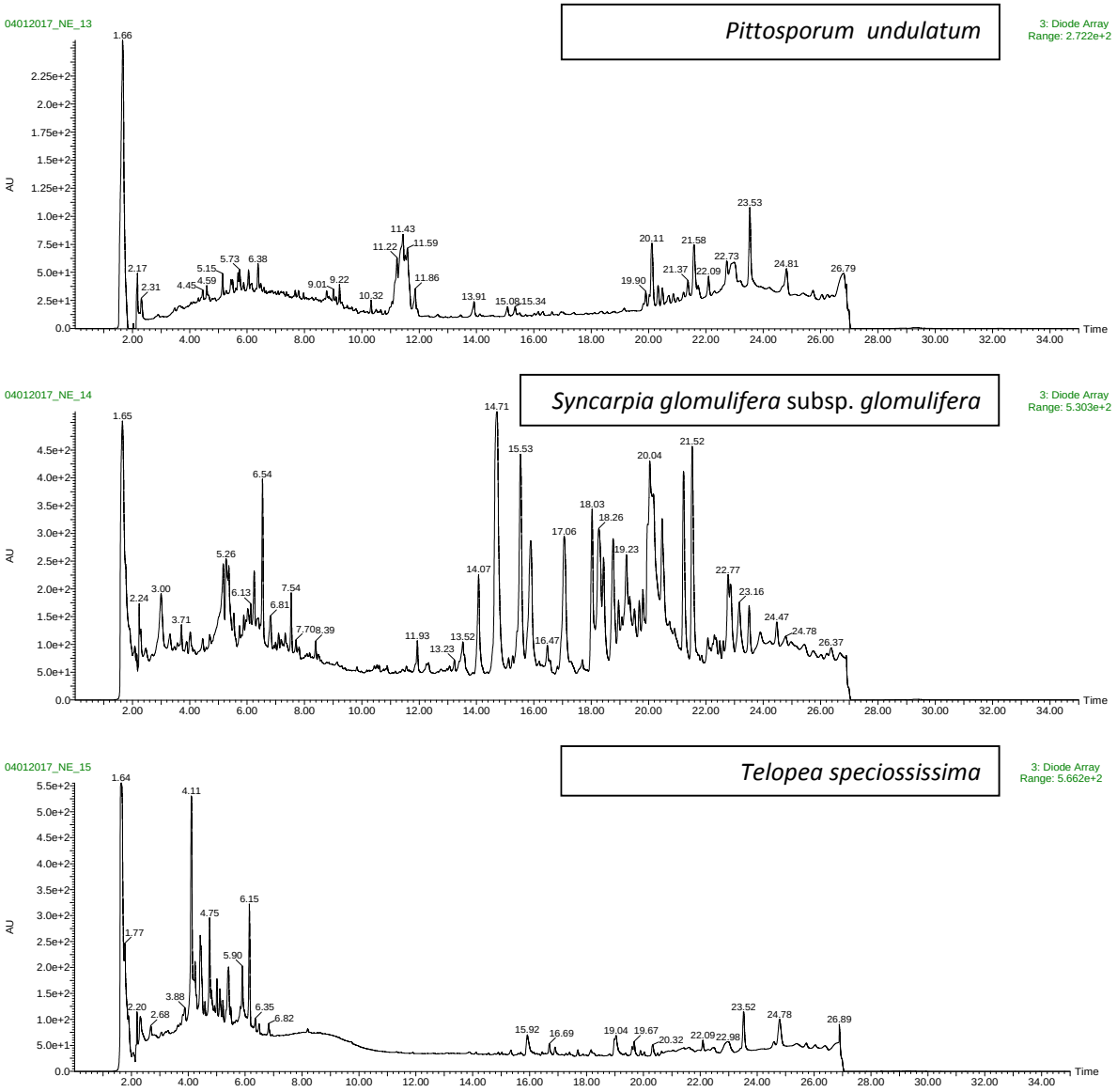

Figure 4-2. LC-MS Profiles of the 15 ethanolic extracts obtained from Australian native plant species

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Chapter 5

Anti-inflammatory Flavonoids and Triterpenoid from *Melaleuca linariifolia* (Myrtaceae)

5.1 Introduction

The plant family Myrtaceae is comprised of 130 genera, which contain more than 3000 species being predominantly distributed in tropical, subtropical, and temperate regions of the world [1]. In Australia *Melaleuca* (Myrtaceae) is considered to be the third most diverse plant genus after *Acacia* and *Eucalyptus* which represents 250 species many being undescribed. As stated in the literature, all known *Melaleuca* species are native to Australia and all except nine are endemic [2,3].

Melaleuca linariifolia is a tree up to 10 m high, cultivated in Australia [4]. It was an important plant to D'harawal people for its medicinal properties, with its use as an anti-inflammatory medicine featuring prominently. Other medicinal uses include covering burns with the inner fine layer of the trunk and fine powder from inner bark was used as good antiseptic. The vapor from the leaves was used as a sedative for crying babies (D'harawal Pharmacopoeia, unpublished).

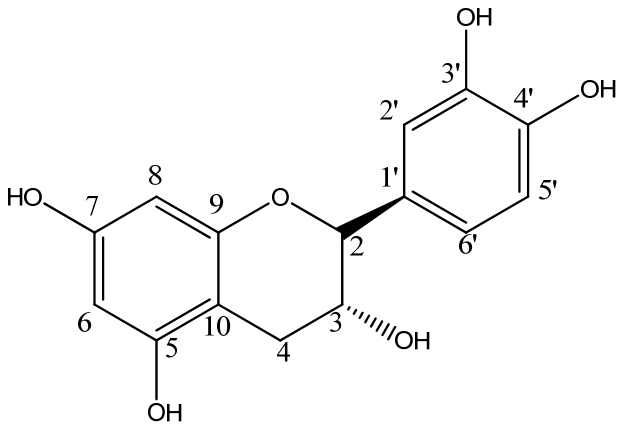
Figure 5-1. Leaves of *Melaleuca linariifolia.*

A number of phytochemical studies carried out on *Melaleuca* species indicate the presence of phenylpropanoids (methyl eugenol, (*E*)-methylisoeugenol), monoterpenoids (mainly, 1,8-cineole, terpinen-4-ol,terpinolene, along with *p*-cymene, *α*-terpinene, *α*-terpineol, *α*-pinene), and sesquiterpenoids (nerolidol, viridiflorol, ledol, *β*-caryophyllene etc.) as the major constituents [5-11]

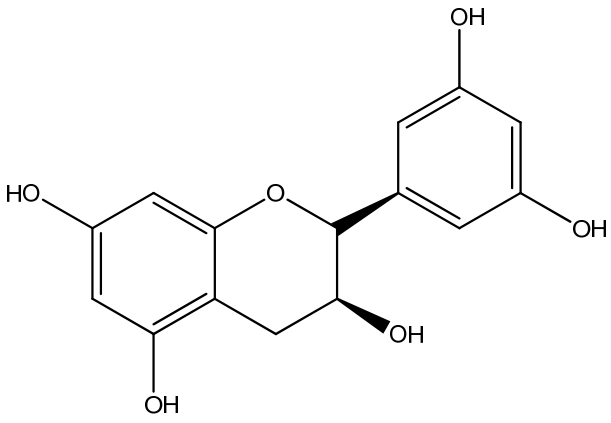
In this study, the crude EtOH leaf extract of *M. linariifolia* exhibited strong anti-inflammatory activity (IC_{50} = 9.74 µg/mL for NO inhibition) in LPS + IFN-γ activated 264.7 RAW macrophages and was selected for investigation to identify bioactive compounds. The fresh leaves of the plant were then sequentially extracted with *n*-hexane, DCM, EtOAc, EtOH, MeOH and water. The EtOAc extract showed the highest

anti-inflammatory activity (IC_{50} = 3.96 µg/mL for NO inhibition) in LPS + IFN-γ activated 264.7 RAW macrophages and was subjected to semi-preparative HPLC for fractionation.

Bioassay-guided fractionation of the extract led to the isolation of three known compounds, **5.1**, **5.2** and **5.3.** The structures of **5.1, 5.2** and **5.3** were confirmed by comparison of their mass spectrometry and NMR data[SI] with literature data.



5.1
3,3',4',5,7-Pentahydroxyflavan



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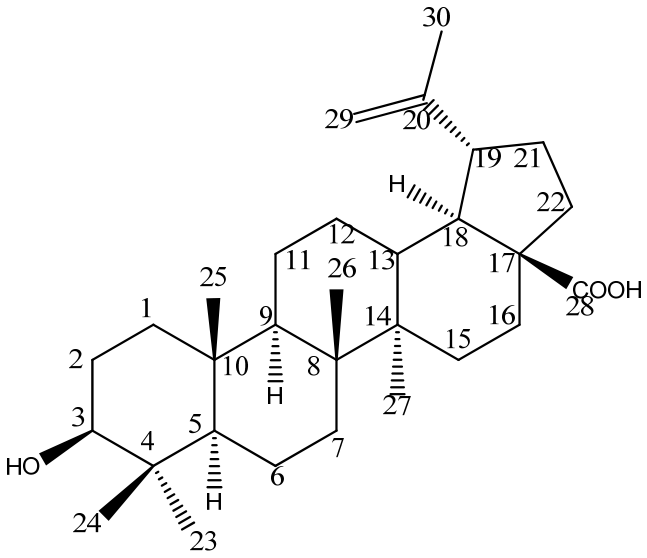
3,3’,5,5’,7- Pentahydroxyflavan



5.3
Betulinic acid

Figure 5-2. Structures of compounds **5.1, 5.2** and **5.3**

Compounds **5.1, 5.2** and **5.3** were evaluated for anti-inflammatory activity in LPS + IFN-γ induced RAW 264.7 macrophages and N11 microglia. In RAW 264.7 macrophages, compounds **5.1** and **5.2** showed moderate activity with IC_{50} values of 72.81 and 39.69 µg/mL respectively for NO inhibition and 58.88 and 80.70 µg/mL for TNF-α inhibition respectively. Both of the compounds did not show any toxicity up to the highest concentration (36 µg/mL) tested. On the other hand, compound **5.3** exhibited strong activity for both NO and TNF-α inhibition with IC_{50} values of 2.73 and 4.11 µg/mL respectively and an LC_{50} value of 9.86 µg/mL.

In N11 microglia, compound **5.1** and **5.2** were also moderate to down regulate NO and the IC_{50} values were 66.25 and 58.05 µg/mL but both compounds were found to be good to down regulate TNF-α with IC_{50} values of 17.34 and 21.84 µg/mL respectively. Compound **5.3** showed similar strong activities in this cell line as compared to RAW macrophages with IC_{50} value of 2.23 and 6.76 µg/mL for NO and TNF-α inhibition respectively and an LC_{50} value of 11.01 µg/mL.

Compound **5.1** and **5.2** were isolated from the genus and compound **5.3** from the species for the first time.

5.2 Results

5.2.1 Extraction and yield

Fresh leaves (130 g) of *Melaleuca linariifolia* (voucher no. AA 922868) were extracted sequentially with six solvents from low to high polarity according to the following: *n*-hexane, DCM, EtOAc, EtOH, MeOH and water in order to separate lipophilic from hydrophilic compounds. A variable range of yields were obtained from the extraction (Table 5.1).

Table 5-1. Yield of the extracts after sequential extraction of fresh leaves from *M. linariifolia*

Extract	**Yield of the extract (g)**
n-Hexane	2.01
DCM	5.91
EtOAc	3.40
EtOH	11.70
MeOH	10.18
Water	3.70
Total	**36.90**

5.2.2 Anti-inflammatory activity of extracts

The *n*-hexane, DCM, EtOAc, EtOH, MeOH and water extracts were tested in LPS + IFN-γ induced RAW 264.7 macrophages to obtain IC_{50} values for NO and TNF-α down regulation and also LC_{50} for cytotoxicity. Due to low solubility of the extracts in the cell culture media, they were diluted in DMSO, leading to a final concentration of up to 0.5% DMSO in cell culture medium. This concentration of DMSO showed a cell viability of less than 10% (data not shown). All six sequential extracts demonstrated significant anti-inflammatory activity, reducing NO production which was less than 32 µg/mL. The IC_{50} obtained for the inhibition of TNF-α production ranged between 19 – 176 µg/mL whereas LC_{50} for cytotoxicity exhibited values between 38 – 381 µg/mL (Figure 5-3 and Table 5-2). The sequential EtOAc extract showed highest activity with IC_{50} of 3.96 and 19.39 µg/mL for suppression of NO and TNF-α production respectively and an LC_{50} of 48.66 µg/mL.

Table 5-2. Anti-inflammatory activity of *M. linariifolia* sequential extracts

Sequential Extract	**Inhibition of NO production (IC_{50} in µg/mL)**	***Inhibition of TNF-α production (IC_{50} in µg/mL)**	**Cytotoxicity (LC_{50} in µg/mL)**
n-Hexane	30.15 ± 4.0	110.20 ± 44.8	221.70 ± 17.2
DCM	31.28 ± 4.0	176.20 ± 90.0	229.10 ± 22.6
EtOAc	3.96 ± 0.5	19.39 ± 7.2	48.66 ± 3.0
EtOH	7.16 ± 0.9	29.91 ± 9.9	76.21 ± 8.6
MeOH	20.70 ± 2.7	67.26 ± 24.1	38.84 ± 4.7
Water	31.48 ± 4.5	165.60 ± 103.9	381.30 ± 32.8

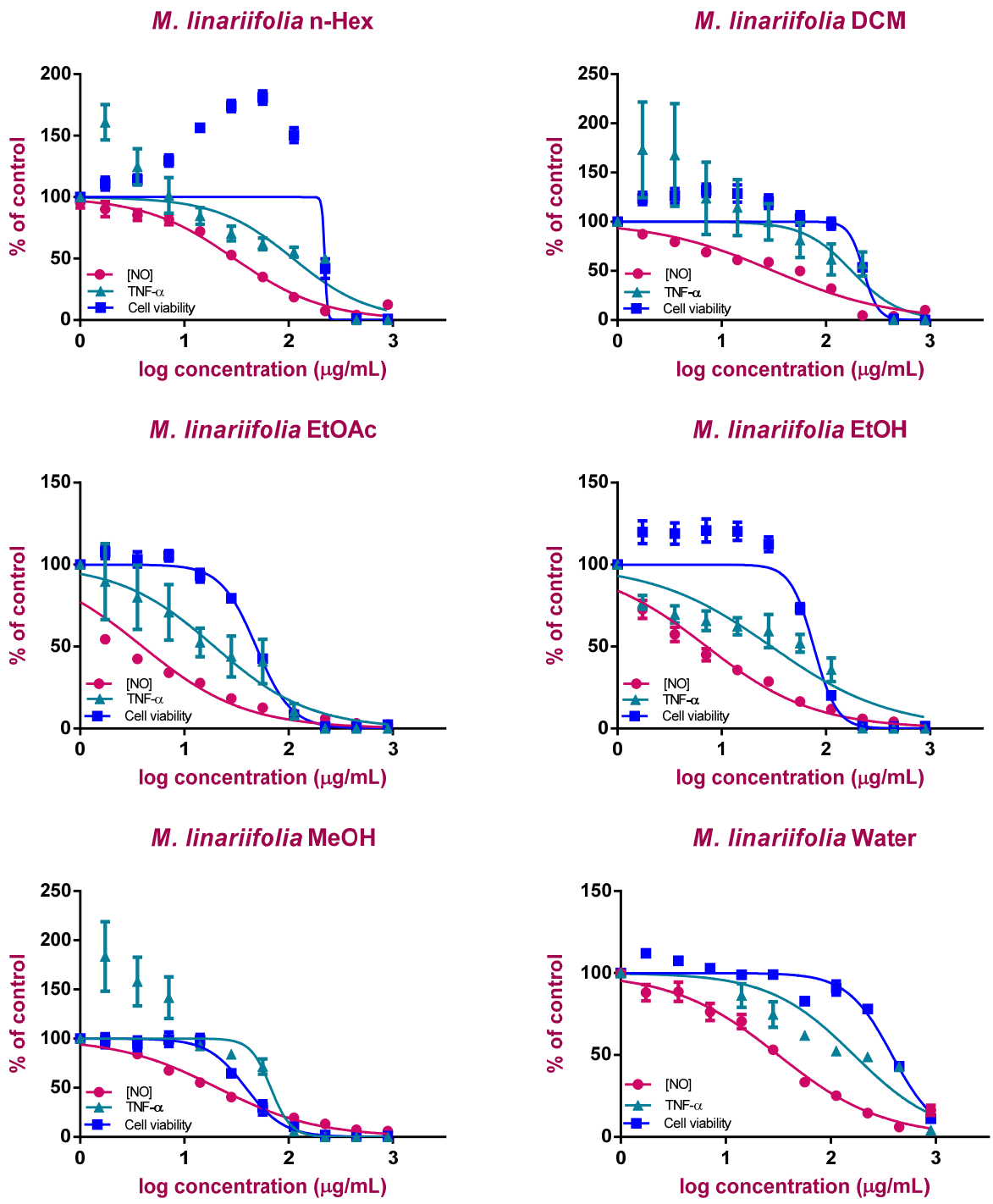
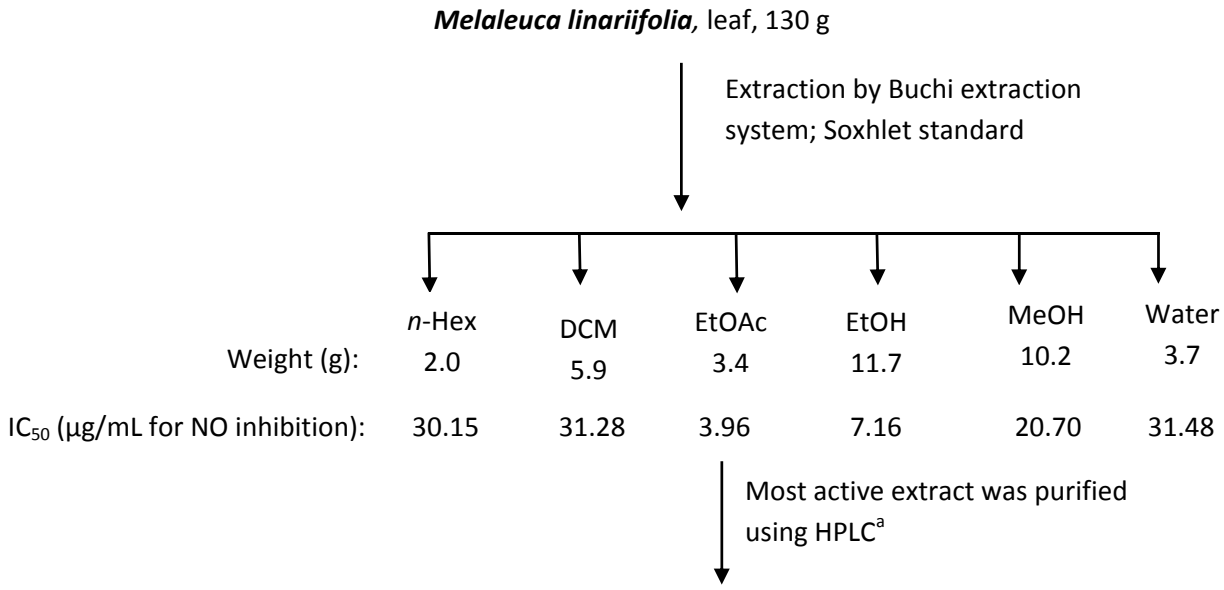


Figure 5-3. Inhibition of LPS and IFN-γ induced NO and TNF-α production and cell viability of *M. linariifolia* sequential extracts in RAW 264.7 macrophages. Data represented as three experiments in quadruplicates (n=12, mean ± SEM). * One experiment in triplicate (n=3, mean ± SEM).

5.2.3 Isolation of active compounds

In order to identify bioactive compounds, the sequential EtOAc extract (IC_{50} = 3.96 µg/mL for NO inhibition) was subjected to HPLC on a phenyl-hexyl column. This process led to the isolation of three known compounds (**5.1, 5.2** and **5.3**) (Scheme 5-1).



(a) Semi-preparative HPLC: Phenyl-Hexyl (250x10 mm; 10 µm), 20-100% MeOH/H_2O (0.01% HCO_2H modifier) over 50 mins, held for 10 mins then to 20% MeOH/H_2O on 60.10 min and held for last 5 minutes.

Scheme 5-1 Bioassay-guided separation of leaf extract of *Melaleuca linariifolia*

5.2.3.1 Identification of compound **5.1**

Compound **5.1** $[\alpha]_D^{25}$ −5.54 (c = 0.012, MeOH), was obtained as yellowish amorphous powder. The protonated molecular peak at *m/z* 291.0875 $[M+H]^+$ (calculated for $C_{15}H_{15}O_6$, 291.0869) corresponded to a molecular formula of $C_{15}H_{14}O_6$ with 9 degrees of unsaturation. The characteristic ^{1}H NMR peaks at H_δ 4.47 (1H, d, *J* = 7.4Hz), 3.81 (1H, ddd, *J* = 13.0, 7.3, 5.7 Hz), 2.65 (1H, dd, *J* = 16.0, 5.4 Hz) and 2.43 (1H, dd, *J* = 16.0, 7.4 Hz) were indicative of H-2, H-3, H-4a and H-4b respectively on the C-ring of a catechin moiety. The ^{1}H NMR spectrum of compound **5.1** (Table 5.1.b[SI]) also showed signals of 3 doublets at H_δ 6.72 (1H, d, *J* = 1.8), 6.68 (1H, d, *J* = 8.0) and 6.58 (1H, dd, *J* = 8.0, 1.5) indicative of H-2', H-5' and H-6' respectively were consistent to a 3',4' disubstituted B ring. Two meta coupled doublets at 5.89 (1H, d, *J* = 2.0 Hz) and 5.68 (1H, d, *J* = 2.0 Hz)] were indicative of H-6 and H-8 respectively were consistent with a 5,7-dioxygenated A ring of flavan. These proton resonances together with their corresponding carbon

signals assigned from HSQC and HMBC spectra (Table 5.1.b[SI]) revealed that compound **5.1** was a 3,3',4',5,7-pentahydroxyflavan. A coupling constant of 13.0 Hz between H-2 and H-3 suggested that these two protons were of *trans* orientation indicative of either (2*S*,3*R*) or (3*R*,2*S*) configurations for compound **5.1**. However the optical rotation value of **5.1** $[\alpha]_D^{25}$ −5.54 (*c* = 0.012, MeOH), was found to be similar to that of (-) catechin [12-16]. Thus the structure of compound **5.1** was determined to be (-)-catechin or 2*S*,3*R*-3,3',4',5,7-pentahydroxyflavan (Figure 5-2).

5.2.3.2 Identification of compound **5.2**

Compound **5.2** $[\alpha]_D^{25}$ +4.51 (c = 0.01, MeOH), was obtained as yellowish amorphous powder. The protonated molecular peak at *m/z* 291.0864 $[M+H]^+$ (calculated for $C_{15}H_{15}O_6$, 291.0869) corresponded to a molecular formula of $C_{15}H_{14}O_6$ with 9 degrees of unsaturation. The ^{1}H NMR spectrum of compound **5.2** (Table 5.2.b[SI]) showed signals at H_δ 4.72 (1H, s), 4.0 (1H, brs), 2.65 (1H, dd, *J* = 16.0, 5.1) and 2.45 (1H, d, *J* = 3.6) which were indicative of H-2, H-3, H-4a and H-4b respectively. The ^{1}H NMR spectrum also exhibited three meta-coupled doublets at H_δ 6.65 (2H, d, *J* = 2.7 Hz) indicative of H-2', H-6' and 6.88 (1H, d, *J* = 2.4 Hz) indicative of H-4', which were attributable to a 3',5' disubstituted B ring of flavane. Two meta-coupled doublets at H_δ 5.88 (1H, d, *J* = 2.4 Hz) and 5.71 (1H, d, *J* = 2.4 Hz), indicative of H-6 and H-8 were consistent with a 5,7-dioxygenated A ring of flavan. These proton resonances together with their corresponding carbon signals assigned from HSQC and HMBC spectra (Table 5.2.b[SI]) revealed that compound 5.2 was a 3,3',5,5',7- pentahydroxyflavan. The 2,3-*cis* configuration of **5.2** was determined on the basis of a broad singlet at H_δ 4.0 for the H-3 and the upfield shift at C_δ 64.6 of C-3 with the 2-phenyl group in a pseudo-equatorial orientation and the 3-hydroxy group in a pseudo-axial orientation indicating the configurations were (2*S*,3*S*) or (2*R*,3*R*). However, the optical rotation value of **5.2** $[\alpha]_D^{25}$ +4.51 (*c* = 0.01, MeOH) was found to be similar to that of 2*S*,3*S*-3,3',5,5',7-pentahydroxyflavan [17,18]. Thus the structure of compound **5.2** was determined to be 2*S*,3*S*-3,3',5,5',7-pentahydroxyflavan (Figure 5-2).

5.2.3.3 Identification of compound **5.3**

Compound **5.3** was obtained as white amorphous powder. The HR-ESI-MS (positive ion mode) of this compound revealed a quasi-molecular ion peak at *m/z* 479.3513 $[M+Na]^+$ (calculated for $C_{30}H_{48}O_3Na$, 479.3501) suggested a molecular formula of $C_{30}H_{48}O_3$ with 7 degrees of unsaturation. The ^{1}H NMR spectrum demonstrated the pattern characteristic of lupane type skeleton. It showed 4 methyl singlets at H_δ 0.65, 0.76, 0.94 and 1.64 (each 3H, 25, 26, 27 and 30 respectively) as well as a singlet at H_δ 0.88 (each 3H, 23 and 24) representing 6 methyl groups in the structure. The terminal methylene protons at H_δ 4.56 and 4.68 (each 1H, s) were assigned to H-29. The rest of the protons observed as multiplates and were overlapping aliphatic CH and CH_2 groups. The ^{1}H NMR, HSQC and HMBC spectra of compound **5.3** (table 5.3.b[SI]) indicated the presence of 1 carbonyl [C_δ 177.5 (C-28)], 6 quaternary [150.7 (C-20), 55.4 (C-17), 42.5 (C-14), 40.8 (C-8), 39.1 (C-4) and 37.4 (C-10)], 6 methine [C_δ 76.5 (C-3), 54.7 (C-5), 49.8 (C-9), 48.8 (C-18), 46.6 (C-19) and 37.5 (C-13)], 11 methylene [C_δ 110.2 (C-29), 37.7 (C-1), 30.4 (C-21), 37.7 (C-7), 31.5 (C-16), 28.3 (C-15), 37.7 (C-22), 29.0 (C-2), 28.0 (C-12), 20.7 (C-11) and 18.0 (C-6)], and 6 methyl [C_δ 28.0 (C-23), 15.7 (C-24), 15.7 (C-25), 15.7 (C-26), 14.0 (C-27) and 18.6 (C-30)] carbons in the

structure. By comparing the mass spectrometry and NMR data[SI], with that of published one, compound **5.3** was identified as the known triterpenoid, betulinic acid (Figure 5-2) [19,20].

5.2.4 Anti-inflammatory activity of identified compounds

Compounds **5.1**, **5.2** and **5.3** were evaluated for anti-inflammatory activity in LPS + IFN-γ induced RAW 264.7 macrophages (Figure 5-4 and Table 5-3) and N11 microglia (Figure 5-5 and Table 5-4).

In RAW 264.7 macrophages, compounds **5.1** and **5.2** showed moderate activity with IC_{50} values of 72.81 and 39.69 µg/mL respectively for NO inhibition and 58.88 and 80.70 µg/mL for TNF-α inhibition respectively. Both of the compounds did not show any toxicity up to the highest concentration (36 µg/mL) tested. On the other hand, compound **5.3** exhibited strong activity for both NO and TNF-α inhibition with IC_{50} values of 2.73 and 4.11 µg/mL respectively and an LC_{50} value of 9.86 µg/mL.

In N11 microglia, compound **5.1** and **5.2** were also moderate to down regulating of NO and the IC_{50} values were 66.25 and 58.05 µg/mL but both compounds were found to be more effective to down regulate TNF-α with IC_{50} values of 17.34 and 21.84 µg/mL respectively. Both of the compounds did not show any toxicity up to the highest concentration (36 µg/mL) tested. Compound **5.3** showed strong activity in this cell line too as compared to RAW macrophages with IC_{50} values of 2.23 and 6.76 µg/mL for NO and TNF-α inhibition respectively and an LC_{50} value of 11.01 µg/mL.

Table 5-3. Anti-inflammatory activity of compounds isolated from *M. linariifolia* in LPS + IFN-γ induced RAW 264.7 macrophages

Compound	**Inhibition of NO production (IC_{50} in µg/mL)**	**Inhibition of TNF-α production (IC_{50} in µg/mL)**	**Cytotoxicity (LC_{50} in µg/mL)**	**% Cytotoxicity**
5.1	72.81 ± 37.9	58.88 ± 35.9	> 36	128.5
5.2	39.69 ± 11.5	80.70 ± 50.1	> 36	131.8
5.3	2.73 ± 0.3	4.11 ± 1.0	9.68 ± 1.1	

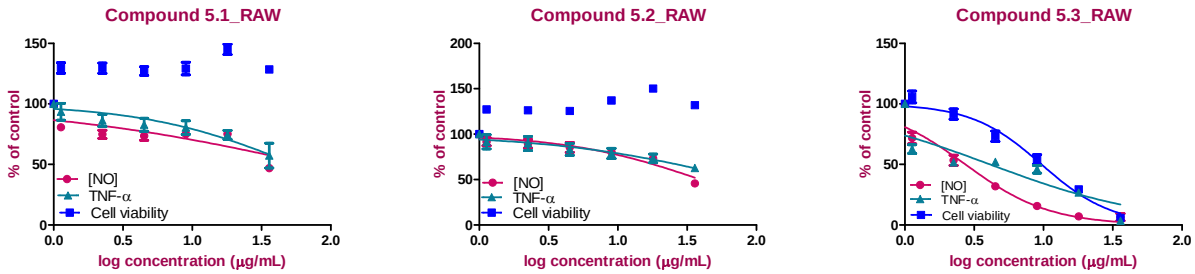


Figure 5-4. Down regulation of LPS and IFN-γ induced NO and TNF-α production and cell viability of isolated compounds from *M. linariifolia* in RAW 264.7 macrophages. Data represented as two experiments in quadruplicates (n=8, mean ± SEM).

Table 5-4. Anti-inflammatory activity of compounds isolated from *M. linariifolia* in LPS + IFN-γ induced N11 microglia

Compound	Inhibition of NO production (IC_{50} in µg/mL)	Inhibition of TNF-α production (IC_{50} in µg/mL)	Cytotoxicity (LC_{50} in µg/mL)	% Cytotoxicity
5.1	66.27 ± 31.3	17.34 ± 3.3	> 36	113.0
5.2	58.05 ± 30.1	21.84 ± 3.6	> 36	129.0
5.3	2.23 ± 0.4	6.76 ± 1.4	11.01 ± 1.4	

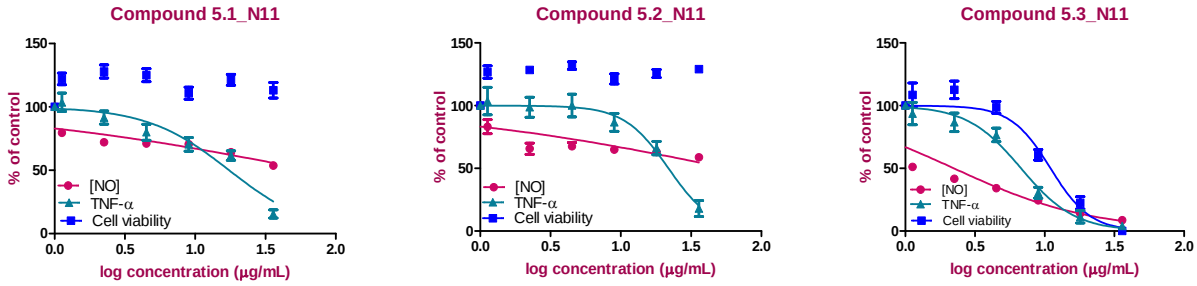


Figure 5-5. Down regulation of LPS and IFN-γ induced NO and TNF-α production and cell viability of isolated compounds from *M. linariifolia* in N11 microglia. Data represented as two experiments in quadruplicates (n=8, mean ± SEM).

5.3 Discussion

Compound **5.1** has four epimers [13] commonly known as catechin and are reported to have anti-oxidant and anti-inflammatory activity in different studies [21-24]. However, compound **5.1** (2*S*, 3*R* - form) showed moderate anti-inflammatory activity with an exception of down regulating TNF-α in N11 cell line which was stronger with an IC_{50} value of 17.34 µg/mL. This finding is not surprising since studies have shown biological activities of many drugs vary extensively among the stereoisomers [25-28].

Compound **5.2** is a constitutional isomer of compound **5.1** and reported to have anti-oxidant activity [18]. The anti-inflammatory activity of compound **5.2** is being reported for the first time. However, this compound is moderately active in both cell lines under investigation, with an exception to down regulate TNF-α in N11 microglia which was strong with an IC_{50} value of 21.84 µg/mL.

Compound **5.3** is a widely studied compound and is reputed for many biological activities including anti-inflammatory activity [20,29-33]. In our study, we also found this compound is very strong to downregulate NO and TNF-α in LPS + IFN-γ induced RAW macrophages with IC_{50} values of 2.73 and 4.11 µg/mL respectively as well as in N11 microglia with IC_{50} values of 2.23 and 6.67 µg/mL respectively. Compound **5.3** is cytotoxic too having an LC_{50} of 9.68 µg/mL in RAW cell and 11.01 µg/mL in N11 cell line.

Compound **5.1** and **5.2** were isolated from the genus and compound **5.3** from the species for the first time.

Table 5-5. Potency and total anti-inflammatory activity of *M. linariifolia* sequential extracts in LPS + IFN-γ induced RAW 264.7 macrophages

Sequential Extract	Potency for NO inhibition(L/g)	Potency for TNF-α inhibition (L/g)	Total anti-inflammatory (NO inhibition) activity of extract (L)	Total anti-inflammatory (TNF-α inhibition) activity of extract (L)
n-Hexane	33.17	9.07	66.67	18.24
Dichloromethane	31.97	5.68	188.94	33.54
Ethyl acetate	252.53	51.57	858.59	175.35
Ethanol	139.66	33.43	1634.08	391.17
Methanol	48.31	14.87	491.79	151.35
Water	31.77	6.04	117.53	22.34
Total anti-inflammatory activity of all extracts (L)			3357.59	792.00

Table 5-6. Potency and total anti-inflammatory (AI) activity of compounds isolated from *M. linariifolia* in LPS + IFN-γ induced RAW 264.7 macrophages

Individual compounds	Potency for NO inhibition(L/g)	Potency for TNF-α inhibition (L/g)	Total anti-inflammatory (NO inhibition) activity of compound (L)	Total anti-inflammatory (TNF-α inhibition) activity of compound(L)
Catechin (**5.1**)	13.73	16.98	0.09	0.11
3,3',5,5',7-Pentahydroxyflavan (**5.2**)	25.20	12.39	0.10	0.05
Betulinic acid (**5.3**)	366.30	243.31	2.93	1.95
Total anti-inflammatory activity of all identified compounds (L)			3.12	2.11
% of total activity			0.09	0.27

The potency (for NO and TNF-α inhibition) of each of the sequential extracts and isolated compounds from *M. linariifolia* leaves were measured as a reciprocal of IC_{50} value (Table 5-5 and 5-6). The total anti-inflammatory activity for both NO and TNF-α inhibition (Table 5-5 and 5-6) of the same extracts and compounds were also obtained by multiplying respective potency and yield of dried extracts and isolated compounds. Thus the total anti-inflammatory activity of all extracts was found to be 3357.59 L for NO inhibition in 130 g leaves of *M. linariifolia* of which 0.09 L, 0.10 L and 2.29 L were obtained by catechin (**5.1**), 3,3',5,5',7-pentahydroxyflavan (**5.2**) and betulinic acid (**5.3**) respectively. Similarly, the total anti-inflammatory activity of all extracts was found to be 792.00 L for TNF-α inhibition in 130 g leaves of which 0.11 L, 0.05 L and 1.95 L were obtained by catechin (**5.1**), 3,3',5,5',7-pentahydroxyflavan (**5.2**) and betulinic acid (**5.3**) respectively.

The compounds were isolated by purifying only the EtOAc extract which was 1.25% of the total extract. Thus the total anti-inflammatory activity of all isolated compounds were calculated 3.92 L and 2.65 L for NO and TNF-α inhibition respectively in the leaves of *M. linarifolia* under investigation.

5.4 Experimental Section

5.4.1 General experimental procedures

Acetonitrile, *n*-hexane, dichloromethane and methanol were supplied by Merck. Ethyl acetate from Fisher Chemical, ethanol from Chem-Supply and water used was Milli-Q water. Bovine serum albumin, lipopolysaccharide (*E. coli* serotype-0127:B8), EDTA, N-(1-napthyl) ethylenediamine dihydrochloride, benzylpenicillin G sodium salt, resazurin sodium salt (10%), streptomycin, sulphanilamide, 3,3',5,5'-tetramethylbenzidine (TMB), trypan blue and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Foetal bovine serum (FBS) and glutamine were purchased from Life Technologies (Mulgrave, VIC, Australia). Murine interferon-γ (IFN-γ) and TNF-α

ELISA kits were purchased from PeproTech Asia (Rehovot, Israel). Citric acid and monosodium dihydrogen carbonate (NaH_2CO_3) were from AJAX Chemicals (Auburn, NSW, Australia). Tween-20 was from Amresco (Solon, Ohio, USA). Methanol, monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), sodium chloride (NaCl) and sulfuric acid (H_2SO_4) were from Merck (Darmstadt, Germany). Sodium carbonate (Na_2CO_3) was BDH brand supplied by Merck Pty. Ltd. (Kilsyth, VIC, Australia).

5.4.2 Experimental procedures for MS, HPLC and NMR

Semi-preparative HPLC was performed using an Agilent 1260 Infinity Series (Santa Clara, CA, USA). NMR spectra were obtained on a Bruker Ascend 400 MHz spectrometer (Bruker Biospin GmbH, Bremen, Germany). TopSpin software was used to analyse the spectral data. ^{1}H NMR spectra were recorded at 400 MHz. The chemical shifts (δ) are expressed in parts per million (ppm) as δ values and coupling constant *J* in Hertz (Hz). COSY, HSQC and HMBC were acquired using the standard Bruker pulse program. The experiments were performed in dimethyl sulfoxide-d_6 (DMSO-d_6) and chemical shifts were calibrated relative to the DMSO solvent peak (^{1}H δ at 2.50 and ^{13}C δ 39.51 ppm). High resolution mass spectrometry (HRMS) was carried out using a Waters Xevo Q-TOF mass spectrometer operating in positive electrospray ionization (ESI) mode.

5.4.3 Plant material

Fresh leaves of *M. linariifolia* (voucher no. AA 922868) were collected in the month of July, 2016 from the 'Australian Botanic Gardens' at Mount Annan, NSW, Australia. A voucher specimen was deposited at the National Herbarium of NSW, Australia.

5.4.4 Extraction and isolation

Approximately 130 g of fresh leaf material from *M. linariifolia* was first cut into small pieces with scissors and then ground to a coarse powder using a hand blender. The coarse powder was then filled into the thimbles of an accelerated solvent extraction system (Buchi B-811, Switzerland) and extracted under standard Soxhlet mode (for 2 × 15 minutes cycles) using six solvents (*n*-hexane, dichloromethane, ethyl acetate, ethanol, methanol and water) sequentially from low to high polarity. The volume of the extracts was reduced to a volume of 2—4 mL using a rotary evaporator and then evaporated to dryness with nitrogen. The sequential ethyl acetate extract was found most potent when screened for anti-inflammatory activity. This crude extract was then resuspended in methanol and subjected directly through reversed phase semi-prep HPLC (Phenyl-Hexyl column, 250 x 10 mm, Phenomenex). Peak detection was performed with a photodiode array detector (PDA) using three detection wavelengths (210 nm, 254 nm and 290 nm). A gradient starting from 20% methanol/water to a 100% methanol (with a constant 0.01% formic acid modifier) over 50 min and held at 100% methanol for 10 min to yield compound **5.1** (t_R = 15.2 min, 6.6 mg), compound **5.2** (t_R = 19.0 min, 4.1 mg) and compound **5.3** (t_R = 53.3 min, 8.0 mg). All isolated compounds were purified to 95% purity or more as judged by HPLC (UV detection) and ^{1}H NMR spectroscopy before determining the bioactivity.

5.4.5 Maintenance and preparation of RAW 264.7 macrophages and N11 microglia

RAW 264.7 macrophages and N11 microglial cells were grown in 175 cm^2 culture flasks on DMEM (Dulbecco's Modified Eagle's Medium) containing 5% FBS (foetal bovine serum) that was supplemented with penicillin (100 U/mL), streptomycin (100 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. Once cells had grown to confluence in the culture flask, they were removed using a rubber policeman cell scraper, as opposed to using trypsin, which can remove membrane-bound receptors. The cell suspension was concentrated by centrifugation for 3 min at 900 rpm and resuspended in a small volume of fresh DMEM (with 1% antibiotics and 5% FBS). Cell densities were estimated using a Neubauer counting chamber. The cell concentration was adjusted with DMEM (with 1% antibiotics and 5% FBS) to obtain 60,000 cells/100 µL cell suspension. 100 µL of cell suspension was then dispensed into the wells of 96-well plates (Eppendorf, Hamburg, Germany). Plates were incubated at 37 °C; 5% CO_2 for 24 h before the activation experiments were carried out.

5.4.6 Activation of RAW 264.7 macrophages and N11 microglia

From each well, the media was removed and replaced with fresh DMEM containing 0.1% FBS. For assays with extracts, a 90 µL volume of the dilutions in DMEM (with 0.1% FBS) was added an hour prior to addition of the activator. Due to the often inconsistent nature of LPS at activating cells, a combination of LPS (10 µg/mL) and IFN-γ (10 U/mL), both in DMEM (with 0.1% FBS), were used for activation. For the sequential extracts a maximum dose of 900 µg/mL was used, and diluted serially by 50% up to a minimum of 10 doses (900, 450, 225, 112.5, 56.25, 28.125, 14.062, 7.031, 3.515, 1.7578 and 0.8789 µg/mL in the wells respectively). A maximum dose of the compounds (**5.1, 5.2** and **5.3**) used is 36 µg/mL, and diluted serially by 50% up to a minimum of 7 doses (36, 18, 9, 4.5, 2.25, 1.12 and 0.56 µg/mL in the wells respectively). After activation, the cells were incubated for 24 h at 37 °C and 5% CO_2 and then NO and TNF-α inhibition, and cell viability were determined. Cells with media alone were used as negative control and activated cells used as positive control. The effects of solvents on readouts were initially determined, but as the anti-inflammatory or cytotoxic effects of the solvents were < 10% even at the highest concentration used, parameters were compared to the "no solvent" controls.

5.4.7 Determination of nitric oxide production by Griess assay

Nitric oxide was determined by Griess reagent quantification of nitrite, one of its stable reaction products. Griess reagent was freshly made up of equal volumes of 1% sulfanilamide and 0.1% naphthylethylene-diamine in 5% HCl. In the presence of nitrite this reagent forms a violet colour. From each well, 50 µL of supernatant was transferred to a fresh 96-well plate and mixed with 50 µL of Griess reagent, and the colour produced was measured at 540 nm in a microplate reader (POLARstar Omega, BMG Labtech, Mornington, Australia). The remaining supernatant from each well was used for a TNF-α assay. The concentration of nitrite was calculated using a standard curve with sodium nitrite (0 to 250 µM), and linear regression analysis.

5.4.8 Determination of cell viability by Alamar blue assay

The Alamar blue assay is a colorimetric assay involving the cellular reduction of resazurin to resorufin. Alamar blue solution [100 µL of 10% Alamar blue (Resazurin) in DMEM medium] was added to each well and incubated at 37 °C for 1-2 h. After incubation, fluorescence was measured (excitation at 544 nm and emission at 590 nm) using a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia). The results were expressed as a percentage of the intensity of that in control cells after background fluorescence was subtracted.

5.4.9 TNF-α determination by ELISA

The supernatants obtained from each well (remaining supernatant after 24 hours of activation) and experiment that gave better readout for NO and Alamar blue assay were used for determination of TNF-α using a commercial sandwich ELISA (Catalog number: 900-K54; Peprotech, USA) according to the manufacturer's protocol. The supernatants were diluted 30 times using diluent (0.1% w/v bovine serum albumin and 0.05% v/v tween-20 in PBS [1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl; pH7.4]). Capture antibody was used at a concentration of 1.25 µg/mL in PBS. To make a standard curve TNF-α (10 ng/mL standard) was diluted serially by 50% up to a minimum of 10 doses (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019 and 0.0097 ng/mL in the wells respectively) and was used as the internal standard. TNF-α was detected with a biotinylated second antibody and an Avidin peroxidase conjugate with TMB as detection reagent. The colour development was monitored at 655 nm, taking readings every 5 min. After about 30 min, the reaction was stopped using 0.5 M sulfuric acid, and the absorbance was measured at 450 nm of measurement filter using a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia). The absorbance data was expressed as a percentage of that of control wells after conversion of the concentrations by using a standard curve constructed with defined concentrations of TNF-α. Curve fitting of this standard curve and extrapolation of experimental data were performed using non-linear regression analysis.

5.4.10 Data presentation and analysis

For sequential extracts, three experiments in quadruplicates (n=12) were combined to determine the IC_{50} (for NO inhibition) and LC_{50} (for cell viability) whereas one experiment in triplicate (n=3) was conducted to obtain IC_{50} for TNF-α inhibition using the dose-response inhibition function in GraphPad Prism version 6.01 (GraphPad Software Incorporated, USA). For purified compounds, two experiments in quadruplicates (n=8) were combined to determine the IC_{50} (for NO and TNF-α inhibition) and LC_{50} (for cell viability) using the dose-response inhibition function in same version of GraphPad.

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Chapter 6

Anti-inflammatory Molecules from *Syncarpia glomulifera* subsp. *glomulifera* (Myrtaceae)

6.1 Introduction

Syncarpia glomulifera subsp. *glomulifera* belongs to the plant family Myrtaceae. It occurs in open forest along the east coast of Australia. Growing from sea level to an altitude of 300 m in NSW and to 900 m in Queensland, it is often a large tree of up to 45 m although 60 m has also been recorded [1].

Figure 6-1. Leaves of *Syncarpia glomulifare* subsp. *glomulifera*

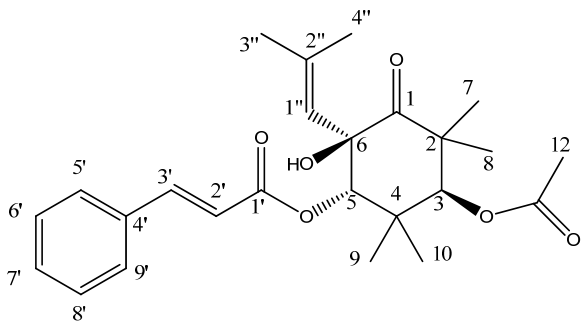
The resin obtained from the plant was used by the D'harawal Aboriginal people to heal soars and ulcers. Isolation of anti-bacterial and cytotoxic triterpenoids from the bark extract of the main species *S. glomulifera* from Paluma, North Queensland, Australia has been previously reported [2]. In another study, the anti-plasmodial compound phloroglucinol has been isolated from the same species [3].

The crude EtOH extract of the leaf of *S. glomulifare* subsp. *glomulifera* exhibited strong anti-inflammatory activity (IC_{50} = 8.25 µg/mL for NO inhibition) in LPS + IFN-γ activated 264.7 RAW macrophages and was selected for investigation to identify bioactive compounds. The fresh leaves of the plant were then sequentially extracted with *n*-hexane, DCM, EtOAc, EtOH, MeOH and water. Sequential EtOAc extract showed the highest anti-inflammatory activity (IC_{50} = 4.72 µg/mL for NO inhibition) in LPS + IFN-γ activated 264.7 RAW macrophages and subjected to HPLC for fractionation.

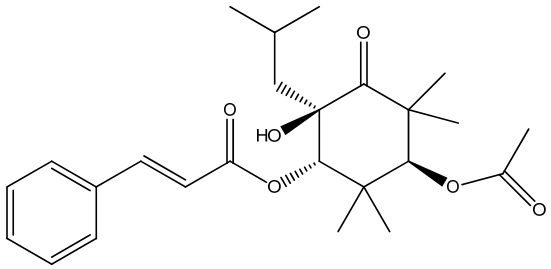
The bioassay-guided fractionation of the extract led to the isolation of two new and three known compounds, **6.1 - 6.5**. The structure of **6.1** was elucidated based on its analyses of MS data, 1D, 2D NMR data as well as by comparison of literature data of the known analogues. The structure of **6.4** was elucidated based on its analyses of MS data and 1D data and by comparison of literature data of the known analogues. On the other hand, the structure of **6.2**, **6.3** and **6.5** were confirmed by comparison of its mass spectrometry and NMR data[SI] with the literature data [4,5].

All of the five compounds **6.1**, **6.2**, **6.3**, **6.4** and **6.5** showed promising anti-inflammatory activity with IC_{50} values of 3.91, 35.15, 2.67, 29.42 and 7.84 µg/mL, respectively in down regulating NO, while IC_{50} values in down regulating TNF-α were 16.90, 32.12, 20.80, 37.57 and 33.35 µg/mL, respectively in LPS + IFN-γ activated 264.7 RAW macrophages. Compound **6.1** and **6.3** did not show any toxicity up to the 2nd highest concentration tested (> 18 µg/mL), whereas compound **6.2**, **6.4** and **6.5** did not show any toxicity with the highest concentration tested (> 36 µg/mL).

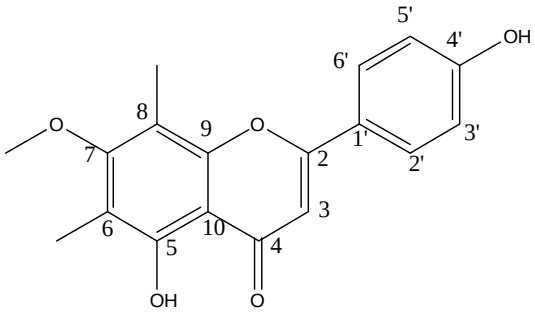
In LPS + IFN-γ activated N11 microglia, compound **6.1** **6.2**, **6.3**, **6.4** and **6.5** gave IC_{50} values of 4.52, 21.17, 3.87, 39.64 and 4.51 µg/mL for NO suppression and IC_{50} values for TNF-α downregulation were 6.50, 27.01, 13.66, 33.06 and 5.46 µg/mL respectively. LC_{50} values for cell viability were 24.31 and 19.05 µg/mL for compound **6.1** and **6.3** respectively whereas compound **6.2**, **6.4** and **6.5** did not show any toxicity with the highest concentration tested (> 36 µg/mL).



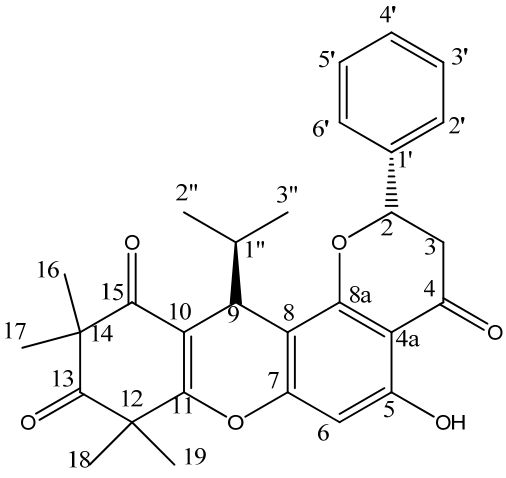
6.1



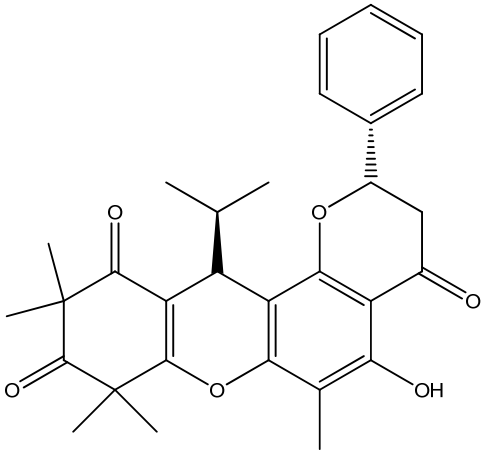
6.2
Tetragocarbone B



6.3
Sideroxylin



6.4



6.5
Lumaflavanone A

Figure 6-2. Structure of compounds **6.1 - 6.5**

6.2 Results

6.2.1 Extraction and yield

Fresh leaves of *S. glomulifera* subsp. *glomulifera* (voucher no. 891962) 180 g were sequentially extracted with six solvents from low to high polarity in order of *n*-hexane, DCM, EtOAc, EtOH, MeOH and water to separate lipophilic from hydrophilic compounds. A variable range of yields were obtained from the extraction (Table 6-1).

Table 6-1. Yield of the extracts after sequential extraction from *S. glomulifera* subsp. *glomulifera*

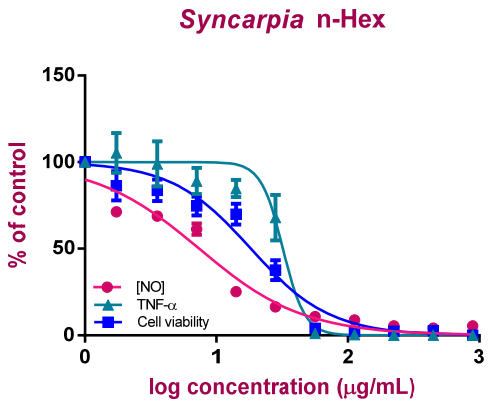
Extract	Yield of the extract (g)
n-Hexane	5.99
DCM	5.81
EtOAc	3.54
EtOH	7.06
MeOH	2.53
Water	3.61
Total	**38.64**

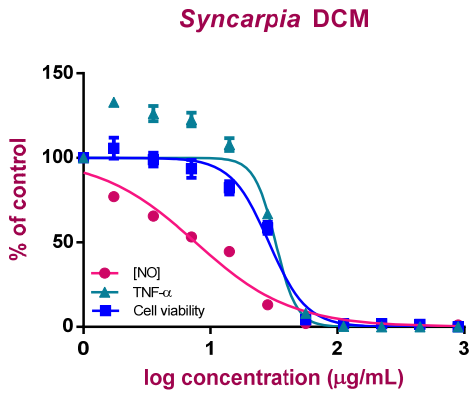
6.2.2 Anti-inflammatory activity of extracts

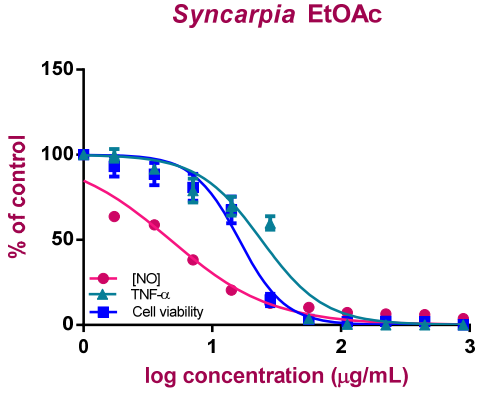
The *n*-hexane, DCM, EtOAc, EtOH, MeOH and water extracts were tested in LPS + IFN-γ induced RAW 264.7 macrophages to obtain IC_{50} values for NO and TNF-α down regulation and also LC_{50} for cytotoxicity. Due to low solubility of the extracts in the cell culture media, they were diluted in DMSO, leading to a final concentration of up to 0.5% DMSO in cell culture medium. This concentration of DMSO showed a cell viability of less than 10% (data not shown). The first four sequential extracts (*n*-hexane, DCM, EtOAc, EtOH) demonstrated significant anti-inflammatory activity to down regulate NO production which were less than16.0 µg/mL. MeOH and water extracts showed moderate effect for NO inhibition (Figure 6.1 and Table 6.2). The sequential EtOAc extract showed the highest activity with IC_{50} of 4.72 and 24.06 µg/mL for suppression of NO and TNF-α production respectively and LC_{50} of 16.22 µg/mL.

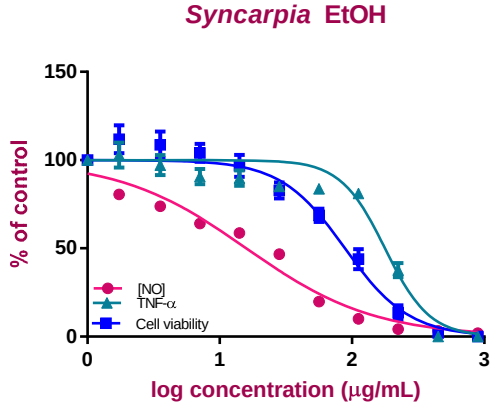
Table 6-2. Anti-inflammatory activity of *S. glomulifera* subsp. *glomulifera* sequential extracts

Sequential Extract	Inhibition of NO production (IC_{50} in µg/mL)	*Inhibition of TNF-α production (IC_{50} in µg/mL)	Cytotoxicity (LC_{50} in µg/mL)
n-Hexane	7.40 ± 0.7	32.08 ± 4.2	18.31 ± 3.3
DCM	7.64 ± 0.6	32.61 ± 5.7	28.88 ± 2.6
EtOAc	4.72 ± 0.4	24.06 ± 3.7	16.22 ± 2.0
EtOH	15.90 ± 1.3	177.6 ± 23.4	88.49 ± 13.5
MeOH	73.95 ± 7.6	271.6 ± 32.6	244.80 ± 49.4
Water	70.56 ± 6.7	208.1 ± 46.3	214.5 ± 26.3









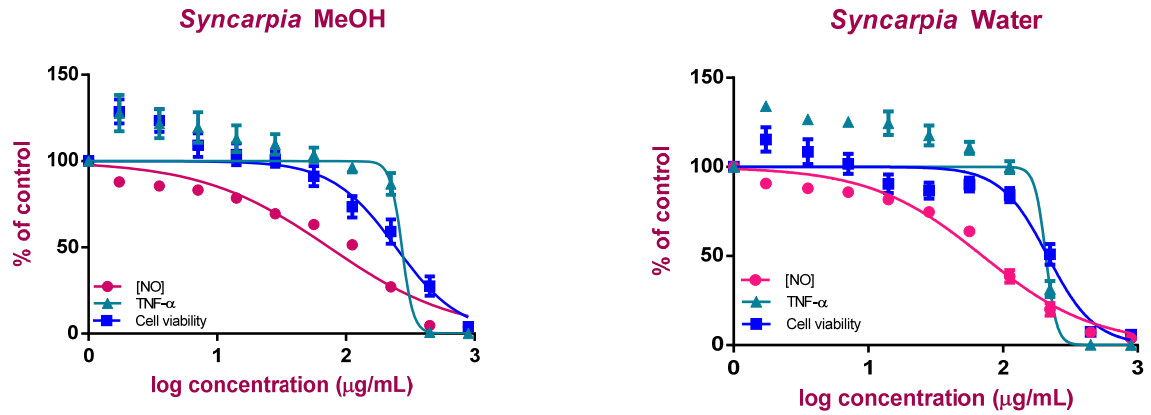


Figure 6-3. Down regulation of LPS and IFN-γ induced NO and TNF-α production and cell viability of *S. glomulifera* subsp. *glomulifera* sequential extracts in RAW 264.7 macrophages. Data represented as three experiments in quadruplicates (n=12, mean ± SEM). *One experiment in triplicate (n=3, mean ± SEM).

6.2.3 Isolation of active compounds

In order to identify bioactive compounds, the sequential EtOAc extract (IC_{50} = 3.96 µg/mL for NO inhibition) was subjected to HPLC on a phenyl-hexyl column. This process led to the isolation of two new (**6.1** and **6.3**) and three known compounds (**6.2**, **6.4** and **6.5**) (Scheme 6-1).

S. glomulifera subsp. *glomulifera,* leaf, 180 g

Extraction by Buchi extraction system; Soxhlet standard

	n-Hex	DCM	EtOAc	EtOH	MeOH	Water
Weight (g):	5.9	5.8	3.5	7.1	2.5	3.6
IC_{50} (µg/mL for NO inhibition):	7.40	7.64	4.72	15.90	73.95	70.57

Most active extract and was purified using HPLC[a]

Compounds **6.1 - 6.5**

(a) Semi-preparative HPLC: Phenyl-Hexyl (250x10 mm; 10 µm), 10-100% MeOH/H_2O (0.01% HCO_2H modifier) over 40 mins, held for 20 mins then to 10% MeOH/H_2O on 60.10 min and held for last 8 minutes.

Scheme 6-1 Bioassay-guided separation of *S. glomulifera* subsp. *glomulifera* leaf extract

6.2.3.1 Identification of compound **6.1**

Compound **6.1** was isolated as a yellowish solid. HRESI (+) MS analysis of compound **6.1** revealed a pseudomolecular ion ($[M + Na]^+$) indicative of a molecular formula ($C_{25}H_{32}O_6Na$) requiring 10 double bond equivalents. The ^{1}H NMR, HSQC and HMBC spectra (Table 6-3) indicated the presence of 3 carbonyl, 5 quaternary, 8 methine, 2 oxymethine and 7 methyl carbons in the structure.

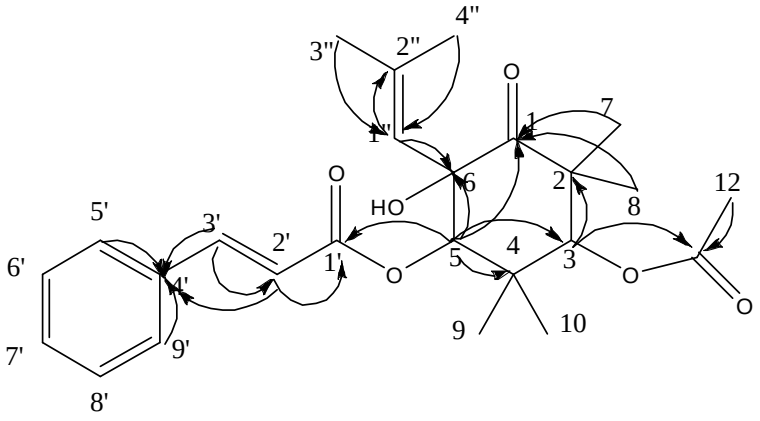


Figure 6-4. Key HMBC correlations of compound **6.1**

The structure of the *trans*-cinnamoyl moiety of compound **6.1** was established by ^{1}H NMR data showing two vicinal olefinic protons H-2' and H-3' [H_δ 6.63 and 7.63, each 1H, d (J_{ax} = 16.0 Hz)] and five aromatic protons at H_δ 7.43 (4H) and H_δ 7.74 (1H). The presence of a cyclohexanone ring of compound **6.1** was suggested by one of the three carbonyls detected at C-1 (C_δ 209.7) and the HMBC correlations of 7- and 8-Me (H_δ 1.10 and 1.23, each 3H, s) to C-1, C-2 and C-3 (C_δ 209.7, 48.2 and 77.4) and of 9- and 10-Me (H_δ 0.94 and 1.21, each 3H, s) to C-3, C-4 and C-5 (C_δ 77.4, 39.2 and 59.4). In addition H-3 is correlated to C-2, C-4 and C-11 (C_δ 170.5) and 12-Me (H_δ 2.21, 3H, s) correlated to C-11 assigning the presence of the acetate fragment at C-3. The oxymethine proton of H-5 is correlated to C-4, C-6 and C-1' (C_δ 165.0) of the cinnamoyl ester group. The side chain moiety of compound **6.1** at C-6 was assigned as an isobutylene group due to the deshielded chemical shift of H-1" (H_δ 5.59, 1H, brs) along with HMBC correlation of H-1" to C-6 (C_δ 77.8), C-2"(C_δ 138.8), C-3" (C_δ 18.8), C-4"(C_δ 26.5) and C-1. Identifications and placements of the cinnamoyl ester, isobutylene and acetyl groups were confirmed by HMBC correlations (Figure 6.4) in addition to comparison of NMR data with those of related compounds [6,7].

There were three stereo centers in the compound **6.1** at C-3, C-5 and C-6. The ^{1}H NMR spectrum of this compound showed 2 singlets at H_δ 5.15 and 5.17 (each 1H, 3 and 5 respectively) indicating that they were not coupled to any other proton. We conducted ROSEY experiment to determine the relative stereochemistry of **6.1** which was also not helpful due to the presence of eight densely congested substituents on the cyclohexanone ring. Therefore, the relative stereochemistry of **6.1** could not be determined by analyzing the NMR data and we attempted chemical modifications. The chemical modifications included acid hydrolysis as well as deacetylation. However, when we analyzed the LC-MS data of the reaction mixtures as well as the purified fractions obtained from the reaction mixtures, we

did not observe the presence of any of the expected products (cinnamoyl ester, substituted cyclohexanone moeity) from chemical modifications. The optical rotation of compound **6.1** ($[\alpha]_D^{25}$ −18.4 (c = 0.12, MeOH)) was found to be similar with its known congeners tetragocarbone A ([α] = - 13.6 (*c* = 0.28, $CHCl_3$)) and tetragocarbone B ([α] = - 17.1 (*c* = 0.14, $CHCl_3$)) (compound **6.2**) [Nishimura, 2016]. The relative stereochemistry of **6.1** can thus be determined as (3*R*,5*R*,6*S*) by comparison of the optical rotation value with that of related known compounds (Figure 6.2).

Table 6-3. NMR data (DMSO-d_6, 400 MHz) for compound **6.1**

Pos	δ_H, Mult (*J* in Hz)	δc *	COSY	HMBC
1	-	209.7		
2	-	48.2		
3	5.15 *s*	77.4		2, 4, 5, 7, 8, 9, 10, 11
4	-	39.2		
5	5.17 *s*	79.4		1, 3, 4, 6, 9, 10, 1', 1"
6	-	77.8		
7	1.10 *s*	26.3		1, 2, 3, 8
8	1.23 *s*	22.5		1, 2, 3, 7
9	0.94 *s*	24.5		3, 4, 5, 10
10	1.21 *s*	22.3		3, 4, 5, 9
11	-	170.5		
12	2.12 *s*	20.1		11
1'	-	165.0		
2'	6.63 *d(16.0)*	117.2	3'	1', 3', 4'
3'	7.63 *d(16.0)*	144.5	2'	1', 2', 4', 5', 9'
4'	-	134.0		
5'	7.43 *d(2.1)*	128.5	6'	2', 3', 4', 6', 7', 8', 9'
6'	7.43 *d(2.1)*	130.3	5', 7'	4', 5', 7', 8', 9'
7'	7.74 *dd(5.6, 2.0)*	127.5	6', 8'	5', 6', 8', 9'
8'	7.43 *d(2.1)*	130.3	7', 9'	4', 5', 6', 7', 9',
9'	7.43 *d(2.1)*	128.5	8'	2', 3', 4', 5', 6', 7', 8'
1"	5.59 *brs*	121.7		6, 2", 3", 4"
2"	-	138.8		
3"	1.69 *s*	18.8		1", 2", 4"
4"	1.65 *s*	26.5		1", 2", 3"

*Assignments supported by HSQC and HMBC experiments.

6.2.3.2 Identification of compound **6.2**

Compound **6.2** was obtained as yellowish solid. Its structure was elucidated as the known compound tetragocarbone B (Figure 6-2) by comparison of its mass spectrometry and NMR data[SI] with the literature data [6,7]. The relative stereochemistry of compound **6.2** has also been determined as (3*R*,5*R*,6*S*) by comparison of the optical rotation value ($[\alpha]_D^{25}$ −22.7 (c = 0.05, MeOH)) with that of the known compound, tetragocarbone B ([α] = - 17.1 (*c* = 0.14, $CHCl_3$)) [Nishimura, 2016] (Figure 6.2).

6.2.3.3 Identification of compound **6.3**

Compound **6.3** was obtained as yellowish solid. Its structure was elucidated as the known compound sideroxylin (Figure 6-2) by comparison of its mass spectrometry and NMR data[SI] with the literature data [4,8,9].

6.2.3.4 Identification of compound **6.4**

Compound **6.4** was isolated as white powder. The protonated molecule peak at m/z 475.2139 $[M+H]^+$ (calc. for $C_{29}H_{31}O_6$, 475.2121) suggested a molecular formula of $C_{29}H_{30}O_6$ with 15 degrees of unsaturation. The ^{1}H NMR spectral data (Table 6-4) was in close agreement with those reported by Labbe et al. [5] for lumaflavanone A, which we have also isolated as our compound **6.5**. However a difference from the known derivative (lumaflavanone A) includes the presence of an aromatic proton at H_δ 6.46 as a singlet instead of a methyl group at C-6 as evident from ^{1}H NMR spectrum[SI].

Compound **6.4** was found to have an optical rotation of zero, indicating that it was isolated as a racemate. It has two stereo centers at C-2 and C-9. This optical inactivity was similar to that of the related compounds including Lumaflavanone A which we isolated as compound **6.5** [Labbe, 2002 and Fugimoto 1995]. Thus the relative stereochemistry of **6.4** was determined as (2S,9*S*) (Figure 6.2).

Table 6-4. ^{1}H NMR data (DMSO-d_6, 400 MHz) for compound **6.4**

Position	δ_H, Mult (*J* in Hz)
2	5.66 *dd(14.0, 3.0)*
3	2.86 *dd(17.0, 2.7)*; 3.41 *d(3.6)*
6	6.46 *s*
9	4.03 *d(3.6)*
16	1.29 *s*
17	1.23 *s*
18	1.52 *s*
19	1.38 *s*
2’	7.56 *dd(8.6, 2.0)*
3’	7.49 *m*
4’	7.44 *t*
5’	7.49 *m*
6’	7.56 *dd(8.6, 2.0)*
1”	1.84 *m*
2”	0.68 *d(7.0)*
3”	0.79 *d(7.0)*
C-5-OH	12.0 *s*

6.2.3.5 Identification of compound **6.5**

Compound **6.5** was isolated as yellowish solid. Its structure was elucidated as the known compound lumaflavanone A (Figure 6-2) by comparison of its mass spectrometry and NMR data[SI] with the literature data [5].

Compound **6.5** was found to have an optical rotation of zero, indicating that it was isolated as a racemate. It has two stereo centers at C-2 and C-9. Its optical inactivity was similar to that of the known compound, Lumaflavanone A [Labbe, 2002]. Thus the relative stereochemistry of **6.5** was determined as (2S,9*S*) (Figure 6.2).

6.2.4 Anti-inflammatory activity of identified compounds

Compounds **6.1** - **6.5** were evaluated for anti-inflammatory activity in LPS + IFN-γ induced RAW 264.7 macrophages (Figure 6-5 and Table 6-5) and N11 microglia (Figure 6-6 and Table 6-6).

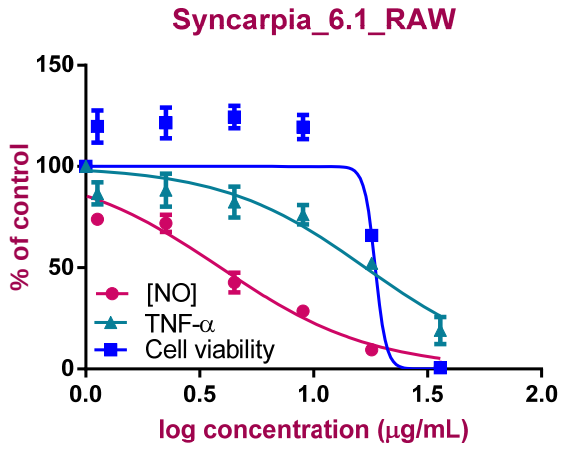
In RAW 264.7 macrophages, compounds **6.1 - 6.5** showed strong anti-inflammatory activity with IC_{50} value of 3.91 ± 0.5, 35.15 ± 7.3, 2.76 ± 0.3, 29.42 ± 2.7 and 7.84 ± 1.3 µg/mL respectively for NO inhibition and 16.90 ± 3.4, 32.12 ± 13.2, 20.80 ± 14.1, 37.57 ± 11.7 and 33.35 ± 3.8 µg/mL for TNF-α inhibition respectively. Compound **6.1** and **6.3** did not show any toxicity with the 2nd highest

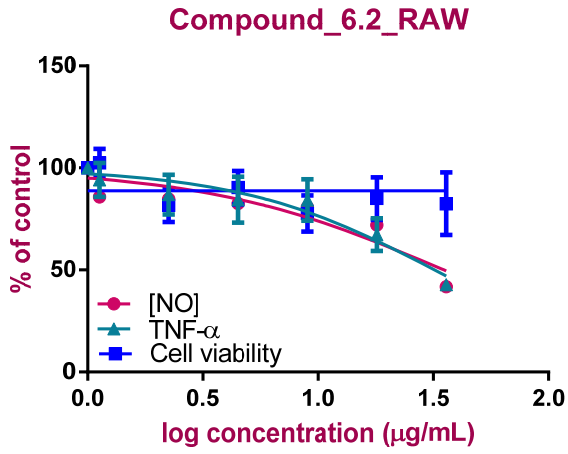
concentration tested (> 18 µg/mL) whereas compound **6.2**, **6.4** and **6.5** did not show any toxicity with the highest concentration tested (> 36 µg/mL).

In N11 microglia, compound **6.1** - **6.5** were also strong to down regulate NO and the IC_{50} values were 4.52 ± 0.8, 35.15 ± 7.3, 3.87 ± 0.5, 39.64 ± 10.4 and 4.51 ± 0.6 µg/mL and the compounds were also found to be very good to down regulate TNF-α with IC_{50} values of 6.50 ± 1.3, 32.12 ± 13.2, 13.66 ± 3.8, 33.06 ± 5.9 and 5.46 ± 0.7 µg/mL respectively. LC_{50} values for cell viability were 24.31 ± 6.1 and 19.05 ± 5.7 µg/mL for compound **6.1** and **6.3** respectively whereas compound **6.2**, **6.4** and **6.5** did not show any toxicity with the highest concentration tested (> 36 µg/mL).

Table 6-5. Anti-inflammatory activity of compounds isolated from *S. glomulifera* subsp. *glomulifera* in LPS + IFN-γ induced RAW 264.7 macrophages

Compound	Inhibition of NO production IC_{50} in µg/mL	Inhibition of TNF- α production IC_{50} in µg/mL	Cytotoxicity LC_{50} in µg/mL	% Cytotoxicity
6.1	3.91 ± 0.5	16.90 ± 3.4	> 18	66.0
6.2	35.15 ± 7.3	32.12 ± 13.2	> 36	82.5
6.3	2.76 ± 0.3	20.80 ± 14.1	> 18	87.8
6.4	29.42 ± 2.7	37.57 ± 11.7	> 36	140.5
6.5	7.84 ± 1.3	33.35 ± 3.8	> 36	77.3





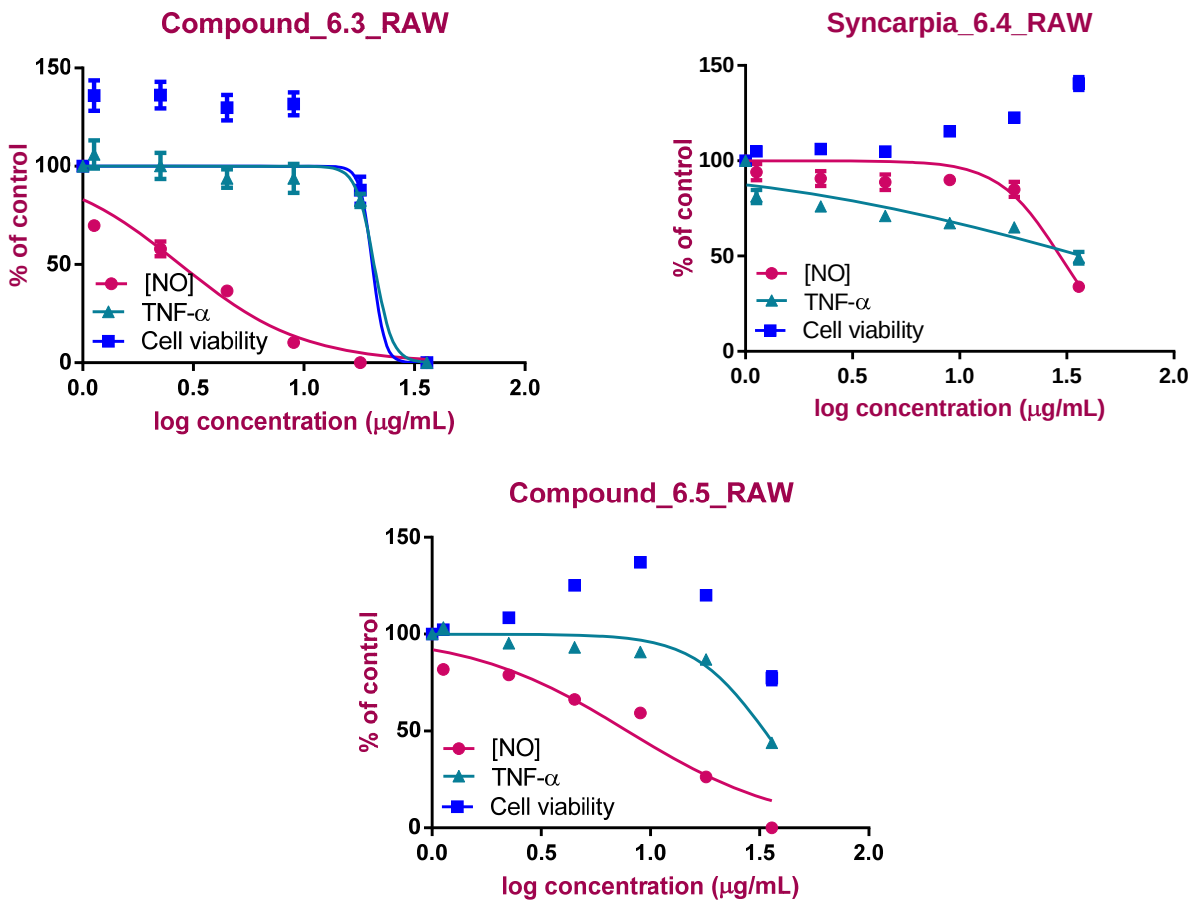


Figure 6-5. Down regulation of LPS and IFN-γ induced NO and TNF-α production and cell viability of isolated compounds from *Syncarpia glomulifera* subsp. *glomulifera* in RAW 264.7 macrophages

Table 6-6. Anti-inflammatory activity of compounds isolated from *S. glomulifera* subsp. *glomulifera* in LPS + IFN-γ induced N11 microglia

Compound	Inhibition of NO production (IC_{50} in μg/mL)	Inhibition of TNF- α production (IC_{50} in μg/mL)	Cytotoxicity (LC_{50} in μg/mL)	% Cytotoxicity
6.1	4.52 ± 0.8	6.50 ± 1.3	24.31 ± 6.1	
6.2	21.17 ± 3.8	27.01 ± 8.5	> 36	143.8
6.3	3.87 ± 0.5	13.66 ± 3.8	19.05 ± 5.7	
6.4	39.64 ± 10.4	33.06 ± 5.9	> 36	155.8
6.5	4.51 ± 0.6	5.46 ± 0.7	> 36	115.2

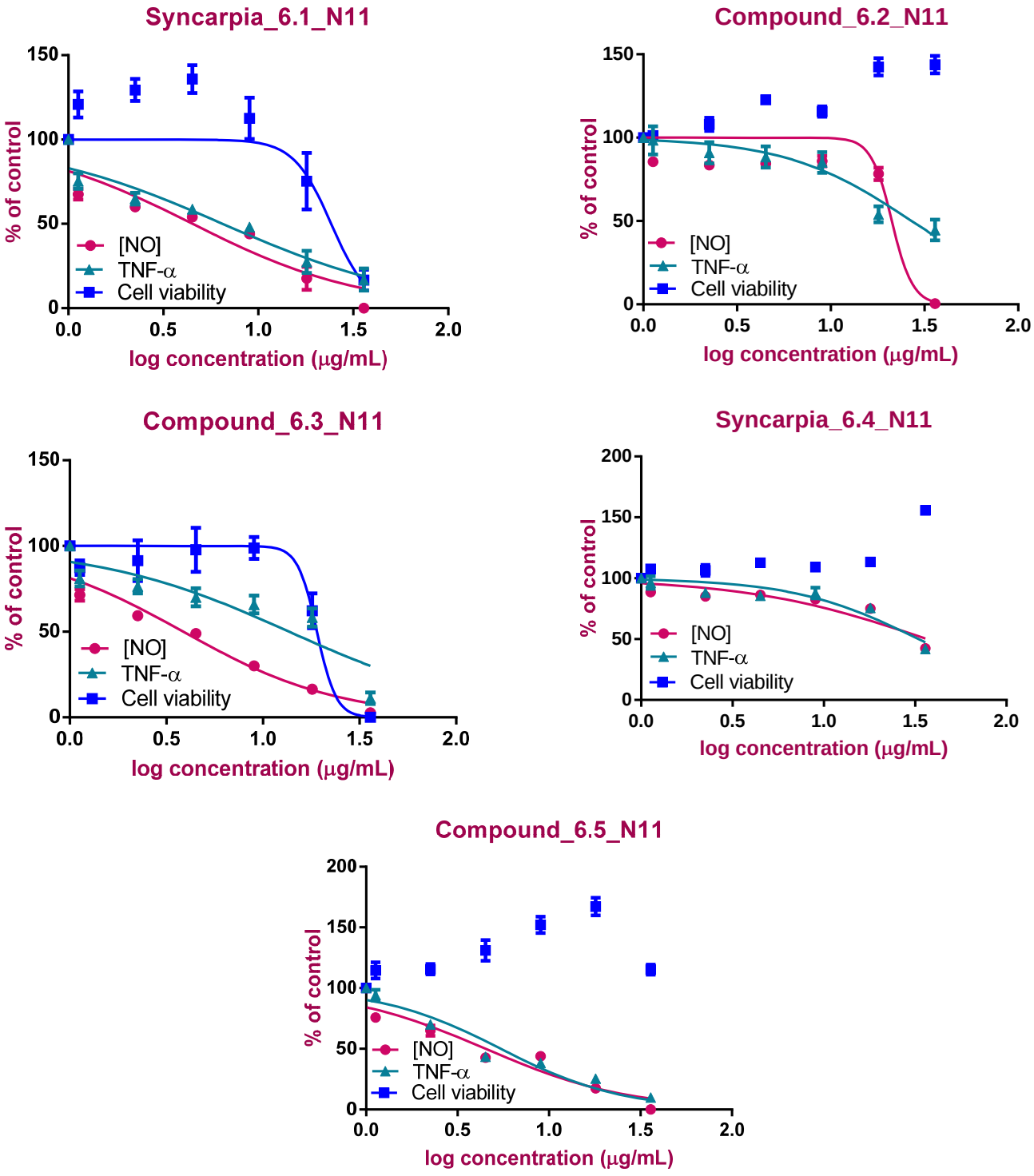


Figure 6-6. Down regulation of LPS and IFN-γ induced NO and TNF-α production and cell viability of isolated compounds from *S. glomulifera* subsp. *glomulifera* in N11 microglia

6.3 Discussion

Compound **6.1** is reported for the first time and this compound demonstrated very promising anti-inflammatory activity as evaluated against both LPS and IFN-γ induced RAW 264.7 macrophage and N11 microglial cell line.

Compound **6.2** is reported by Nishimura et al in 2016 but there is no report on the anti-inflammatory activity of compound **6.2.** In our study, compound **6.2** exhibited good anti-inflammatory activity as found against both LPS and IFN-γ induced RAW 264.7 macrophage and N11 microglial cell line.

The monomeric phloroglucinol derivatives, compound **6.1** and **6.2** contains isobutylene and butylene group at position 6 respectively in the structure (Figure 6-2). Compound **6.1** is found to be several folds more active regarding NO and TNF-α inhibition in both cell lines as compared to compound **6.2** indicating that the double bond of isobutylene is important for better activity of the compound (**6.1**).

Compound **6.3** is reported from several myrtaceous plants and also from other plant families [10-12]. This compound is commonly known as sideroxylin and has been reported to exhibit antimicrobial [13] and nematicidal [14] activity. This compound has also been evaluated for neuroprotective effect on PC12 cells against Aβ induced toxicity, where it did not show any protective effect [4]. However in our study, compound **6.3** has demonstrated promising activity towards down regulation of NO and TNF-α in both RAW 264.7 macrophages and N11 microglia, which is being reported for the first time.

Compound **6.4** is reported for the first time and this compound also exhibited good anti-inflammatory activity as found against both LPS and IFN-γ induced RAW 264.7 macrophage and N11 microglial cell line.

There is only one report on compound **6.5,** isolated from fresh leaves of *Luma chequen* (Myrtaceae) where this compound has been tested for its antifeedant and fungistatic activity as well as toxicity [5]. However, there is no report on the anti-inflammatory activity of compound **6.5** and in our study the compound showed strong anti-inflammatory activity by downregulating NO and TNF-α in both LPS and IFN-γ induced RAW 264.7 macrophage and N11 microglial cell line.

Compound **6.5** is different from compound **6.4** due to the presence of a methyl group at position 6 of the aromatic ring in the structure (Figure 6-2). However, compound **6.5** is much more active to supress NO in both cell line and TNF-α in N11 microglia compared to compound **6.4** indicating that the substitution of the aromatic proton with methyl group contributing for the activity of the compound (**6.5**).

Table 6-7. Potency and total anti-inflammatory activity of *S. glomulifera* subsp. *glomulifera* sequential extracts in LPS + IFN-γ induced RAW 264.7 macrophages

Sequential Extract	Potency for NO inhibition(L/g)	Potency for TNF-α inhibition (L/g)	Total anti-inflammatory (NO inhibition) activity of extract (L)	Total anti-inflammatory (TNF-α inhibition) activity of extract (L)	Sequential Extract
n-Hexane	135.14	31.17	809.46	186.72	*n*-Hexane
Dichloromethane	130.89	30.67	760.47	178.17	Dichloromethane
Ethyl acetate	211.86	41.56	750.00	147.13	Ethyl acetate
Ethanol	62.89	5.63	444.03	39.75	Ethanol
Methanol	13.52	3.68	34.21	9.32	Methanol
Water	14.17	4.81	51.16	17.35	Water
Total anti-inflammatory activity of all extracts (L)			2849.33	578.43	

Table 6-8. Potency and total anti-inflammatory (AI) activity of compounds isolated from *S. glomulifera* subsp. *glomulifera* in LPS + IFN-γ induced RAW 264.7 macrophages

Individual compounds	Potency for NO inhibition(L/g)	Potency for TNF-α inhibition (L/g)	Total anti-inflammatory (NO inhibition) activity of compound (L)	Total anti-inflammatory (TNF-α inhibition) activity of compound(L)
Compound **6.1**	255.75	59.17	2.56	0.59
Tetragocarbon B (**6.2**)	28.45	31.13	0.07	0.07
Sideroxylin (**6.3**)	362.32	48.08	1.09	0.14
Compound **6.4**	33.99	26.62	0.11	0.09
Lumaflavanone A (**6.5**)	127.55	29.99	0.41	0.10
Total anti-inflammatory activity of all identified compounds (L)			4.23	0.99
% of total activity			0.15	0.17

The potency (for NO and TNF-α inhibition) of each of the sequential extracts and isolated compounds from *S. glomulifera* subsp. *glomulifera* leaves were measured as a reciprocal of IC_{50} value (Table 6-7 and 6-8). The total anti-inflammatory activity for both NO and TNF-α inhibition (Table 6-7 and 6-8) of the same extracts and compounds were also obtained by multiplying respective potency and yield of dried extracts and isolated compounds. Thus the total anti-inflammatory activity of all extracts was found to

be 2849.33 L for NO inhibition in 180 g leaves of *S. glomulifera* subsp. *glomulifera* of which 2.56 L, 0.07 L, 1.09 L, 0.11 L and 0.41.26 L were obtained by compound **6.1**, tetragocarbon B (**6.2**), sideroxylin (**6.3**), compound **6.4** and lumaflavanone A (**6.5**) respectively. Similarly, the total anti-inflammatory activity of all extracts was found to be 578.43 L for TNF-α inhibition in 180 g leaves of 0.59 L, 0.07 L, 0.14 L, 0.09 L and 0.10 L were obtained by compound **6.1**, tetragocarbon B (**6.2**), sideroxylin (**6.3**), compound **6.4** and lumaflavanone A (**6.5**) respectively.

The compounds were isolated by purifying only the EtOAc extract which was 1.01% of the total extract. Thus the total anti-inflammatory activity of all isolated compounds were calculated 4.27 L and 1.00 L for NO and TNF-α inhibition respectively in the leaves of *S. glomulifera* subsp. *glomulifera* under investigation.

6.4 Experimental Section

6.4.1 General experimental procedures

Acetonitrile, *n*-hexane, dichloromethane and methanol were supplied by Merck. Ethyl acetate from Fisher Chemical, ethanol from Chem-Supply and water used was Milli-Q water. Bovine serum albumin, lipopolysaccharide (*E. coli* serotype-0127:B8), EDTA, N-(1-napthyl) ethylenediamine dihydrochloride, benzylpenicillin G sodium salt, resazurin sodium salt (10%), streptomycin, sulphanilamide, 3,3',5,5'-tetramethylbenzidine (TMB), trypan blue and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Foetal bovine serum (FBS) and glutamine were purchased from Life Technologies (Mulgrave, VIC, Australia). Murine interferon-γ (IFN-γ) and TNF-α ELISA kits were purchased from PeproTech Asia (Rehovot, Israel). Citric acid and monosodium dihydrogen carbonate (NaH_2CO_3) were from AJAX Chemicals (Auburn, NSW, Australia). Tween-20 was from Amresco (Solon, Ohio, USA). Methanol, monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), sodium chloride (NaCl) and sulfuric acid (H_2SO_4) were from Merck (Darmstadt, Germany). Sodium carbonate (Na_2CO_3) was BDH brand supplied by Merck Pty. Ltd. (Kilsyth, VIC, Australia).

6.3.2 Experimental procedures for MS, HPLC and NMR

Semi-preparative HPLC was performed using an Agilent 1260 Infinity Series (Santa Clara, CA, USA). NMR spectra were obtained on a Bruker Ascend 400 MHz spectrometer (Bruker Biospin GmbH, Bremen, Germany). TopSpin software was used to analyze the spectral data. ^{1}H NMR spectra were recorded at 400 MHz. The chemical shifts (δ) are expressed in parts per million (ppm) as δ values and coupling constant *J* in Hertz (Hz). COSY, HSQC and HMBC were acquired using the standard Bruker pulse program. The experiments were performed in deuterated solvents and the chemical shifts were calibrated relative to the DMSO solvent peak (^{1}H δ at 2.50 and ^{13}C δ 39.51 ppm) and $CHCl_3$ solvent peak (^{1}H δ at 7.24 and ^{13}C δ 77.37 ppm). HRMS was carried out using a Waters Xevo Q-TOF mass spectrometer operating in the positive ESI mode.

6.4.3 Plant material

Fresh leaves of *Syncarpia glomulifera* subsp. *glomulifera* (voucher no. 891962) were collected in the month of July, 2016 from the 'Australian Botanic Gardens' at Mount Annan, NSW, Australia. A voucher specimen was deposited at the National Herbarium of NSW, Australia.

6.4.4 Extraction and isolation

Approximately 180 g of fresh leaf material from *Syncarpia glomulifera* subsp. *glomulifera* was first cut into small pieces with scissors and then ground to a coarse powder using a hand blender. The coarse powder was then filled into the thimbles of an accelerated solvent extraction system (Buchi B-811, Switzerland) and extracted under standard Soxhlet mode (for 2 × 15 minutes cycles) using six solvents (*n*-hexane, dichloromethane, ethyl acetate, ethanol, methanol and water) sequentially from low to high polarity. The volume of the extracts was reduced to a volume of 2—4 mL using a rotary evaporator and then evaporated to dryness with nitrogen. The sequential ethyl acetate extract was found most potent when screened for anti-inflammatory activity. This crude extract was then resuspended in methanol and subjected directly through reversed phase semi-prep HPLC (Phenyl-Hexyl column, 250 x 10 mm, Phenomenex). Peak detection was performed with a photodiode array detector (PDA) using three detection wavelengths (210 nm, 254 nm and 290 nm). A gradient starting from 10% methanol/water to a 100% methanol (with a constant 0.01% formic acid modifier) over 40 min and held at 100% methanol for 20 min to yield compound **6.1** (t_R = 42.9 min, 10.0 mg), compound **6.2** compound (t_R = 43.5 min, 2.3 mg), **6.3** (t_R = 46.5 min, 3.0 mg), compound **6.4** (t_R = 51.5 min, 3.2 mg) and compound **6.5** (t_R = 53.1 min, 3.2 mg). All isolated compounds were purified to 95% purity or more as judged by HPLC (UV detection) and ^{1}H NMR spectroscopy before determining the bioactivity.

6.4.5 Maintenance and preparation of RAW 264.7 macrophage and N11 microglia

RAW 264.7 macrophages and N11 microglial cells were grown in 175 cm^2 culture flasks on DMEM (Dulbecco's Modified Eagle's Medium) containing 5% FBS (fetal bovine serum) that was supplemented with penicillin (100 units/mL), streptomycin (100 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. Once cells had grown to confluence in the culture flask, they were removed using a rubber policeman cell scraper, as opposed to using trypsin, which can remove membrane-bound receptors. The cell suspension was concentrated by centrifugation for 3 min at 900 rpm and resuspended in a small volume of fresh DMEM (with 1% antibiotics and 5% FBS). Cell densities were estimated using a Neubauer counting chamber. The cell concentration was adjusted with DMEM (with 1% antibiotics and 5% FBS) to obtain 60,000 cells/100 µL cell suspension. 100 µL of cell suspension was then dispensed into the wells of 96-well plates (Eppendorf, Hamburg, Germany). Plates were incubated at 37 °C; 5% CO_2 for 24 h before the activation experiments were carried out.

6.4.6 Activation of RAW 264.7 macrophage and N11 microglia

From each well, the media was removed and replaced with fresh DMEM containing 0.1% FBS. For assays with extracts, a 90 µL volume of the dilutions in DMEM (with 0.1% FBS) was added an hour prior to

addition of the activator. Due to the often inconsistent nature of LPS at activating cells, a combination of LPS (10 µg/mL) and IFN-γ (10 u/mL), both in DMEM (with 0.1% FBS), were used for activation. For the sequential extracts a maximum dose of 900 µg/mL was used, and diluted serially by 50% up to a minimum of 10 doses (900, 450, 225, 112.5, 56.25, 28.125, 14.062, 7.031, 3.515, 1.7578 and 0.8789 µg/mL in the wells respectively). A maximum dose of the isolated compounds (**6.1 - 6.5**) used was 36 µg/mL, and diluted serially by 50% up to a minimum of 7 doses (36, 18, 9, 4.5, 2.25, 1.12 and 0.56 µg/mL in the wells respectively), After activation, the cells were incubated for 24 h at 37 °C and 5% CO_2 and then NO and TNF-α inhibition, and cell viability were determined. Cells with media alone were used as negative control and activated cells used as positive control. The effects of solvents on readouts were initially determined, but as the anti-inflammatory or cytotoxic effects of the solvents were <10% even at the highest concentration used, parameters were compared to the "no solvent" controls.

6.4.7 Determination of nitric oxide production by Griess assay

Nitric oxide was determined by Griess reagent quantification of nitrite, one of its stable reaction products. Griess reagent was freshly made up of equal volumes of 1% sulfanilamide and 0.1% naphthylethylene-diamine in 5% HCl. In the presence of nitrite this reagent forms a violet colour. From each well, 50 µL of supernatant was transferred to a fresh 96-well plate and mixed with 50 µL of Griess reagent, and the colour produced was measured at 540 nm in a microplate reader (POLARstar Omega, BMG Labtech, Mornington, Australia). The remaining supernatant from each well was used for a TNF-α assay. The concentration of nitrite was calculated using a standard curve with sodium nitrite (0 to 250 µM), and linear regression analysis.

6.4.8 Determination of cell viability by Alamar blue assay

The Alamar Blue assay is a colorimetric assay involving the cellular reduction of resazurin to resorufin. Alamar Blue solution [100 µL of 10% Alamar Blue (resazurin) in DMEM medium] was added to each well and incubated at 37 °C for 1-2 h. After incubation, fluorescence was measured (excitation at 544 nm and emission at 590 nm) using a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia). The results were expressed as a percentage of the intensity of that in control cells after background fluorescence was subtracted.

6.4.9 TNF-α determination by ELISA

The supernatants obtained from each well (remaining supernatant after 24 hours of activation) and experiment that gave better readout for NO and Alamar blue assay were used for determination of TNF-α using a commercial sandwich ELISA (Catalog number: 900-K54; Peprotech, USA) according to the manufacturer's protocol. The supernatants were diluted 30 times using diluent (0.1% w/v bovine serum albumin and 0.05% v/v tween-20 in PBS [1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl; pH7.4]). Capture antibody was used at a concentration of 1.25 µg/mL in PBS. To make a standard curve TNF-α (10 ng/mL standard) was diluted serially by 50% up to a minimum of 10 doses (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019 and 0.0097 ng/mL in the wells respectively) and was used as the internal standard. TNF-α was detected with a biotinylated second antibody and an Avidin peroxidase conjugate with TMB as detection reagent. The colour development was monitored at 655 nm, taking

readings every 5 min. After about 30 min, the reaction was stopped using 0.5 M sulfuric acid, and the absorbance was measured at 450 nm of measurement filter using a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia). The absorbance data was expressed as a percentage of that of control wells after conversion of the concentrations by using a standard curve constructed with defined concentrations of TNF-α. Curve fitting of this standard curve and extrapolation of experimental data were performed using non-linear regression analysis.

6.4.10 Spectroscopic properties

Compound **6.1**: yellowish solid; $[\alpha]_D^{25}$ −18.4 (*c* = 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 280 (4.02); ^{1}H and δ_C NMR data, see Table 6.3; HRESIMS (pos.): *m/z* $[M+Na]^+$ 451.2115 (calculated for $C_{25}H_{32}O_6Na$, 451.2097).

Compound **6.4**: yellowish solid; $[\alpha]_D^{25}$ 0 (*c* = 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 257 (3.15), 296 (3.22); ^{1}H NMR data, see Table 6.4; HRESIMS (pos.): *m/z* $[M+H]^+$ 475.2139 (calculated for $C_{29}H_{31}O_6$, 451.2121).

6.4.11 Data presentation and analysis

For sequential extracts, three experiments in quadruplicates (n=12) were combined to determine the IC_{50} (for NO inhibition) and LC_{50} (for cell viability) whereas one experiment in triplicate (n=3) was conducted to obtain IC_{50} for TNF-α inhibition using the dose-response inhibition function in GraphPad Prism version 6.01 (GraphPad Software Incorporated, USA). For purified compounds, two experiments in quadruplicates (n=8) were combined to determine the IC_{50} (for NO and TNF-α inhibition) and LC_{50} (for cell viability) using the dose-response inhibition function in same version of GraphPad.

6.5 References

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Chapter 7

Screening of *Baeckea ramosissima* subsp. *ramosissima* and *B. imbricata* (Myrtaceae) Leaf Extracts for Anti-inflammatory activity

7.1 Introduction

Baeckea spp. belong to the plant family Myrtaceae and are usually shrubs or small trees [1]. According to A. R. Bean the genus *Baeckea* comprises 14 species and except *B. frutescens* L. all other species are confined to Australia. *Baeckea* is found abundantly in coastal areas and tablelands of south-eastern Australia [2]. *B. frutescens* is widely distributed in Malaysia, China, Hong Kong as well as in Australia [3].

Figure 7-1. Leaves of *Baeckea ramosissima* subsp. *ramosissima*

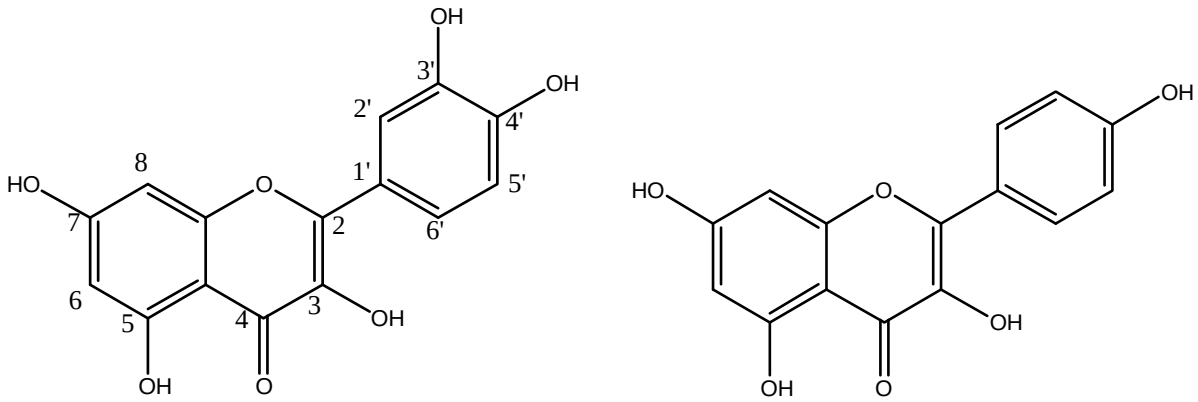
Baeckea genus is also recognized as *Euryomyrtus*. *B. frutescens* L. is the most widely studied *Baeckea* species either for chemical constituents [4-7] or for biological activities [8,9]. A broad range of compounds have been isolated from this *Baeckea* spp. for example, chromones and chromanones [10], chromone c-glycosidees [11], flavanones [12], bi-flavonoides [13-15], phloroglucinols and acylphloroglucinols [16-18], tasmanone-based meroterpenoids [19] etc. with cytotoxic, anti-bacterial as well as anti-inflammatory activities. Bioassay guided fractionation of *B. gunniana* led to isolation of four ursane and oleanane triterpenoids which has been reported to inhibit DNA polymerase β [20].

In the present study, *B. ramosissima* subsp. *ramosissima* and *B. imbricata* have been investigated for their anti-inflammatory activity as we did not find any report on these two *Baeckea* spp. regarding their biological activity or chemical profiling.

The crude EtOH extracts of the leaves of *B. ramosissima* subsp. *ramosissima* and *B. imbricata* exhibited strong anti-inflammatory activity (IC_{50} = 15.68 and 16.78 µg/mL respectively for NO inhibition) in LPS + IFN-γ activated 264.7 RAW macrophages and were selected for investigation to identify bioactive compounds. The fresh leaves of the two plants were then sequentially extracted with *n*-hexane, DCM, EtOAc, EtOH, MeOH and water. The sequential EtOAc extract from both plants showed the highest anti-inflammatory activity for NO inhibition (IC_{50} = 9.33 and 8.98 µg/mL for *Baeckea ramosissima* subsp. *ramosissima* and *B. imbricata* respectively) in LPS + IFN-γ activated 264.7 RAW macrophages and were subjected to HPLC for fractionation.

Figure 7-2. Leaves of *B. imbricata*

Bioassay-guided fractionation of the EtOAc extract from *B. ramosissima* subsp. *ramosissima* led to the isolation of two known compounds, **7.1** and **7.2** and their structures were confirmed by comparison of their mass spectrometry and NMR data[SI] with literature data.



7.1
3,5,7,4',5'-Pentahydroxyflavone
or Quercetin

7.2
3,5,7,4'-Tetrahydroxyflavone
or Kaempferol

Figure 7-3. Structures of compounds **7.1** and **7.2**

Compounds **7.1** and **7.2** were evaluated for anti-inflammatory activity in LPS + IFN-γ induced RAW 264.7 macrophages and N11 microglia. In RAW 264.7 macrophages, compounds **7.1** and **7.2** showed strong activity with IC_{50} value of 10.95 and 9.18 µg/mL respectively for NO inhibition and 17.35 and 11.26 µg/mL for TNF-α inhibition respectively.

In N11 microglia, compound **7.1** and **7.2** were also good to down regulate NO and the IC_{50} values were 19.71 and 16.06 µg/mL but both compounds were found to be good to down regulate TNF-α with IC_{50} values of 8.84 and 8.12 µg/mL respectively.

Compounds **7.1** and **7.2** did not show any toxicity in either of the cell lines up to the highest concentration (36 µg/mL) tested.

Compound **7.1** and **7.2** were isolated from the genus for the first time.

7.2 Results

7.2.1 Extraction and yield

Fresh leaves (230 g) of *Baeckea ramosissima* subsp. *ramosissima* (voucher no. AD 823136) and *B. imbricata* (120 g) (voucher no. AC 942545) were sequentially extracted with six solvents from low to high polarity which were *n*-hexane, DCM, EtOAc, EtOH, MeOH and water in order to separate lipophilic from hydrophilic compounds. A variable range of yields were obtained from the extraction (Table 7-1 and 7-2).

Table 7-1. Yield of the extracts after sequential extraction from *Baeckea ramosissima* subsp. *ramosissima*

Extract	**Yield of the extract (g)**
n-Hexane	2.47
DCM	4.51
EtOAc	3.27
EtOH	13.34
MeOH	8.40
Water	9.78
Total	**41.77**

Table 7-2. Yield of the extracts after sequential extraction from *B. imbricata*

Extract	**Yield of the extract (g)**
n-Hexane	1.99
DCM	2.64
EtOAc	2.30
EtOH	5.50
MeOH	4.73
Water	1.34
Total	**18.50**

7.2.2 Anti-inflammatory activity of extracts

The *n*-hexane, DCM, EtOAc, EtOH, MeOH and water extracts were tested in LPS + IFN-γ induced RAW 264.7 macrophages to obtain IC_{50} values for NO and TNF-α down regulation and also LC_{50} for cytotoxicity. Due to low solubility of the extracts in the cell culture media, they were diluted in DMSO, leading to a final concentration of up to 0.5% DMSO in cell culture medium. This concentration of DMSO showed a cell viability of less than 10% (data not shown). All of the sequential extracts with organic solvents (*n*-hexane, DCM, EtOAc, EtOH and MeOH) demonstrated significant anti-inflammatory activity to down regulate NO production with IC_{50} values ranging from 9.3 to 33.0 µg/mL for *B. ramosissima* subsp. *ramosissima*. The sequential EtOAc extract showed highest activity with IC_{50} of 9.33 and 6.96 µg/mL for suppression of NO and TNF-α production respectively and LC_{50} of 44.99 µg/mL for this *Baeckea* spp (Table-3 and Figure 3).

On the other hand, the sequential extracts with organic solvents (*n*-hexene, DCM, EtOAc and EtOH) obtained from *B. imbricata* exhibited anti-inflammatory activity to down regulate NO production with IC_{50} values ranging from 8.9 to 40.4 µg/mL. The sequential MeOH extract showed moderate activity (IC_{50} = 81.27 for NO suppresion). Similar to *B. ramosissima* subsp. *ramosissima*, the sequential EtOAc extract showed highest activity with IC_{50} of 8.98 and 8.96 µg/mL for suppression of NO and TNF-α production respectively and LC_{50} of 33.55 µg/mL for *B. imbricata* (Table-4 and Figure 4).

Table 7-3. Anti-inflammatory activity of *Baeckea ramosissima* subsp. *ramosissima* sequential extracts

Sequential Extract	**Inhibition of NO production (IC_{50} in µg/mL)**	***Inhibition of TNF-α production (IC_{50} in µg/mL)**	**Cytotoxicity (LC_{50} in µg/mL)**
n-Hexane	25.21 ± 2.8	27.60 ± 8.2	141.10 ± 50.8
DCM	13.85 ± 1.5	6.88 ± 2.7	44.18 ± 9.6
EtOAc	9.33 ± 1.1	6.96 ± 1.8	44.99 ± 9.7
EtOH	28.65 ± 2.8	33.17 ± 11.49	194.80 ± 42.4
MeOH	32.82 ± 3.7	86.03 ± 22.26	209.00 ± 74.1
Water	80.95 ± 8.3	133.0 ± 63.8	748.0 ± 287.7

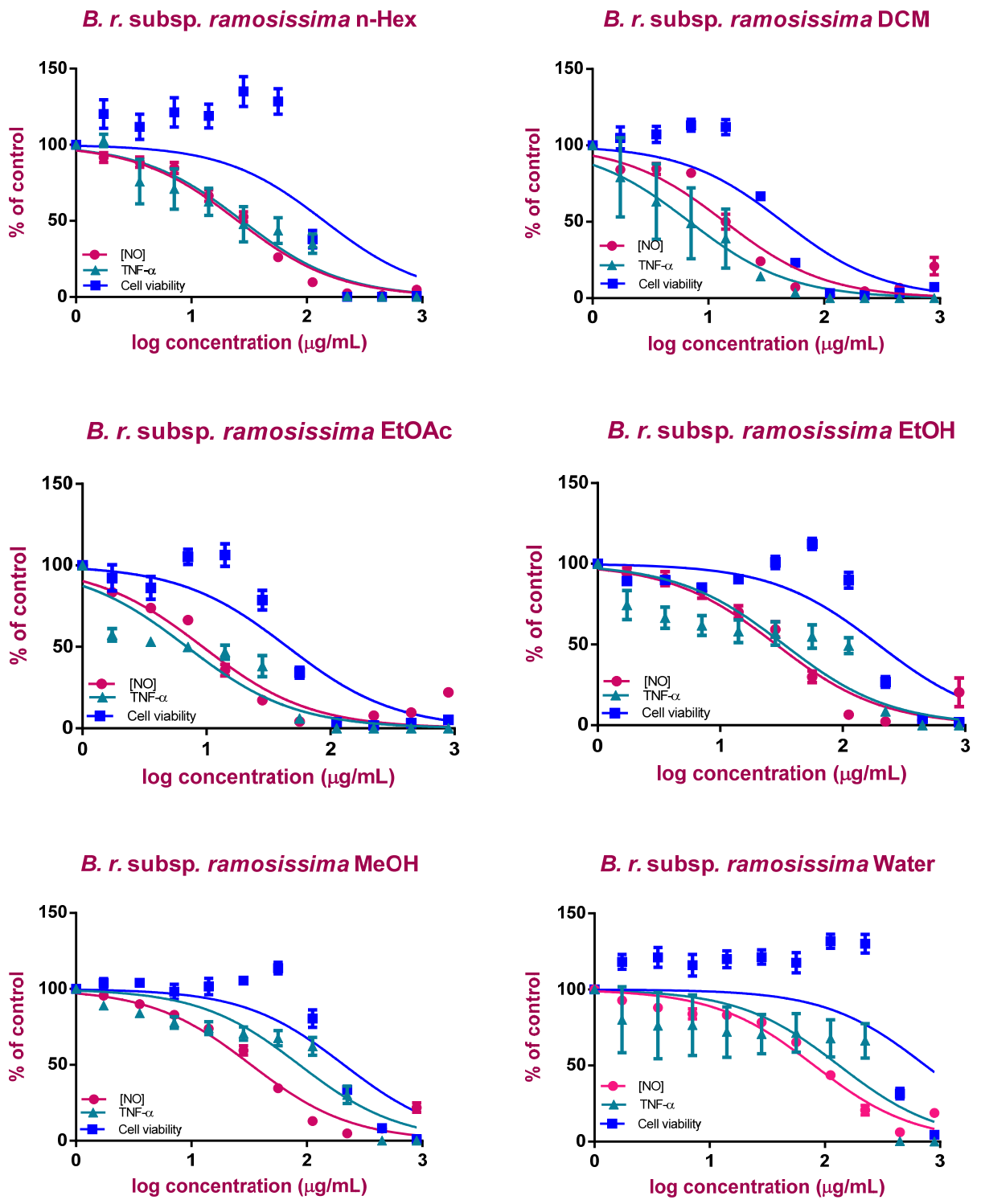


Figure 7-4. Down regulation of LPS and IFN-γ induced NO and TNF-α production and cell viability of *Baeckea ramosissima* subsp. *ramosissima* sequential extracts in RAW 264.7 macrophages. Data represented as three independent experiments in quadruplicates (n=12, mean ± SEM). *One experiment in triplicate (n=3, mean ± SEM).

Table 7-4. Anti-inflammatory activity of *B. imbricata* sequential extracts

Sequential Extract	**Inhibition of NO production (IC_{50} in µg/mL)**	***Inhibition of TNF-α production (IC_{50} in µg/mL)**	**Cytotoxicity (LC_{50} in µg/mL)**	**% Cytotoxicity**
n-Hexane	40.40 ± 4.9	65.77 ± 32.9	> 225	89.4
DCM	13.17 ± 1.6	29.68 ± 14.5	17.03 ± 2.7	
EtOAc	8.98 ± 0.7	8.96 ± 2.7	33.55 ± 4.9	
EtOH	17.64 ± 2.5	57.36 ± 17.5	65.45 ± 9.8	
MeOH	81.27 ± 17.2	57.36 ± 17.5	76.10 ± 19.3	
Water	71.01 ± 8.4	355.40 ± 176.2	> 450	65.7

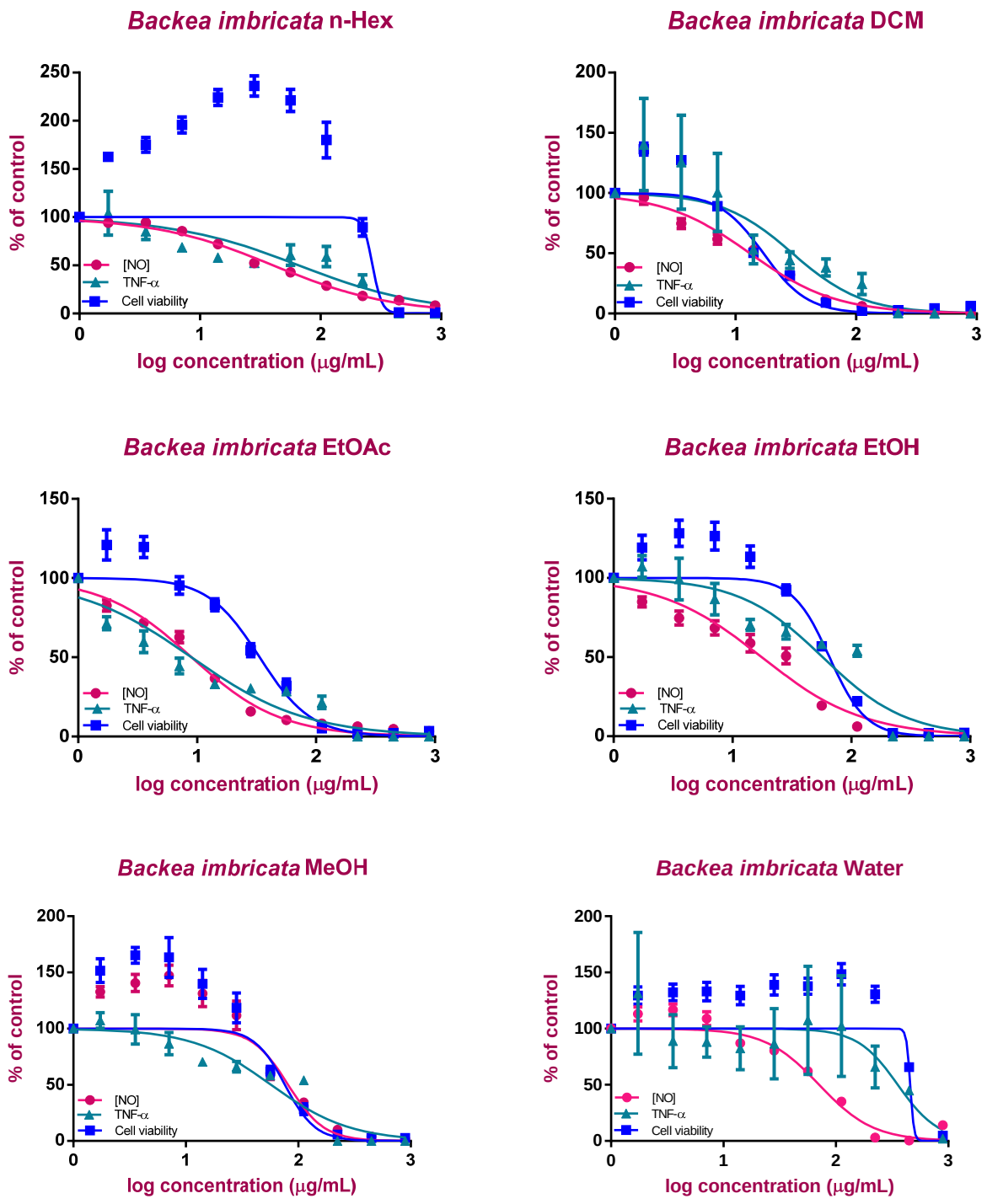
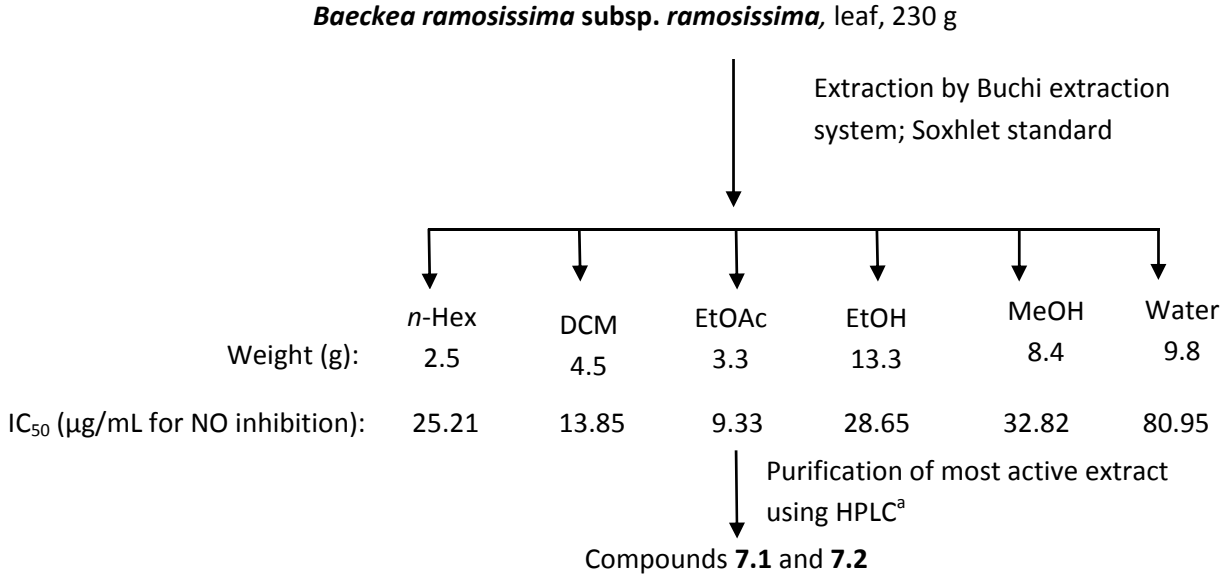


Figure 7-5. Down regulation of LPS and IFN-γ induced NO and TNF-α production and cell viability of *B. imbricata* sequential extracts in RAW 264.7 macrophages

Data represented as three independent experiments in quadruplicates (n=12, mean ± SEM). *One experiment in triplicate (n=3, mean ± SEM).

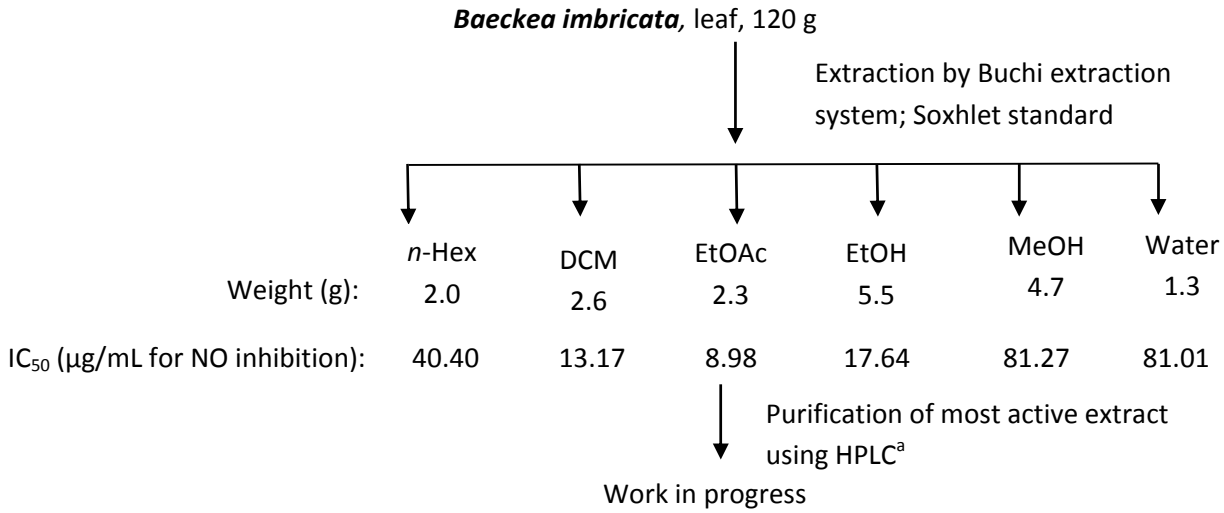
7.2.3 Isolation of active compounds

In order to identify bioactive compounds, the sequential EtOAc extract (IC_{50} = 9.33 and 8.98 µg/mL for NO inhibition for *B. ramosissima* subsp. *ramosissima* and *B. imbricata* respectively) was subjected to HPLC on a phenyl-hexyl column.



(a) Semi-preparative HPLC: Phenyl-Hexyl (250x10 mm; 10 µm), 10-100% MeOH/H_2O (0.01% HCO_2H modifier) over 40 mins, held for 15 mins then to 10% MeOH/H_2O on 57 min and held for last 3 minutes.

Scheme 7-1 Bioassay-guided separation of leaf extract of *B. ramosissima* subsp. *ramosissima*



(a) Semi-preparative HPLC: Phenyl-Hexyl (250x10 mm; 10 µm), 10-100% MeOH/H_2O (0.01% HCO_2H modifier) over 30 mins, held for 10 mins then to 10% MeOH/H_2O on 42 min and held for last 3 minutes.

Scheme 7-2 Bioassay-guided separation of leaf extract of *B. imbricata*

7.2.3.1 Identification of compound **7.1**

Compound **7.1** was obtained as a yellow solid. Its structure was dereplicated as the known quercetin or 3,5,7,4',5'-pentahydroxyflavone (Figure 7-2) by comparison of its mass spectrometry and NMR data[SI] with the literature data [21].

7.2.3.2 Identification of compound **7.2**

Compound **7.2** was obtained as a yellow solid. Its structure was dereplicated as the known kaempferol or 3,5,7,4'-tetrahydroxyflavone (Figure 7-2) by comparison of its mass spectrometry and NMR data[SI] with the literature data [22] .

7.2.4 Anti-inflammatory activity of identified compounds

Compounds **7.1** and **7.2** were evaluated for anti-inflammatory activity in LPS + IFN-γ induced RAW 264.7 macrophages (Figure 7-6 and Table 7-5) and N11 microglia (Figure 7-7 and Table 7-6).

In RAW 264.7 macrophages, compounds **7.1** and **7.2** showed strong activity with IC_{50} value of 10.95 and 9.18 µg/mL respectively for NO inhibition and 17.35 and 11.26 µg/mL for TNF-α inhibition respectively. Both of the compounds did not show any toxicity up to the highest concentration (36 µg/mL) tested.

In N11 microglia, compound **7.1** and **7.2** were also good to down regulate NO and the IC_{50} values were 19.71 and 16.06 µg/mL but both compounds were found to be stronger to down regulate TNF-α with IC_{50} values of 8.84 and 8.12 µg/mL respectively. Both of the compounds did not show any toxicity up to the highest concentration (36 µg/mL) tested.

Table 7-5. Anti-inflammatory activity of compounds isolated from *B. ramosissima* subsp. *ramosissima* in LPS + IFN-γ induced RAW 264.7 macrophages

Compound	Inhibition of NO production (IC_{50} in µg/mL)	Inhibition of TNF-α production (IC_{50} in µg/mL)	Cytotoxicity (LC_{50} in µg/mL)	% Cytotoxicity
7.1	10.95 ± 1.7	17.35 ± 4.3	> 36	211.3
7.2	9.18 ± 1.0	11.26 ± 2.3	> 36	155.5

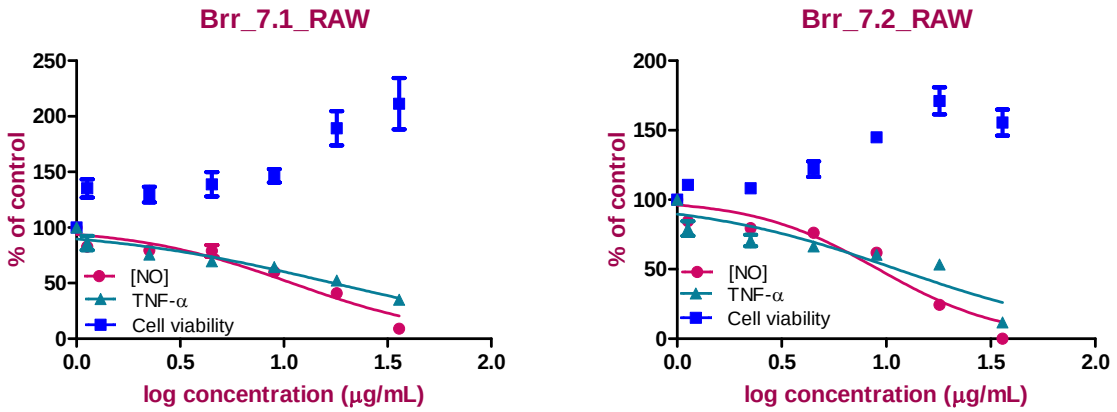


Figure 7-6. Down regulation of LPS and IFN-γ induced NO and TNF-α production and cell viability of isolated compounds from *B. ramosissima* subsp. *ramosissima* in RAW 264.7 macrophages. Data represented as two experiments in quadruplicates (n=8, mean ± SEM).

Table 7-6. Anti-inflammatory activity of compounds isolated from *B. ramosissima* subsp. *ramosissima* in LPS + IFN-γ induced N11 microglia

Compound	Inhibition of NO production (IC_{50} in µg/mL)	Inhibition of TNF-α production (IC_{50} in µg/mL)	Cytotoxicity (LC_{50} in µg/mL)	% Cytotoxicity
7.1	19.71 ± 2.0	8.84 ± 1.9	> 36	135.1
7.2	16.06 ± 1.2	8.12 ± 0.9	> 36	127.5

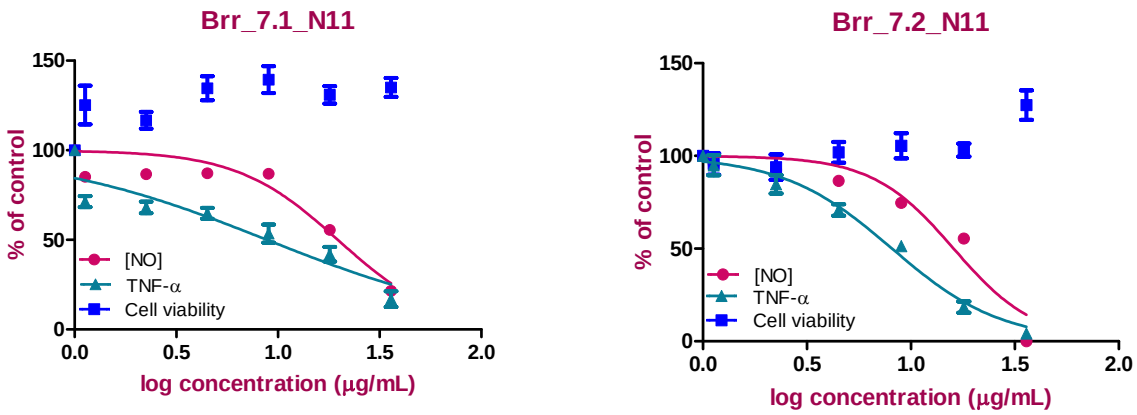


Figure 7-7. Down regulation of LPS and IFN-γ induced NO and TNF-α production and cell viability of isolated compounds from *B. ramosissima* subsp. *ramosissima* in N11 microglia. Data represented as two experiments in quadruplicates (n=8, mean ± SEM)..

7.3 Discussion

Sequential ethyl acetate extracts from both *B. ramosissima* subsp. *ramosissima* and *B. imbricata* were highly potent towards downregulation of both NO or TNF-α in LPS + IFN-γ induced RAW macrophages, which indicates that these two extracts are rich in anti-inflammatory constituents.

Compound **7.1** is commonly known as quercetin and is reported to have anti-oxidant, hepatoprotective, anti-cancer, anti-HIV, antidiabetic and anti-inflammatory activities in a number of studies [23-26]. In our study, we also found this compound is very good to downregulate NO and TNF-α in LPS + IFN-γ induced RAW macrophages with IC_{50} values of 10.95 and 17.35 µg/mL respectively as well as in N11 microglia with IC_{50} values of 19.71 and 8.84 µg/mL respectively.

Compound **7.2** is commonly known as kaempferol and is reported to have anti-oxidant, antidiabetic, diuretic as well as anti-inflammatory activities [23,27,28]. In our study, we also found this compound is very good to downregulate NO and TNF-α in LPS + IFN-γ induced RAW macrophages with IC_{50} values of 9.18 and 11.26 µg/mL respectively as well as in N11 microglia with IC_{50} values of 16.06 and 8.12 µg/mL respectively. Compounds **7.1** and **7.2** were found non-toxic in both of the cell lines up to the highest concentration (36 µg/mL) tested. Isolation and characterization of other active constituents from both of the *Baeckea* spp. are ongoing.

Table 7-7. Potency and total anti-inflammatory activity of *B. ramosissima* subsp. *ramosissima* sequential extracts in LPS + IFN-γ induced RAW 264.7 macrophages

Sequential Extract	Potency for NO inhibition(L/g)	Potency for TNF-α inhibition (L/g)	Total anti-inflammatory (NO inhibition) activity of extract (L)	Total anti-inflammatory (TNF-α inhibition) activity of extract (L)
n-Hexane	39.67	36.23	97.98	89.49
Dichloromethane	72.20	145.35	325.63	655.52
Ethyl acetate	107.18	143.68	350.48	469.83
Ethanol	34.90	30.15	465.62	402.17
Methanol	30.47	11.62	255.94	97.64
Water	12.35	7.52	120.82	73.53
Total anti-inflammatory activity of all extracts (L)			1616.47	1788.19

Table 7-8. Potency and total anti-inflammatory (AI) activity of compounds isolated from *B. ramosissima* subsp. *ramosissima* in LPS + IFN-γ induced RAW 264.7 macrophages

Individual compounds	Potency for NO inhibition(L/g)	Potency for TNF-α inhibition (L/g)	Total anti-inflammatory (NO inhibition) activity of compound (L)	Total anti-inflammatory (TNF-α inhibition) activity of compound(L)
Quercetin (**7.1**)	91.32	57.64	0.26	0.16
Kaempferol (**7.2**)	108.93	88.81	0.33	0.27
Total anti-inflammatory activity of all identified compounds (L)			0.58	0.43
% of total activity			0.04	0.02

The potency (for NO and TNF-α inhibition) of each of the sequential extracts and isolated compounds from *B. ramosissima* subsp. *ramosissima* leaves were measured as a reciprocal of IC_{50} value (Table 7-7 and 7-8). The total anti-inflammatory activity for both NO and TNF-α inhibition (Table 7-7 and 7-8) of the same extracts and compounds were also obtained by multiplying respective potency and yield of dried extracts and isolated compounds. Thus the total anti-inflammatory activity of all extracts was found to be 1616.47 L for NO inhibition in 230 g leaves of *B. ramosissima* subsp. *ramosissima* of which 0.26 L and 0.33 L were obtained by quercetin (**7.1**) and kaempferol (**7.2**) respectively. Similarly, the total anti-inflammatory activity of all extracts was found to be 1788.19 L for TNF-α inhibition in 230 g leaves of which 0.16 L and 0.27 L were obtained by quercetin (**7.1**) and kaempferol (**7.2**) respectively.

The compounds were isolated by purifying only the EtOAc extract which was 1.37% of the total extract and 25% of this extract was purified using HPLC. Thus the total anti-inflammatory activity of all isolated compounds were calculated 3.18 L and 2.34 L for NO and TNF-α inhibition respectively in the leaves of *B. ramosissima* subsp. *ramosissima* under investigation.

7.4 Experimental Section

7.4.1 General experimental procedures

Acetonitrile, *n*-hexane, dichloromethane and methanol were supplied by Merck. Ethyl acetate from Fisher Chemical, ethanol from Chem-Supply and water used was Milli-Q water. Bovine serum albumin, lipopolysaccharide (*E. coli* serotype-0127:B8), EDTA, N-(1-napthyl) ethylenediamine dihydrochloride, benzylpenicillin G sodium salt, resazurin sodium salt (10%), streptomycin, sulphanilamide, 3,3',5,5'-tetramethylbenzidine (TMB), trypan blue and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Foetal bovine serum (FBS) and glutamine were purchased from Life Technologies (Mulgrave, VIC, Australia). Murine interferon-γ (IFN-γ) and TNF-α ELISA kits were purchased from PeproTech Asia (Rehovot, Israel). Citric acid and monosodium dihydrogen carbonate (NaH_2CO_3) were from AJAX Chemicals (Auburn, NSW, Australia). Tween-20 was from Amresco (Solon, Ohio, USA). Methanol, monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), sodium chloride (NaCl) and sulfuric acid (H_2SO_4) were from Merck (Darmstadt, Germany). Sodium carbonate (Na_2CO_3) was BDH brand supplied by Merck Pty. Ltd. (Kilsyth, VIC, Australia).

7.3.2 Experimental procedures for MS, HPLC and NMR

Semi-preparative HPLC was performed using an Agilent 1260 Infinity Series (Santa Clara, CA, USA). NMR spectra were obtained on a Bruker Ascend 400 MHz spectrometer (Bruker Biospin GmbH, Bremen, Germany). TopSpin software was used to analyse the spectral data. ^{1}H NMR spectra were recorded at 400 MHz. The chemical shifts (δ) are expressed in parts per million (ppm) as δ values and coupling constant J in Hertz (Hz). The experiments were performed in dimethyl sulfoxide-d_6 (DMSO-d_6) and chemical shifts were calibrated relative to the DMSO solvent peak (^{1}H δ at 2.50 and ^{13}C δ 39.51 ppm). High resolution mass spectrometry (HRMS) was carried out using a Waters Xevo Q-TOF mass spectrometer operating in positive electrospray ionization (ESI) mode.

7.4.3 Plant material

Fresh leaves of *Baeckea ramosissima* subsp. *ramosissima* (voucher no. AD 823136) and *B. imbricata* (voucher no. AC 942545) were collected in the month of July, 2016 from the 'Australian Botanic Gardens' at Mount Annan, NSW, Australia. The voucher specimens were deposited at the National Herbarium of NSW, Australia.

7.4.4 Extraction and isolation

Fresh leaf material from *Baeckea ramosissima* subsp. *ramosissima* (230 g) *and B. imbricata* (120 g) was first cut into small pieces with scissors and then ground to a coarse powder using a hand blender. The coarse powder was then filled into the thimbles of an accelerated solvent extraction system (Buchi B-811, Switzerland) and extracted under standard Soxhlet mode (for 2 × 15 minutes cycles) using six solvents (*n*-hexane, dichloromethane, ethyl acetate, ethanol, methanol and water) sequentially from low to high polarity. The volume of the extracts was reduced to ca. 2—4 mL using a rotary evaporator and then evaporated to dryness with nitrogen. The sequential ethyl acetate extract was found most

potent for both plants when screened for anti-inflammatory activity. These crude extracts were then resuspended in methanol and subjected directly through reversed phase semi-prep HPLC (Phenyl-Hexyl column, 250 x 10 mm, Phenomenex). Peak detection was performed with a photodiode array detector (PDA) using three detection wavelengths (210 nm, 254 nm and 290 nm). A gradient starting from 10% methanol/water to a 100% methanol (with a constant 0.01% formic acid modifier) over 40 min and held at 100% methanol for 15 min was applied for *B. ramosissima* subsp. *ramosissima.* to yield compound **7.1** (t_R = 32.3 min, 2.8 mg) and compound **7.2** (t_R = 34.6 min, 3.0 mg). On the other hand, a gradient starting from 10% methanol/water to a 100% methanol (with a constant 0.01% formic acid modifier) over 30 min and held at 100% methanol for 10 min was applied for *B. imbricata*. All isolated compounds were purified to 95% purity or more as judged by HPLC (UV detection) and ^{1}H NMR spectroscopy before determining the bioactivity.

7.4.5 Maintenance and preparation of RAW 264.7 macrophages and N11 microglia

RAW 264.7 macrophage and N11 microglial cells were grown in 175 cm^2 culture flasks on DMEM (Dulbecco's Modified Eagle's Medium) containing 5% FBS (fetal bovine serum) that was supplemented with penicillin (100 u/mL), streptomycin (100 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. Once cells had grown to confluence in the culture flask, they were removed using a rubber policeman cell scraper, as opposed to using trypsin, which can remove membrane-bound receptors. The cell suspension was concentrated by centrifugation for 3 min at 900 rpm and resuspended in a small volume of fresh DMEM (with 1% antibiotics and 5% FBS). Cell densities were estimated using a Neubauer counting chamber. The cell concentration was adjusted with DMEM (with 1% antibiotics and 5% FBS) to obtain 60,000 cells/100 µL cell suspension. 100 µL of cell suspension was then dispensed into the wells of 96-well plates (Eppendorf, Hamburg, Germany). Plates were incubated at 37 °C; 5% CO_2 for 24 h before the activation experiments were carried out.

7.4.6 Activation of RAW 264.7 macrophages and N11 microglia

From each well, the media was removed and replaced with fresh DMEM containing 0.1% FBS. For assays with extracts, a 90 µL volume of the dilutions in DMEM (with 0.1% FBS) was added an hour prior to addition of the activator. Due to the often inconsistent nature of LPS at activating cells, a combination of LPS (10 µg/mL) and IFN-γ (10 U/mL), both in DMEM (with 0.1% FBS), were used for activation. For the sequential extracts a maximum dose of 900 µg/mL was used, and diluted serially by 50% up to a minimum of 10 doses (900, 450, 225, 112.5, 56.25, 28.125, 14.062, 7.031, 3.515, 1.7578 and 0.8789 µg/mL in the wells respectively). A maximum dose of the compounds (**7.1** and **7.2**) used is 36 µg/mL, and diluted serially by 50% up to a minimum of 7 doses (36, 18, 9, 4.5, 2.25, 1.12 and 0.56 µg/mL in the wells respectively). After activation, the cells were incubated for 24 h at 37 °C and 5% CO_2 and then NO and TNF-α inhibition, and cell viability were determined. Cells with media alone were used as negative control and activated cells used as positive control. The effects of solvents on readouts were initially determined, but as the anti-inflammatory or cytotoxic effects of the solvents were < 10% even at the highest concentration used, parameters were compared to controls without solvent.

7.4.7 Determination of nitric oxide production by Griess assay

Nitric oxide was determined by Griess assay for quantification of nitrite, one of its stable reaction products. Griess reagent was freshly made up of equal volumes of 1% sulfanilamide and 0.1% naphthylethylene-diamine in 5% HCl. In the presence of nitrite this reagent forms a violet colour. From each well, 50 µL of supernatant was transferred to a fresh 96-well plate and mixed with 50 µL of Griess reagent, and the colour produced was measured at 540 nm in a microplate reader (POLARstar Omega, BMG Labtech, Mornington, Australia). The remaining supernatant from each well was used for a TNF-α assay. The concentration of nitrite was calculated using a standard curve with sodium nitrite (0 to 250 µM), and linear regression analysis.

7.4.8 Determination of cell viability by Alamar blue assay

The Alamar Blue assay is a colorimetric assay involving the cellular reduction of resazurin to resorufin. Alamar Blue solution [100 µL of 10% Alamar Blue (Resazurin) in DMEM medium] was added to each well and incubated at 37 °C for 1-2 h. After incubation, fluorescence was measured (excitation at 544 nm and emission at 590 nm) using a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia). The results were expressed as a percentage of the intensity of that in control cells after background fluorescence was subtracted.

7.4.9 TNF-α determination by ELISA

The supernatants obtained from each well (remaining supernatant after 24 hours of activation) and experiment that gave better readout for NO and Alamar blue assay were used for determination of TNF-α using a commercial sandwich ELISA (Catalog number: 900-K54; Peprotech, USA) according to the manufacturer's protocol. The supernatants were diluted 30 times using diluent (0.1% w/v bovine serum albumin and 0.05% v/v tween-20 in PBS [1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl; pH7.4]). Capture antibody was used at a concentration of 1.25 µg/mL in PBS. To make a standard curve TNF-α (10 ng/mL standard) was diluted serially by 50% up to a minimum of 10 doses (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019 and 0.0097 ng/mL in the wells respectively) and was used as the internal standard. TNF-α was detected with a biotinylated second antibody and an Avidin peroxidase conjugate with TMB as detection reagent. The colour development was monitored at 655 nm, taking readings every 5 min. After about 30 min, the reaction was stopped using 0.5 M sulfuric acid, and the absorbance was measured at 450 nm of measurement filter using a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia). The absorbance data was expressed as a percentage of control wells after conversion of the concentrations by using a standard curve constructed with defined concentrations of TNF-α. Curve fitting of this standard curve and extrapolation of experimental data were performed using non-linear regression analysis.

7.4.10 Data presentation and analysis

For sequential extracts, three experiments in quadruplicates (n=12) were combined to determine the IC_{50} (for NO inhibition) and LC_{50} (for cell viability) whereas one experiment in triplicate (n=3) was conducted to obtain IC_{50} for TNF-α inhibition using the dose-response inhibition function in GraphPad

Prism version 6.01 (GraphPad Software Incorporated, USA). For purified compounds, two experiments in quadruplicates (n=8) were combined to determine the IC_{50} (for NO and TNF-α inhibition) and LC_{50} (for cell viability) using the dose-response inhibition function in same version of GraphPad.

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Chapter 8
Summary and Future Perspective

8.1 Summary and Future Perspective

Persistent inflammation contributes to multiple age-related chronic conditions including musculoskeletal and neurodegenerative diseases, cardiovascular diseases, asthma, rheumatoid arthritis and inflammatory bowel disease. More recently, chronic neuroinflammation has been attributed to Parkinson's and Alzheimer's disease, autism-spectrum and obsessive-compulsive disorders [1,2]. Plants having medicinal values are recognised as the oldest and most widespread form of medication throughout the world. On the basis of ethnobotanical knowledge the anti-inflammatory activity of several plant extracts and isolated compounds has already been scientifically demonstrated in a number of literature studies [3,4].

In a continuing search for anti-inflammatory natural products with potential for longer-term clinical development, more than ten plants were selected for bioassay guided investigation after screening of a total of 32 plants for the suppression of nitric oxide and TNF-α production induced by lipopolysaccharide (LPS) and interferon gamma (IFN-γ) in RAW 264.7 macrophages. Cytotoxicity of the crude extracts was also examined using an Alamar blue cell viability assay.

The plants studied here were chosen on the basis of their traditional use to treat inflammatory conditions by the D'harawal Aboriginal people of the Campbelltown region (Southwest Sydney, Australia). Five of the selected plants were fractionated to yield three new and twelve known compounds as summarised in Tables 8-1 and 8-2. Even though many known molecules were characterised, the anti-inflammatory activity for many of them have not been reported previously. These findings provide the scientific base for the claim of the Aboriginal D'harawal people regarding their knowledge of medicinal plants. This body of work is contributing towards the recognition of the Aboriginal knowledge as well as preserving the knowledge for future generations and at the same time giving additional value to it.

Substantial purification procedures were followed to obtain proper NMR spectroscopic data for structure elucidation as well as reliable biological assay results for each of the isolated compounds. However, purification and structure identification of the compounds along with extensive biological assays presented a challenge due to limited amount of starting material. Again as we described in Chapter 2 and Chapter 4, according to traditional D'harawal knowledge, the amount of the anti-inflammatory compounds in the plants is dependent on the plant's environment especially on other plants that are growing around the particular plant required. But for the present study, we collected the plant material from random trees in the botanical garden.

Identification of other anti-inflammatory compounds from the selected plants is in progress.

The next ideal step would be intervention trials for the pure compounds in animal models followed by humans. This could be done with a single compound or a combination of two compounds or more. Any adverse effect from the compounds needs to be documented in addition to the drug efficacy.

Table 8-1 Summary of Anti-inflammatory Natural Products Isolated and Tested in RAW 264.7 Macrophages

Compound	Plant	IC_{50} µg/mL (NO inhibition)	IC_{50} µg/mL (TNF-α inhibition)	LC_{50} µg/mL (Cytotoxicity)	New/Known
1	*Eucalyptus viminalis*	44.0 ± 9.0	41.0 ± 23.8	> 36	New
2	*Eucalyptus viminalis*	47.0 ± 7.8	38.3 ± 14.8	> 36	Known
3	*Eucalyptus viminalis*	37.6 ± 4.9	43.2 ± 18.4	> 36	Known
4 (hydrolysed product of 1)	*Eucalyptus viminalis*	18.7 ± 0.5	21.5 ± 4.4	54.4 ± 21.4	Known
3a.1	*Eucalyptus bosistoana*	95.74 ± 14.4	51.56 ± 4.6	> 36	Known
5.1	*Melaleuca linariifolia*	72.81 ± 37.9	58.88 ± 35.9	> 36	Known
5.2	*Melaleuca linariifolia*	39.69 ± 11.5	80.70 ± 50.1	> 36	Known
5.3	*Melaleuca linariifolia*	2.73 ± 0.3	4.11 ± 1.0	9.68 ± 1.1	Known
6.1	*Syncarpia glomulifera* subsp. *glomulifera*	3.91 ± 0.5	16.90 ± 3.4	> 18	New
6.2	*Syncarpia glomulifera* subsp. *glomulifera*	35.15 ± 7.3	32.12 ± 13.2	> 36	Known
6.3	*Syncarpia glomulifera* subsp. *glomulifera*	2.76 ± 0.3	20.80 ± 14.1	> 18	Known
6.4	*Syncarpia glomulifera* subsp. *glomulifera*	29.42 ± 2.7	37.57 ± 11.7	> 36	New
6.5	*Syncarpia glomulifera* subsp. *glomulifera*	7.84 ± 1.3	33.35 ± 3.8	> 36	Known
7.1	*Baeckea ramosissima* subsp. *ramosissima*	10.95 ± 1.7	17.35 ± 4.3	> 36	Known
7.2	*Baeckea ramosissima* subsp. *ramosissima*	9.18 ± 1.0	11.26 ± 2.3	> 36	Known

Table 8-2 Summary of Anti-inflammatory Natural Products Isolated and Tested in N11 Microglial cells

Compound	Plant	IC_{50} µg/mL (NO inhibition)	IC_{50} µg/mL (TNF-α inhibition)	LC_{50} µg/mL (Cytotoxicity)	New/Known
1	*Eucalyptus viminalis*	43.4 ± 7.3	20.4 ± 10.5	> 36	New
2	*Eucalyptus viminalis*	34.1 ± 4.2	34.3 ± 18.1	> 36	Known
3	*Eucalyptus viminalis*	21.8 ± 3.7	19.0 ± 7.1	> 36	Known
4 (hydrolysed product of **1**)	*Eucalyptus viminalis*	10.2 ± 1.0	17.0 ± 5.0	> 22.5	Known
3a.1	*Eucalyptus bosistoana*	44.31 ± 6.1	35.50 ± 11.3	> 36	Known
5.1	*Melaleuca linariifolia*	66.27 ± 31.3	17.34 ± 3.3	> 36	Known
5.2	*Melaleuca linariifolia*	58.05 ± 30.1	21.84 ± 3.6	> 36	Known
5.3	*Melaleuca linariifolia*	2.23 ± 0.4	6.76 ± 1.4	11.01 ± 1.4	Known
6.1	*Syncarpia glomulifera* subsp. *glomulifera*	4.52 ± 0.8	6.50 ± 1.3	24.31 ± 6.1	New
6.2	*Syncarpia glomulifera* subsp. *glomulifera*	21.17 ± 3.8	27.01 ± 8.5	> 36	Known
6.3	*Syncarpia glomulifera* subsp. *glomulifera*	3.87 ± 0.5	13.66 ± 3.8	19.05 ± 5.7	Known
6.4	*Syncarpia glomulifera* subsp. *glomulifera*	39.64 ± 10.4	33.06 ± 5.9	> 36	New
6.5	*Syncarpia glomulifera* subsp. *glomulifera*	4.51 ± 0.6	5.46 ± 0.7	> 36	Known
7.1	*Baeckea ramosissima* subsp. *ramosissima*	19.71 ± 2.0	8.84 ± 1.9	> 36	Known
7.2	*Baeckea ramosissima* subsp. *ramosissima*	16.06 ± 1.2	8.12 ± 0.9	> 36	Known

8.2 References

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Supporting Information (SI)

Chapter 2: Supporting Information

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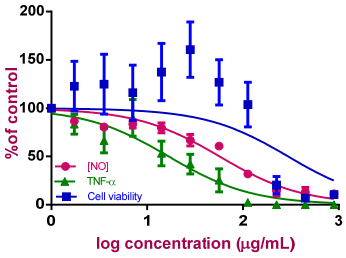

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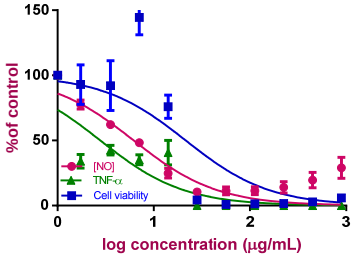

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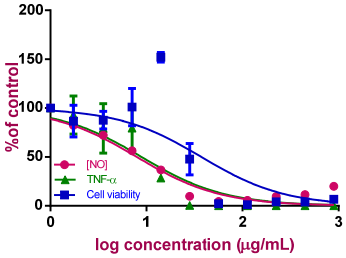

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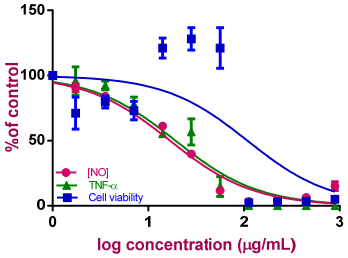

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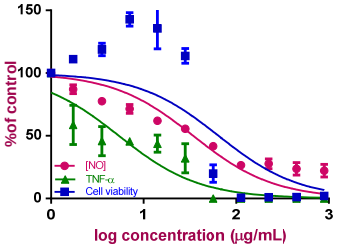

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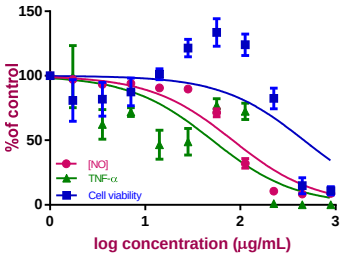

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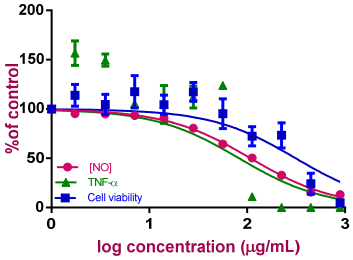

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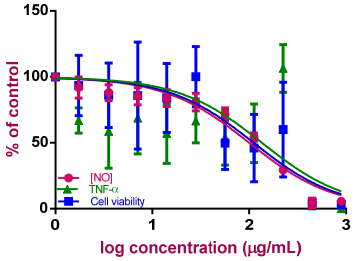

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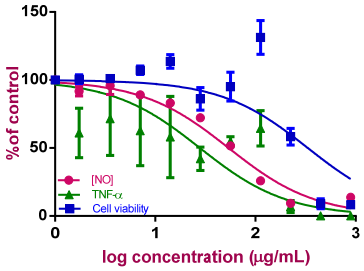

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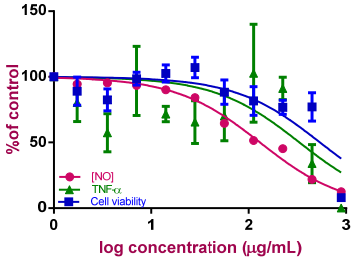


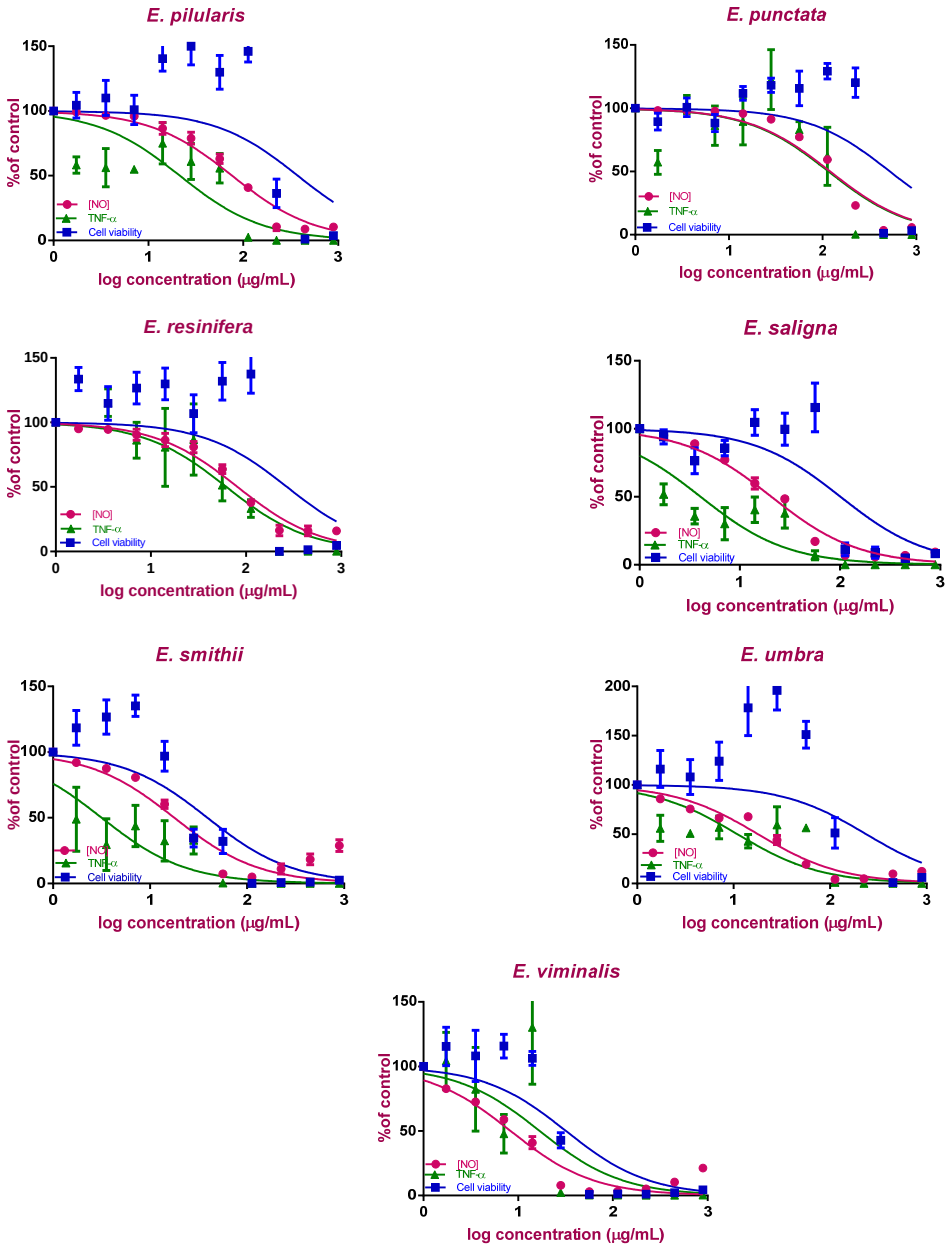
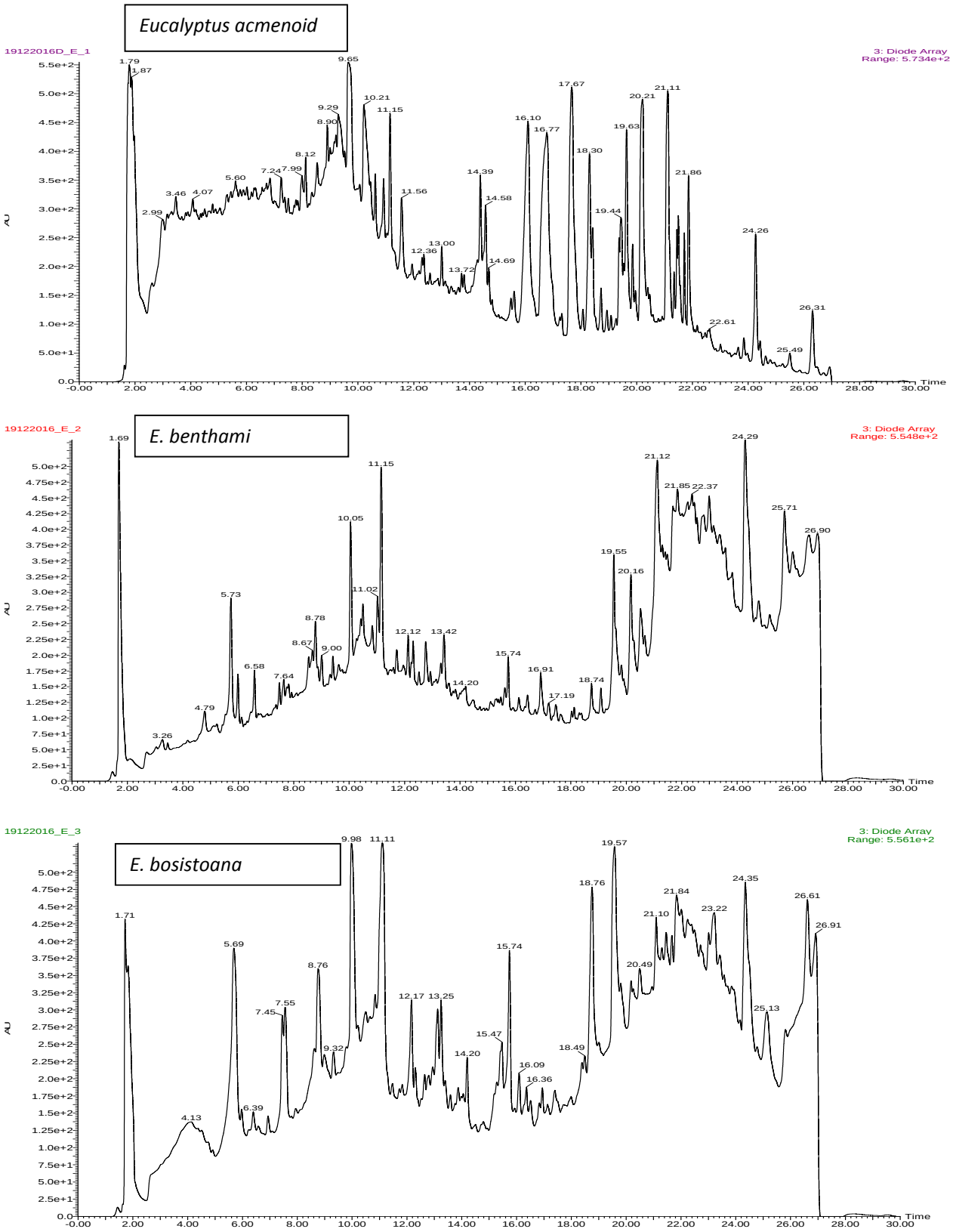
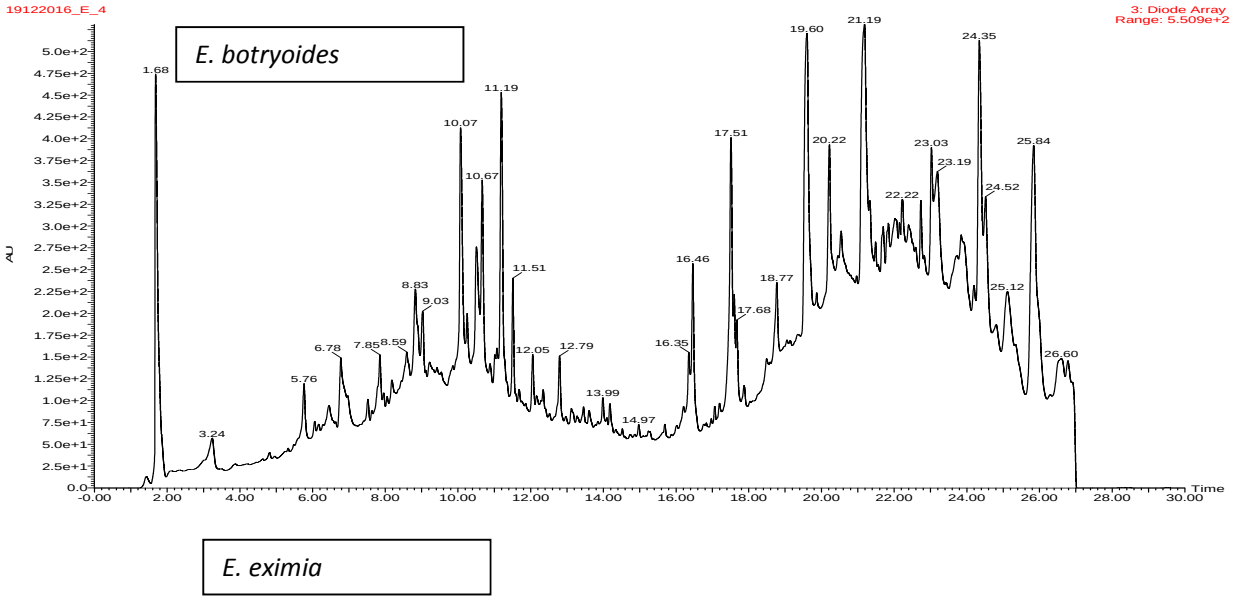


Figure: Down regulation of LPS and IFN-γ induced production of pro-inflammatory markers by ethanolic extracts from seventeen *Eucalyptus* spp. Results are represented as mean ± SEM of three experiments assayed in triplicate for downregulation of nitric oxide production and cell viability and for down regulation of TNF-α production one experiment assayed in triplicate.

2.1 Photo Diode Array (PDA) Spectrum of 17 *Eucalyptus* spp. screened for anti-inflammatory activity.

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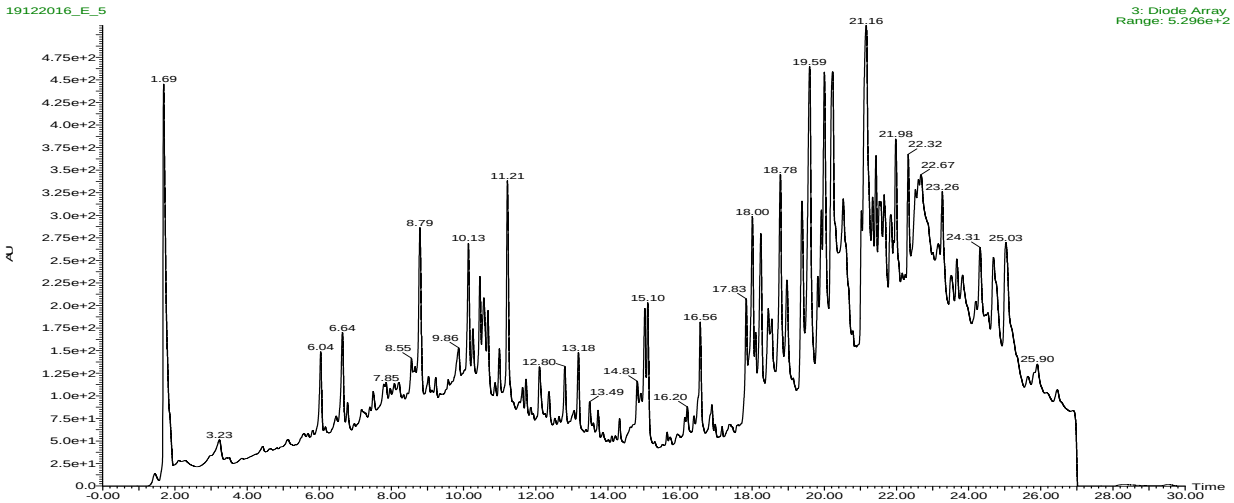
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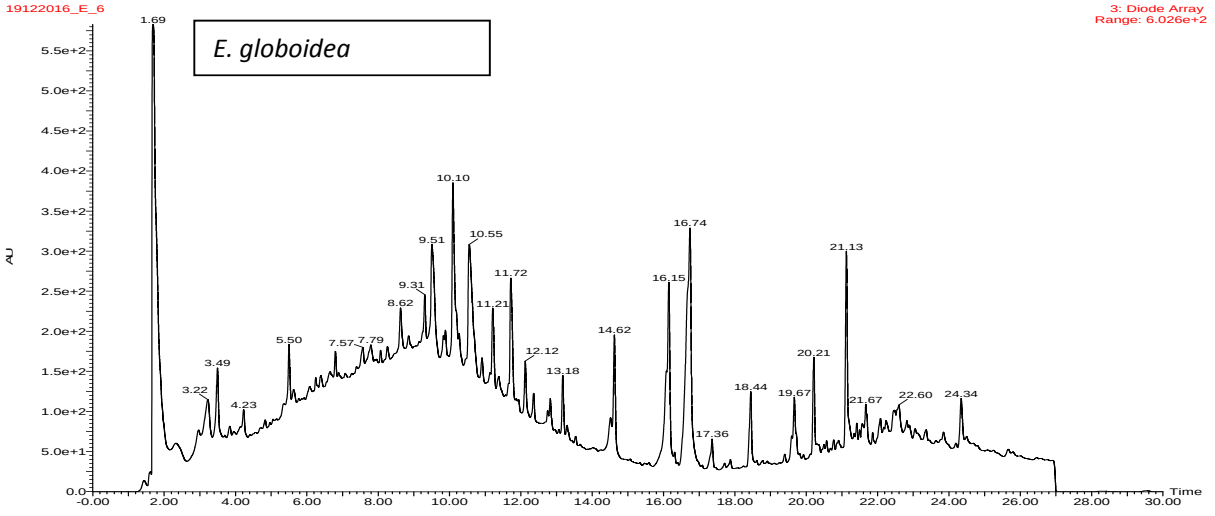
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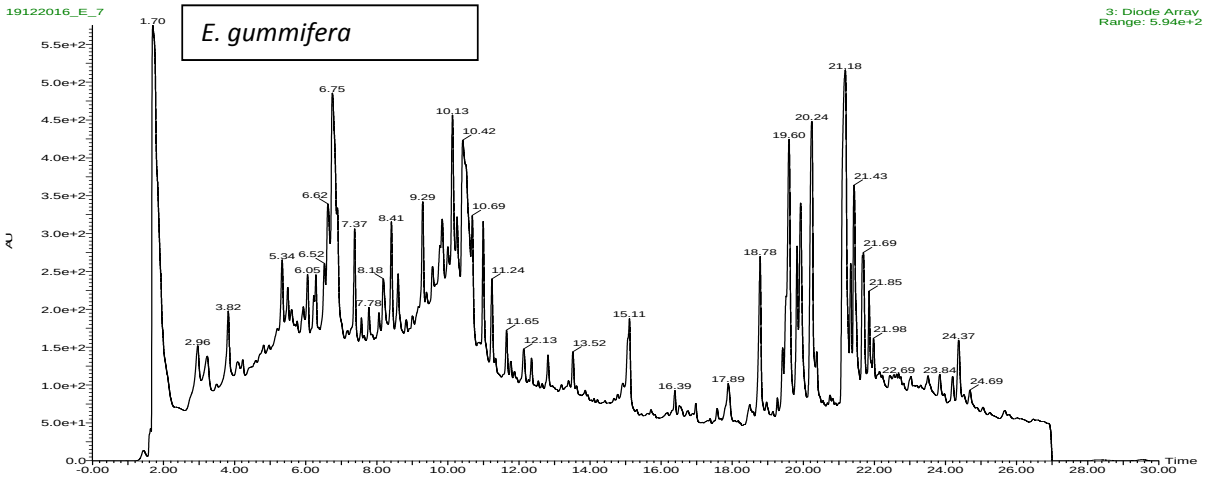
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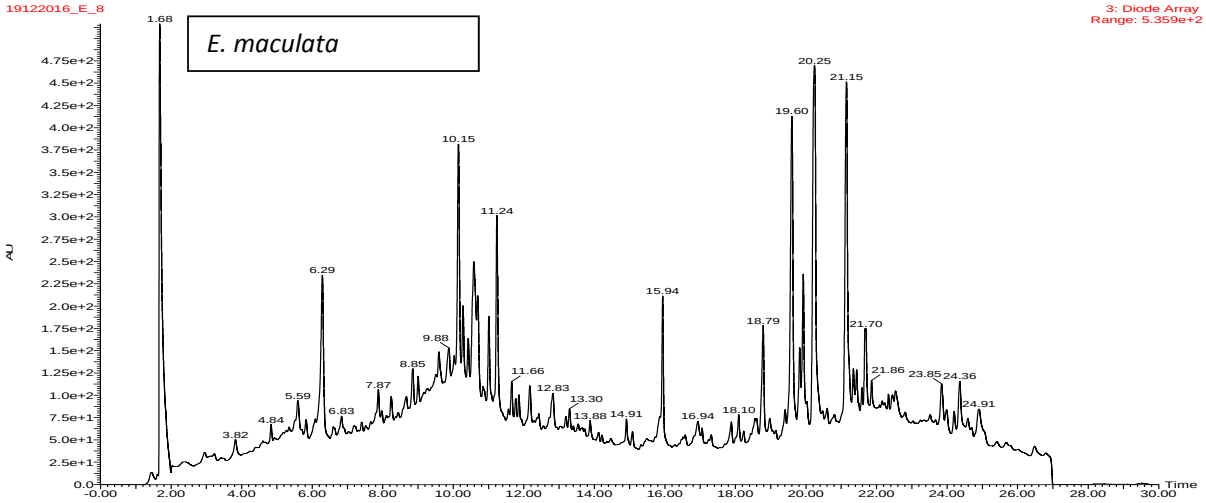
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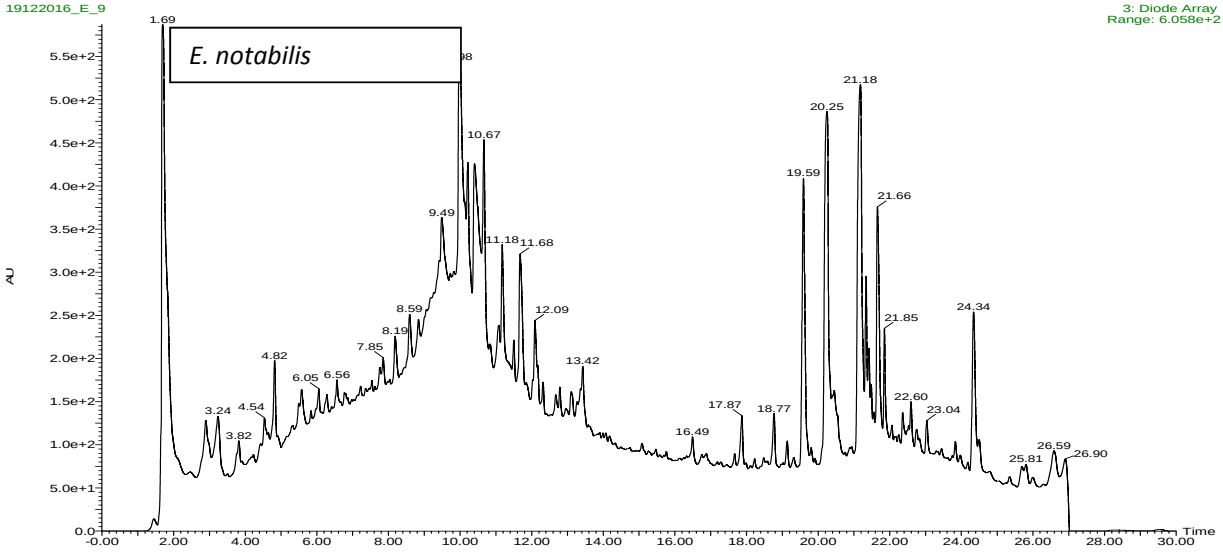
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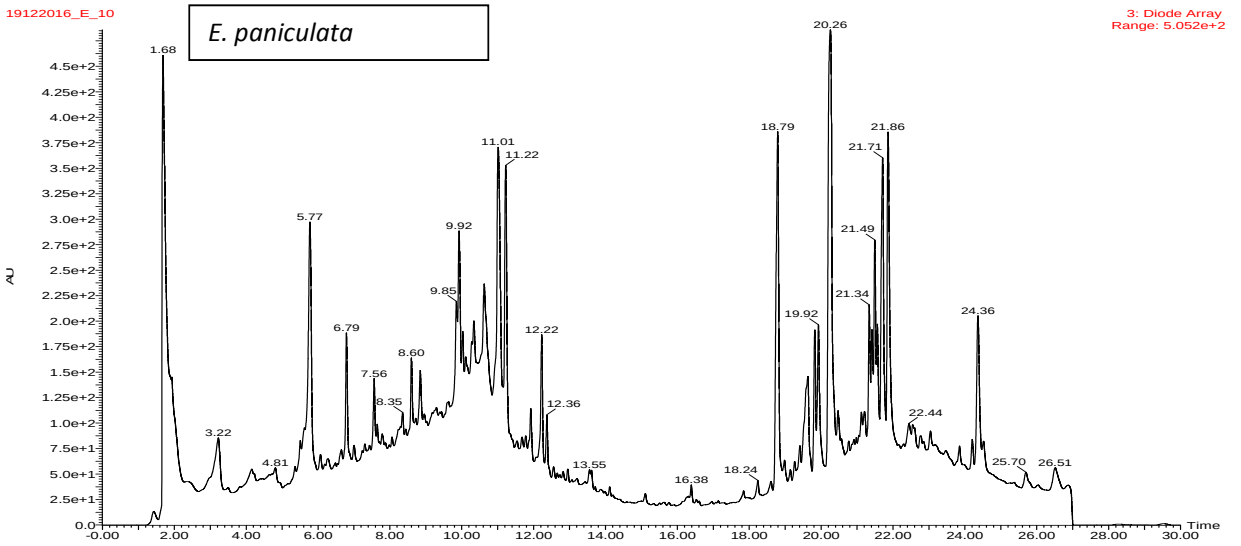
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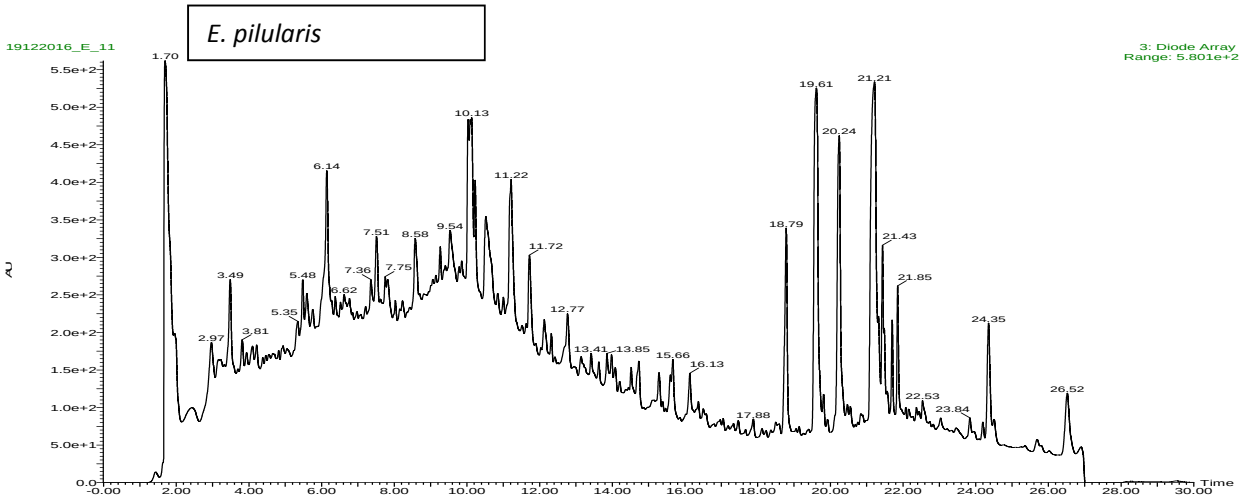
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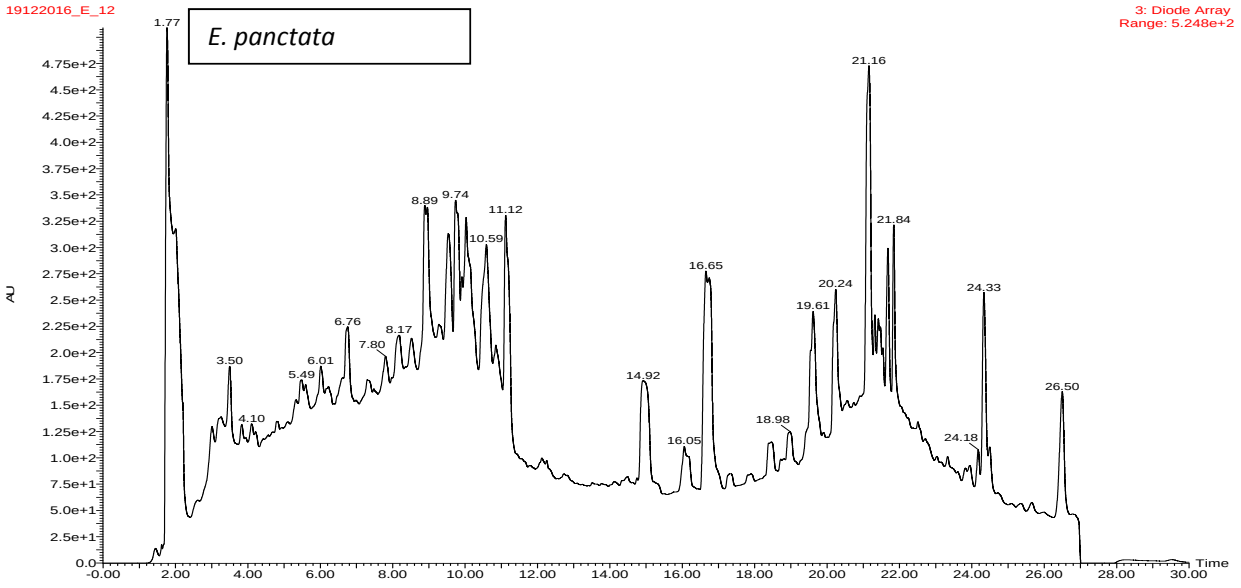
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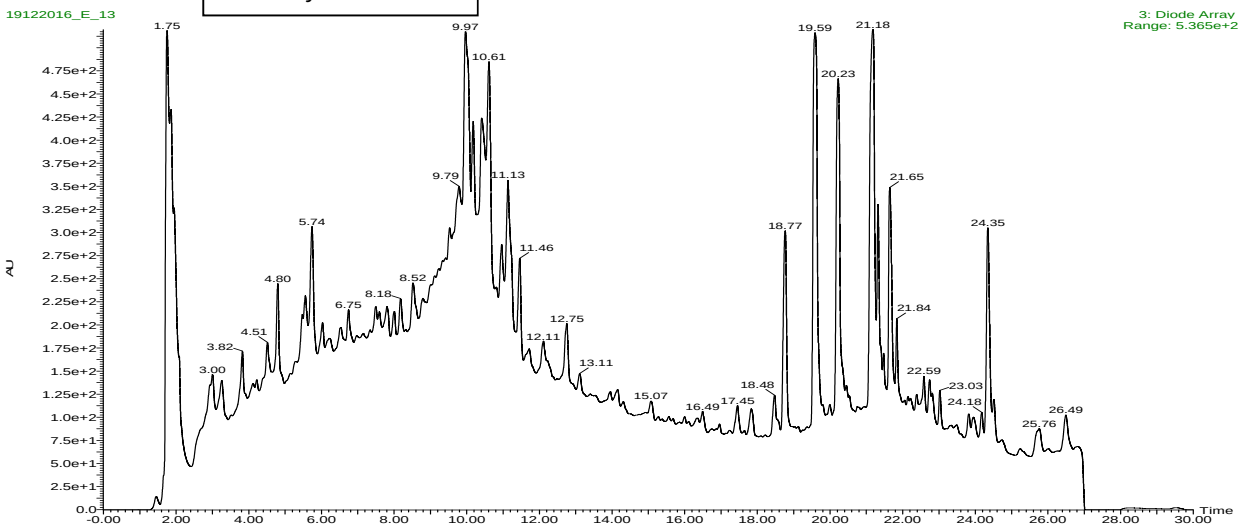

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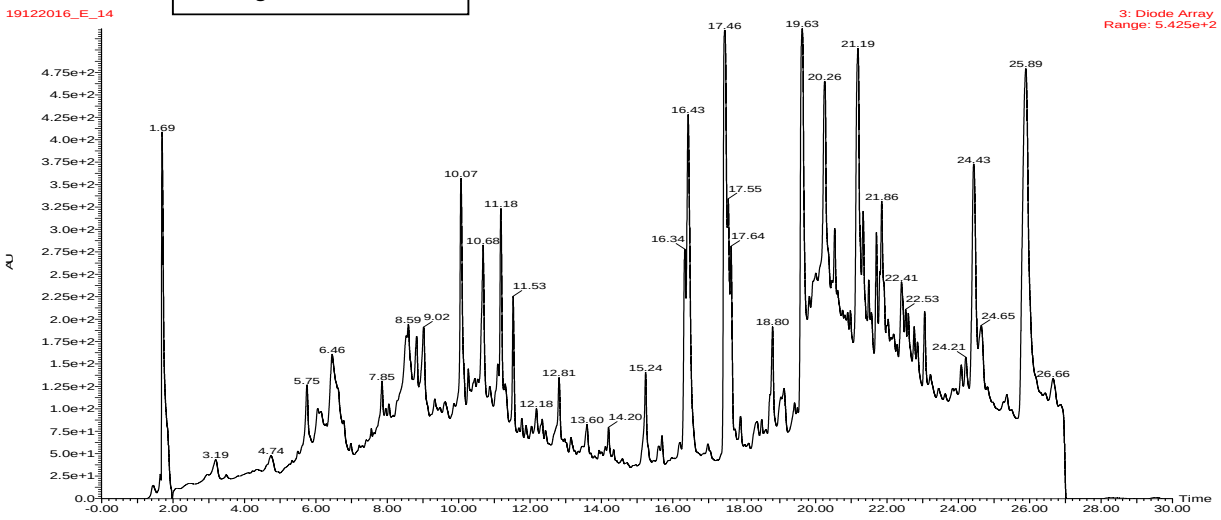

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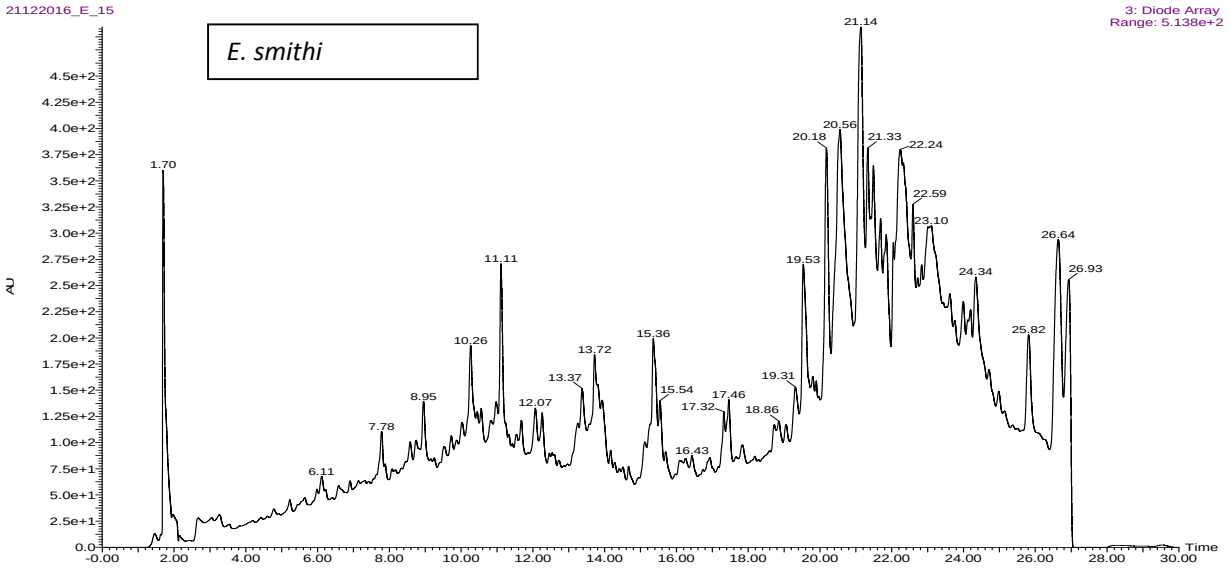

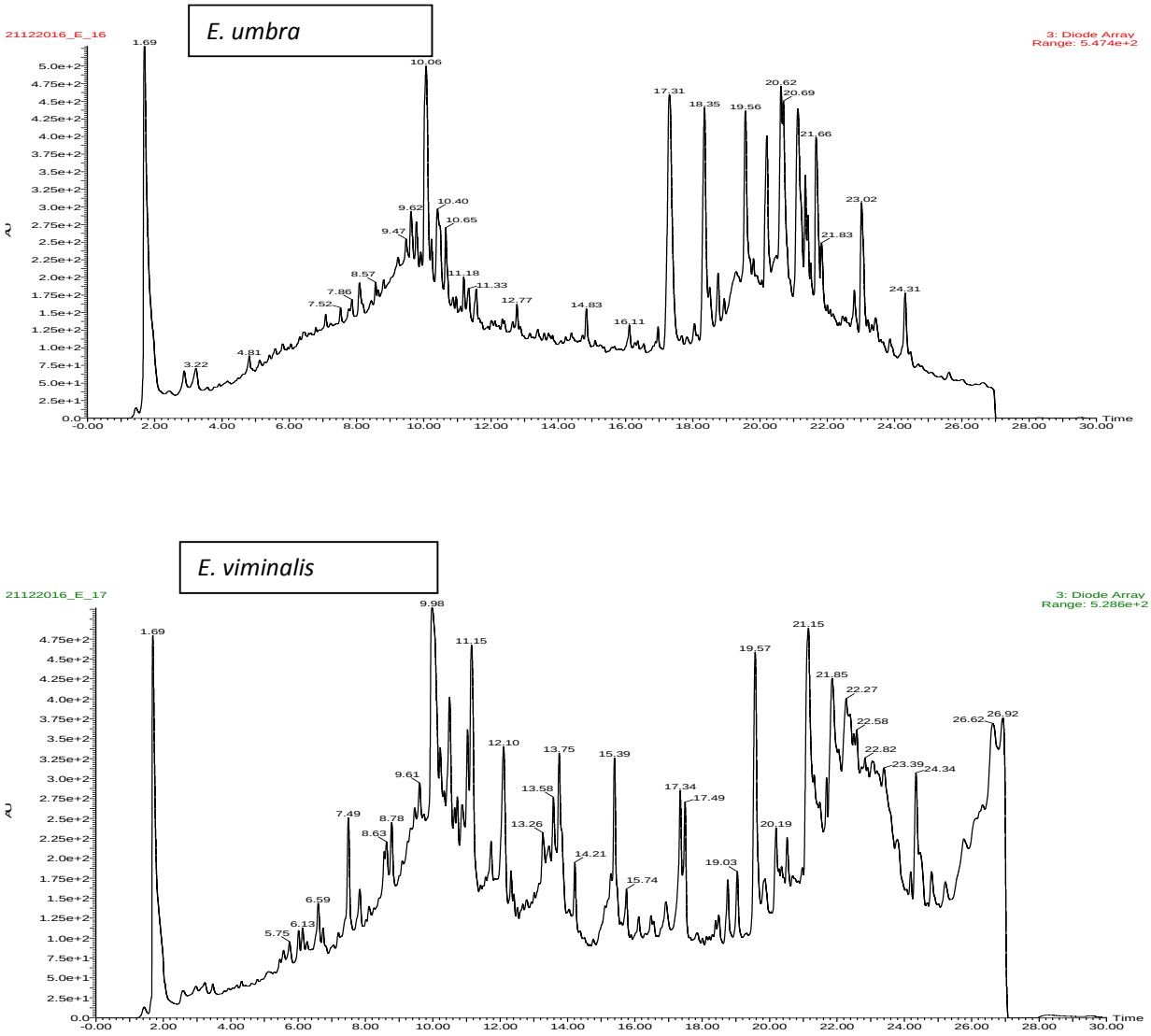

***LCMS Condition:** Aquity UPLC column (2.1x 150 mm, 1.7 µm), eluting 0.2 mL/min, 5% to 95% methanol over 20 minutes and held for 10 minutes detecting at 210 and 254 nm (DAD).

Chapter 3: Supporting Information

A new anti-inflammatory chromone from the leaves of *Eucalyptus viminalis*

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[b]NICM Health Research Institute, Western Sydney University, Penrith NSW, Australia

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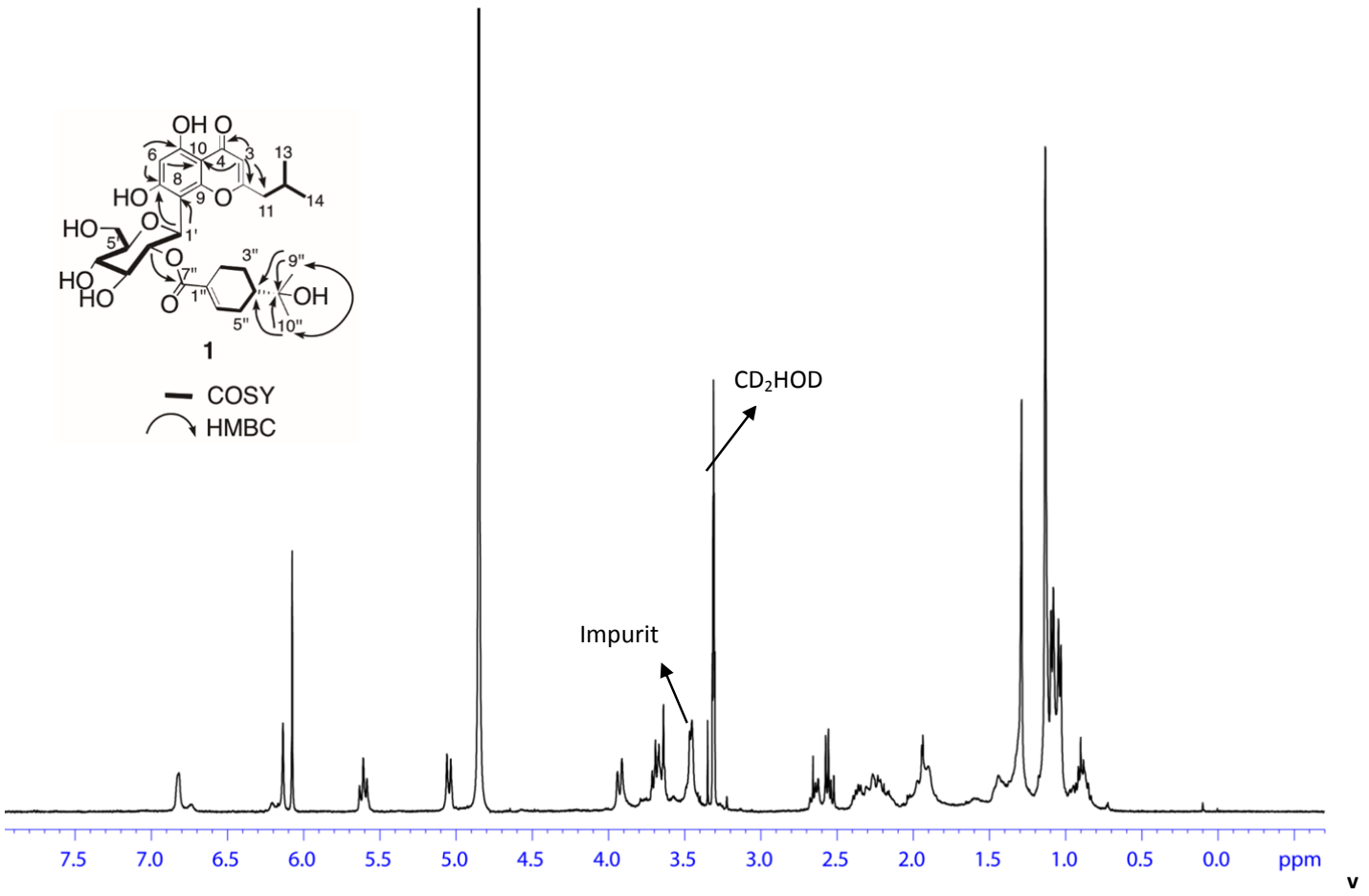


Figure S1. ^{1}H NMR spectrum (400 MHz, MeOD) for compound **1**

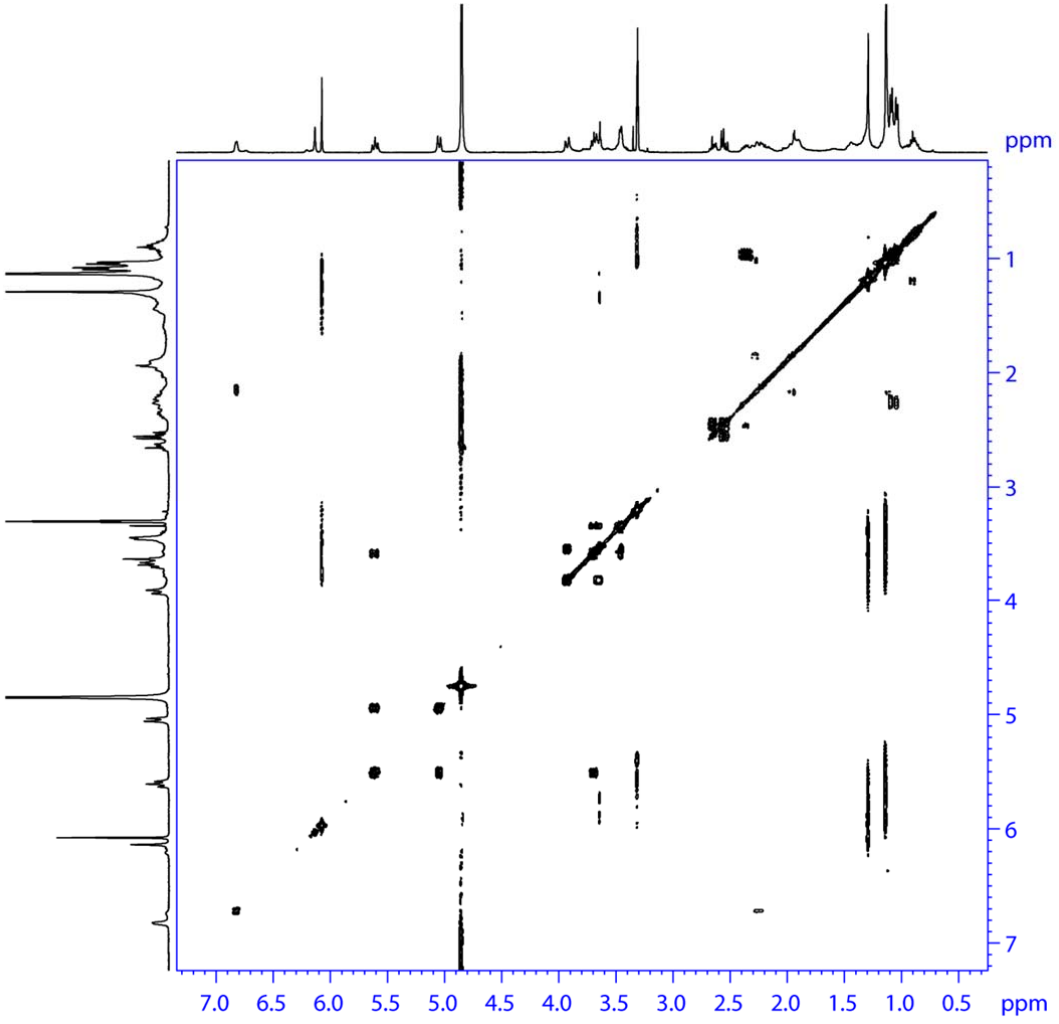


Figure S2. COSY spectrum (400 MHz, MeOD) for compound **1**

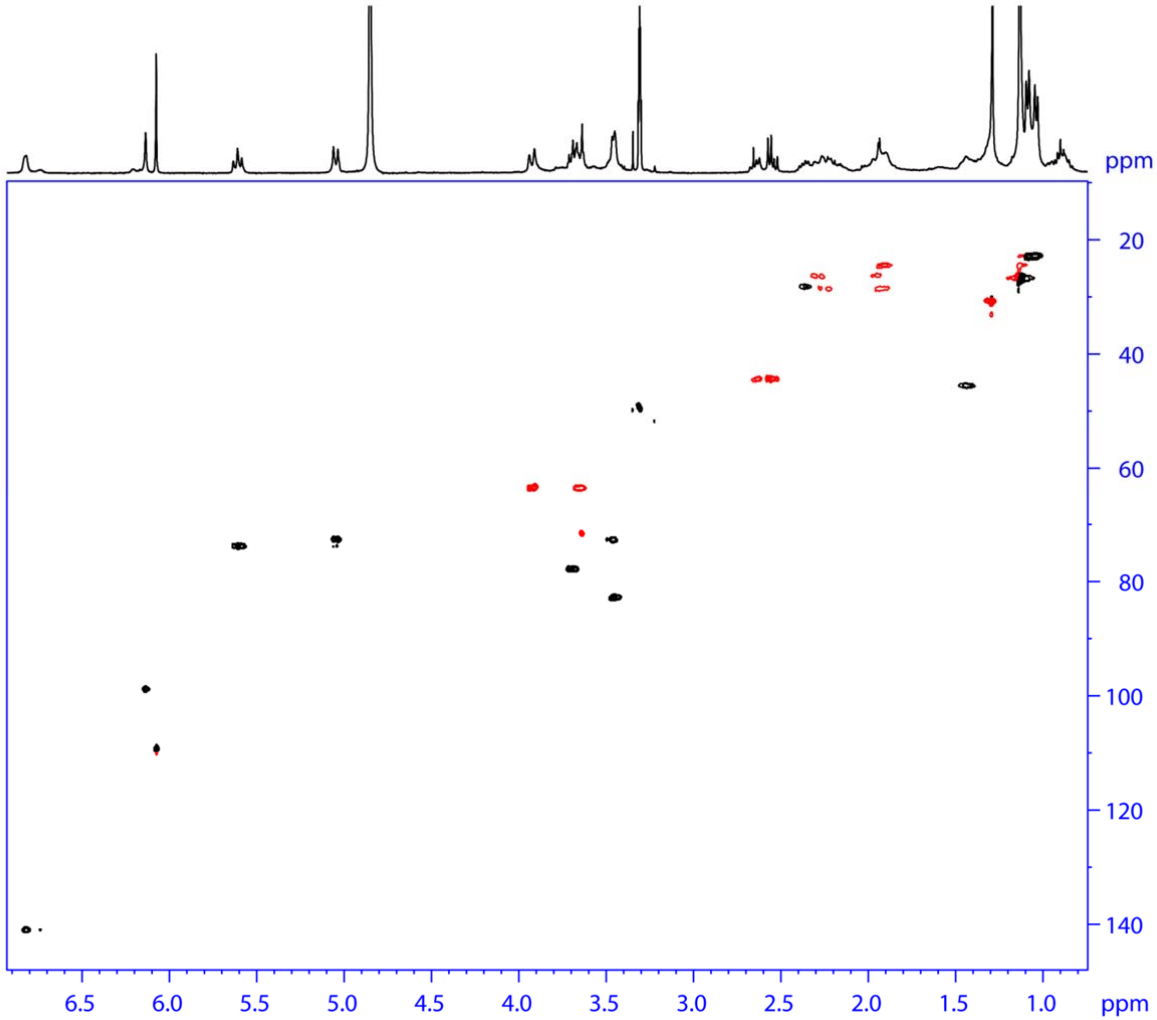


Figure S3. HSQC spectrum (400 MHz, MeOD) for compound **1**

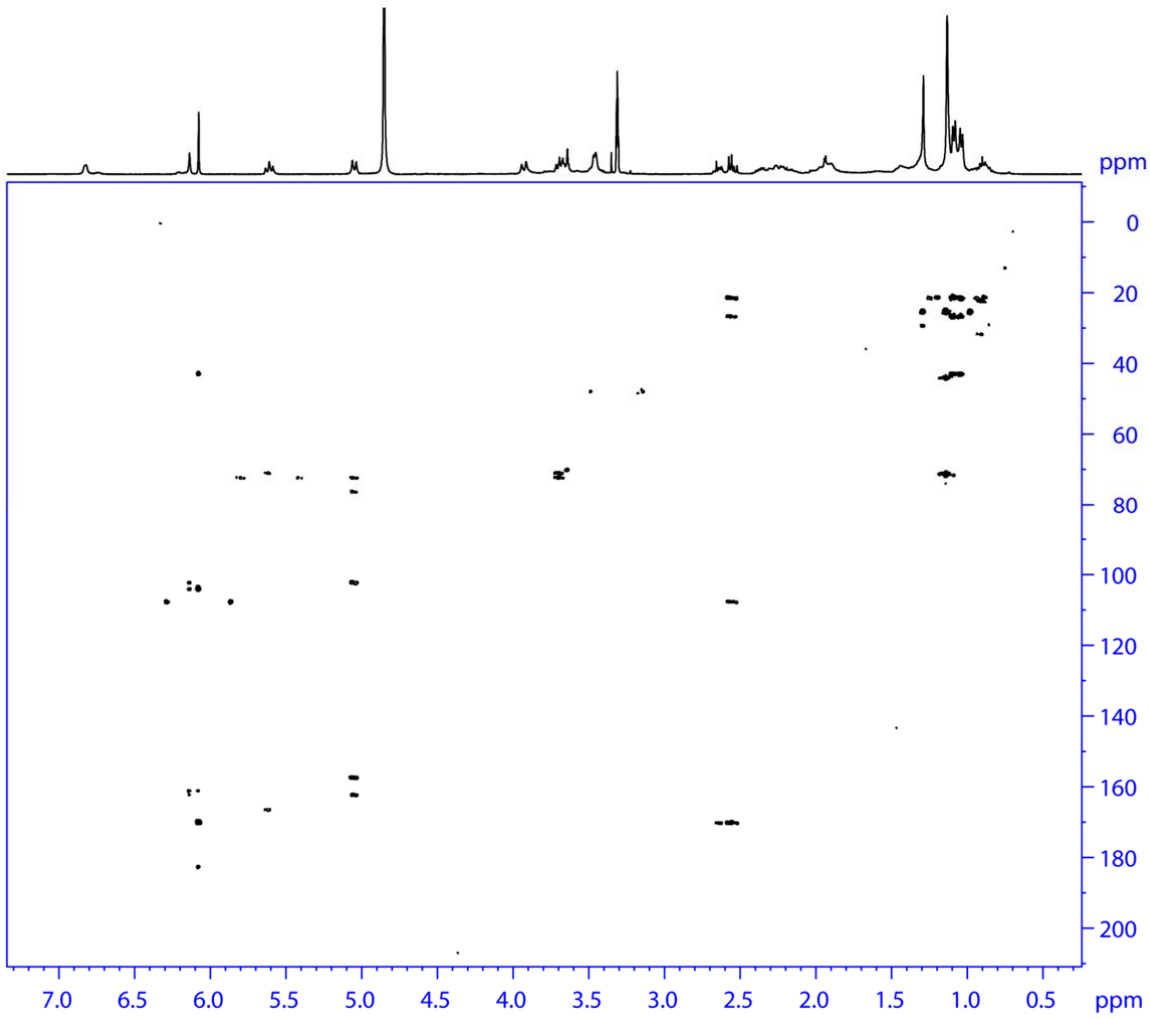


Figure S4. HMBC spectrum (400 MHz, MeOD) for compound **1**

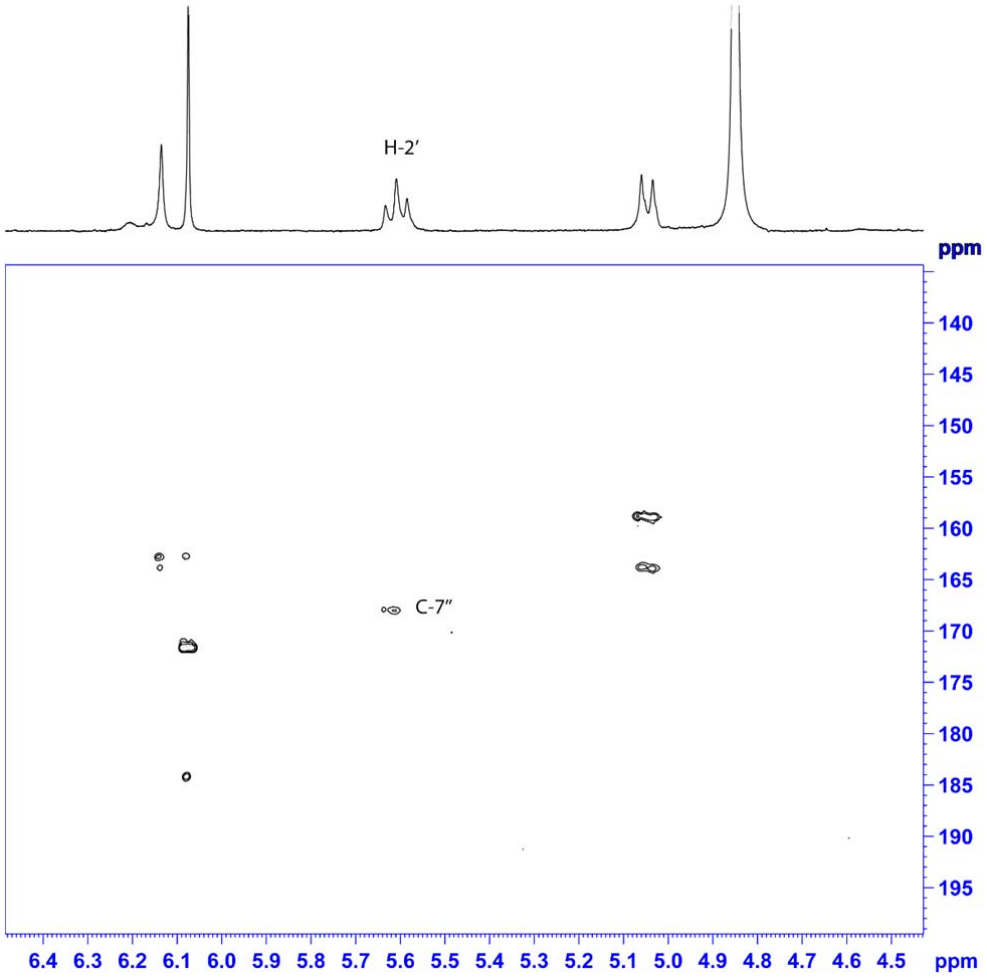


Figure S5. HMBC spectrum (expansion) (400 MHz, MeOD) for compound **1**

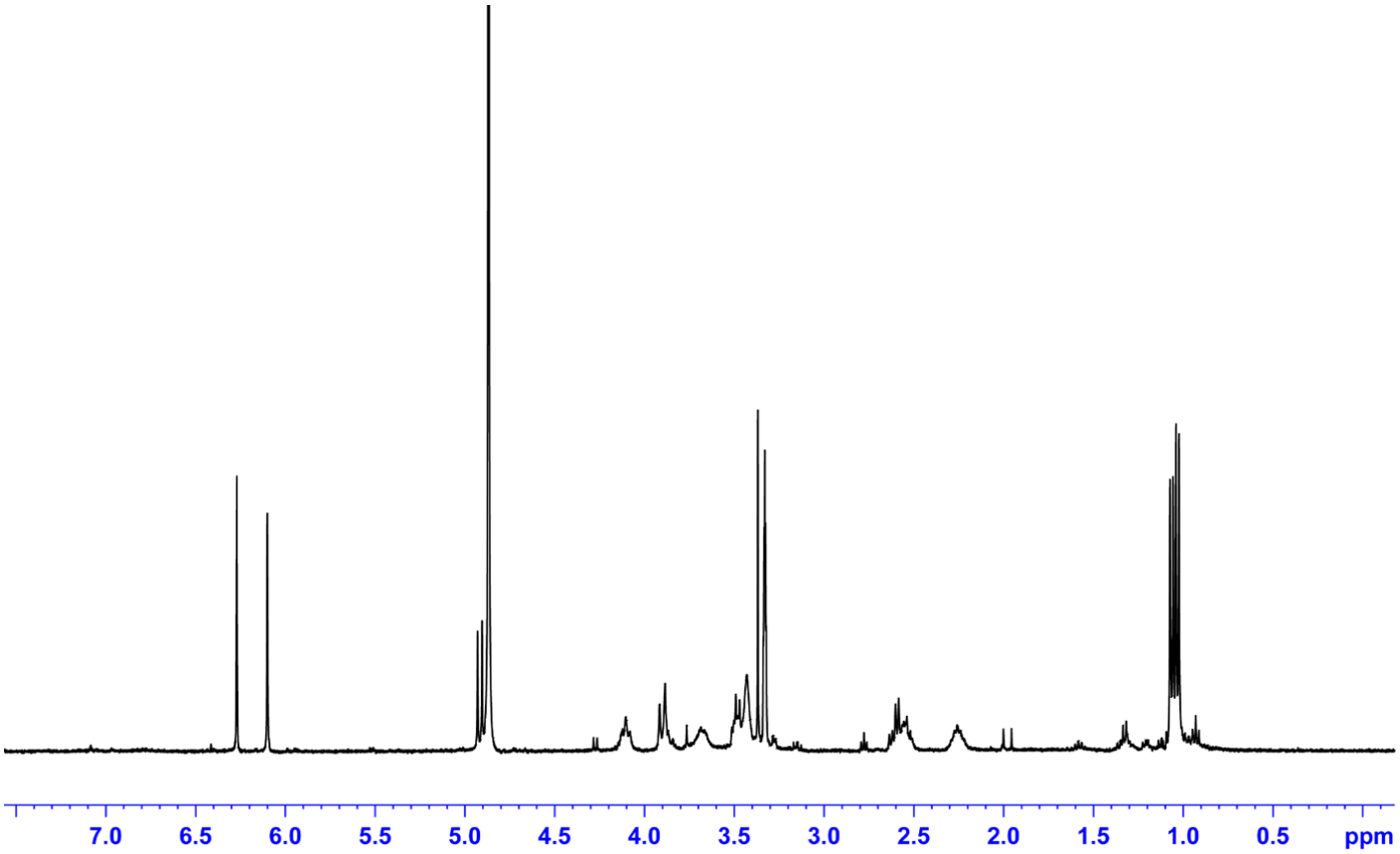


Figure S6. ^{1}H NMR spectrum (400 MHz, MeOD) for compound **2**

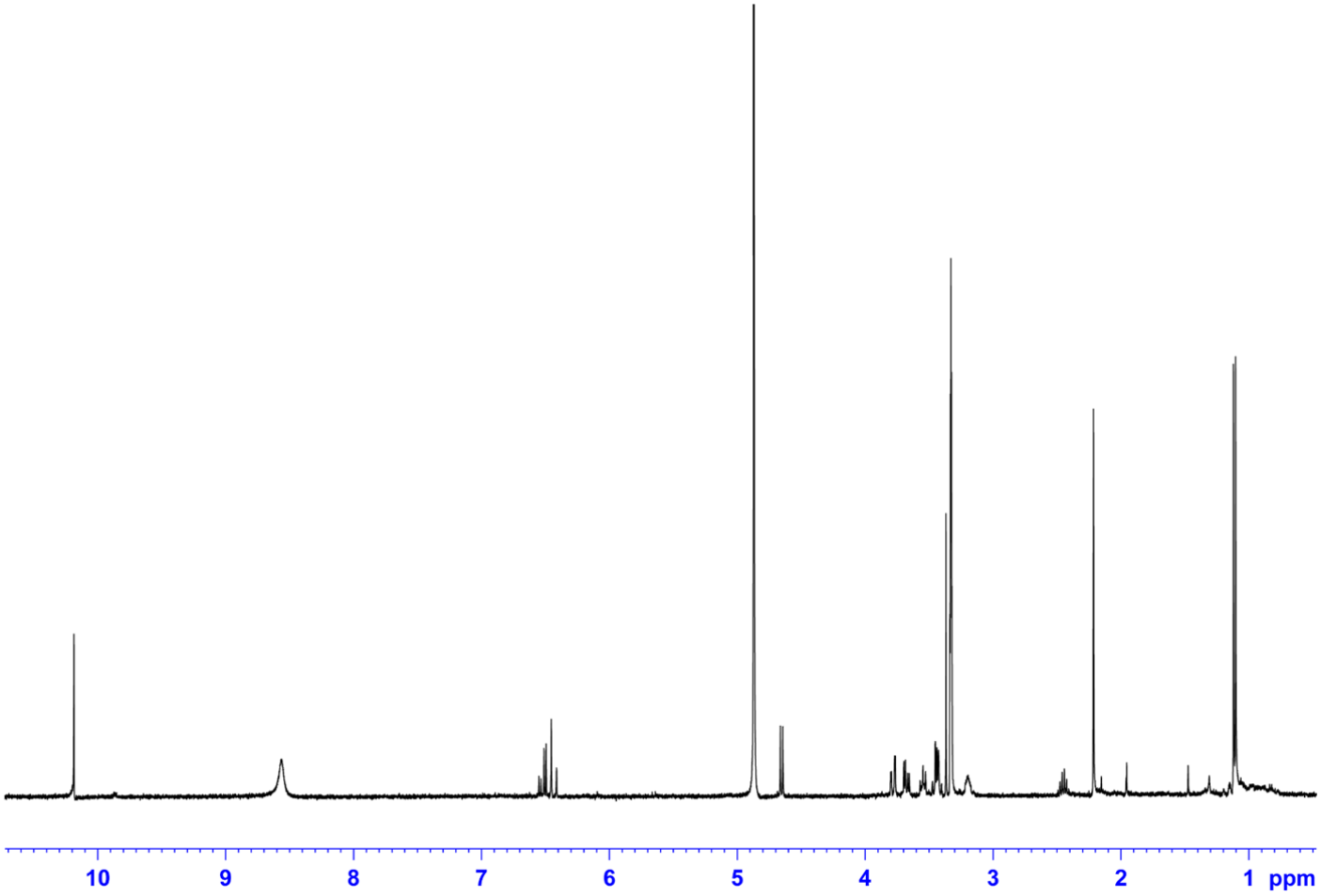


Figure S7. ^{1}H NMR spectrum (400 MHz, MeOD) for compound **3**

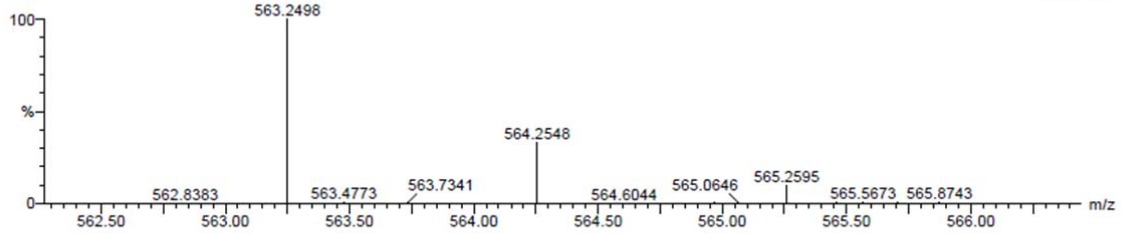
Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
128 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 13-40 H: 0-1000 O: 0-200

EvF-8Pos
170605_EvF-8 92 (0.862)

1: TOF MS ES+
8.30e+003



Minimum:				-1.5			
Maximum:		5.0	10.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
563.2498	563.2492	0.6	1.1	10.5	92.4	0.0	C29 H39 O11

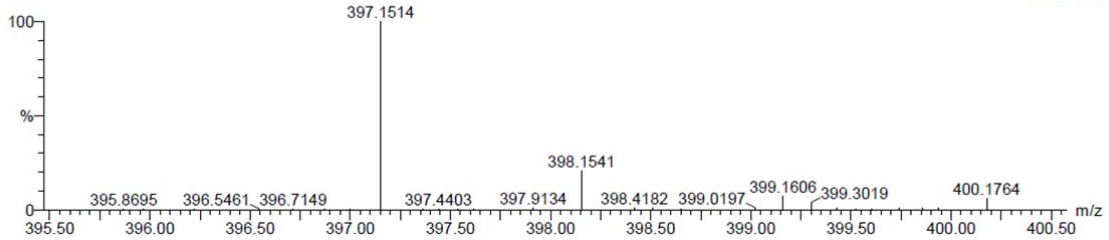
Figure S8. HRMS spectrum for compound **1**

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
39 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 13-20 H: 0-1000 O: 0-200
EvF-5Pos
170605_EvF-5b 30 (0.293)

1: TOF MS ES+
6.82e+002



Minimum:				-1.5			
Maximum:		5.0	10.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
397.1514	397.1499	1.5	3.8	7.5	50.4	0.0	C19 H25 O9

Figure S9. HRMS spectrum for compound **2**

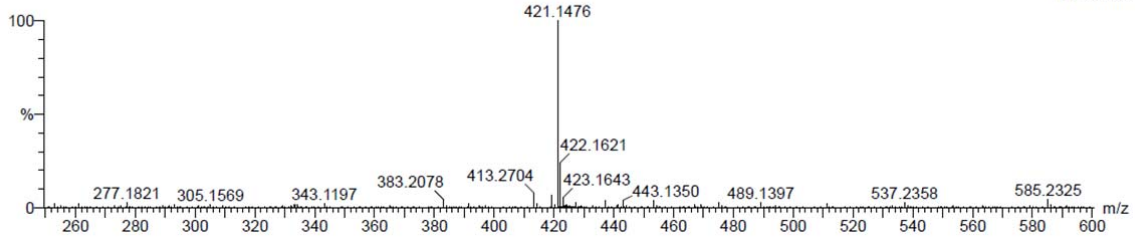
Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
89 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 18-20 H: 0-1000 O: 0-200 Na: 0-2

EvF-10Pos
170605_EvF-10 37 (0.362)

1: TOF MS ES+
1.23e+005

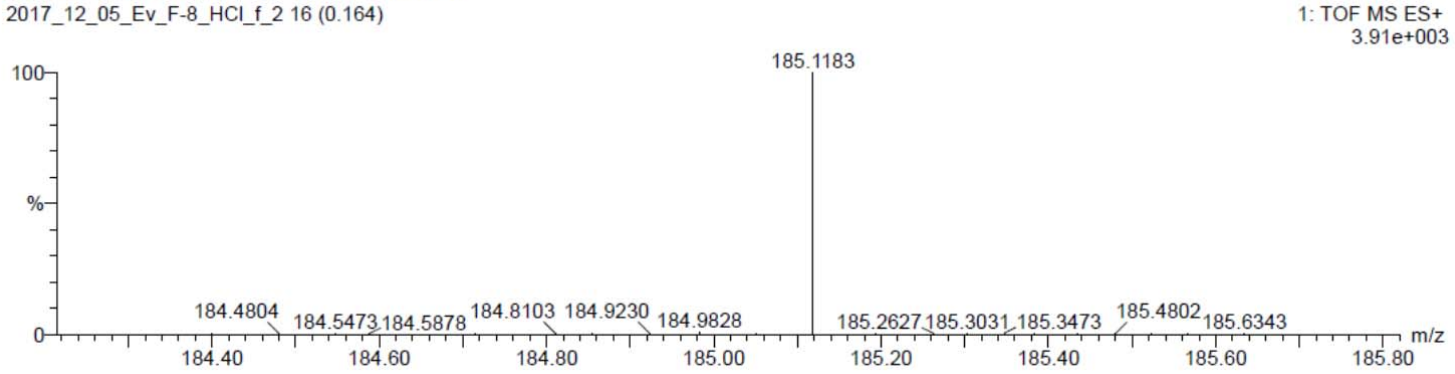


Minimum:				-1.5			
Maximum:		5.0	10.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
421.1476	421.1475	0.1	0.2	6.5	230.2	0.0	C19 H26 O9 Na

Figure S10. HRMS spectrum for compound **3**

Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
61 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-26 H: 0-1000 O: 0-200 Na: 0-2



Minimum:				-1.5			
Maximum:		5.0	10.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
185.1183	185.1178	0.5	2.7	2.5	70.3	0.0	C10 H17 O3

Figure S11. HRMS spectrum for compound **4**

Table S1. Antiinflammatory activity for compounds 1-3 and (4/1a) (oleuropeic acid) in RAW 264.7 Macrophages

NO & TNF-α down regulation and Cell Viability of Isolated Compounds from *E. viminalis* in LPS & IFN-γ induced In **RAW 264.7 Macrophages**

E. viminalis **Compounds**	**Inhibition of NO production IC_{50} in µg/mL**	**Inhibition of TNF- α production IC_{50} in µg/mL**	**Cytotoxicity LC_{50} in µg/mL**	**% Cytotoxicity**
1	44.00 ± 9.0	40.98 ± 23.8	>36	131.4
2	46.97 ± 7.8	38.27 ± 14.8	>36	145.7
3	37.61 ± 4.9	43.17 ± 18.4	>36	144.0
4	18.68 ± 0.5	21.48 ± 4.4	54.48 ± 21.4	144.0

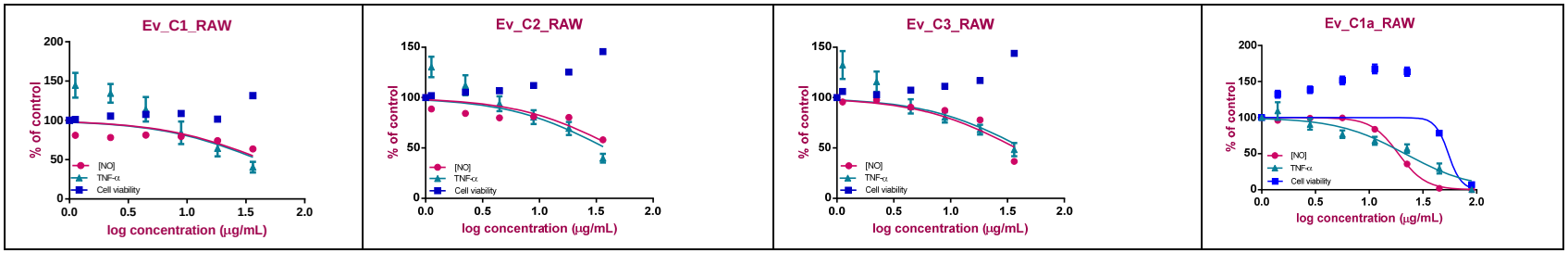
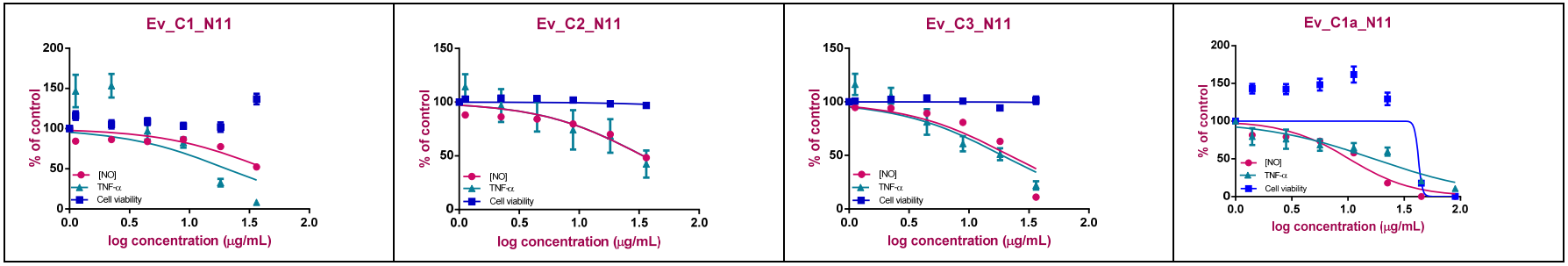


Table S2. Antiinflammatory activity for compounds 1-3 and (4/1a) (oleuropeic acid) in N-11 Microglia

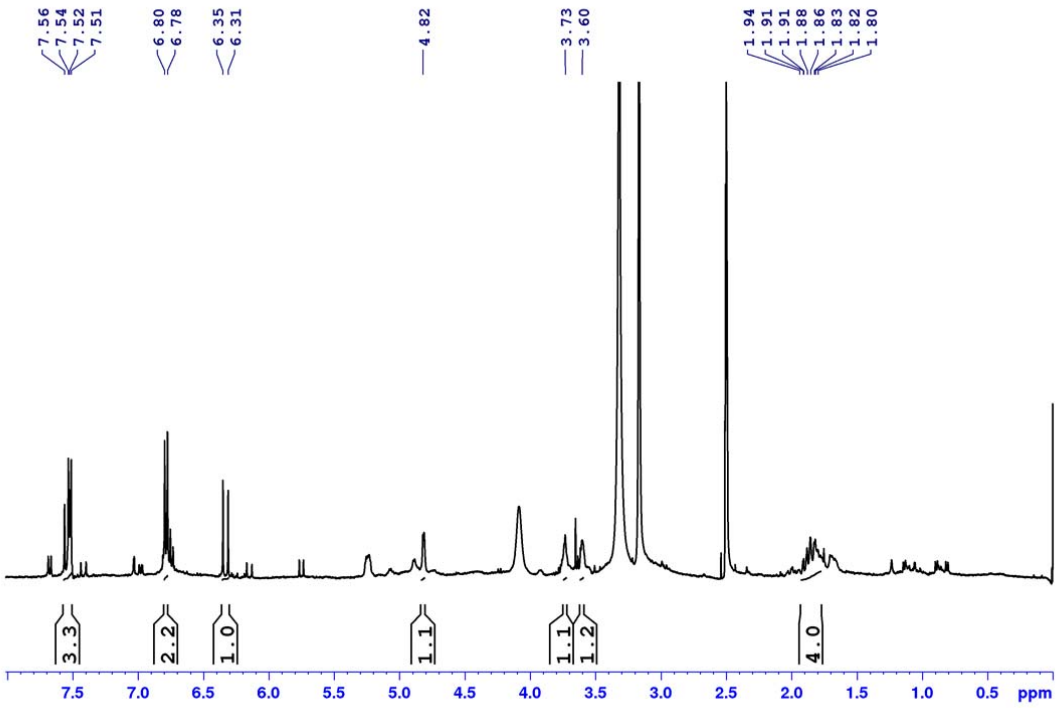
NO & TNF-α down regulation and Cell Viability of Isolated Compounds from *E. viminalis* in LPS & IFN-γ induced **N-11 Microglia**

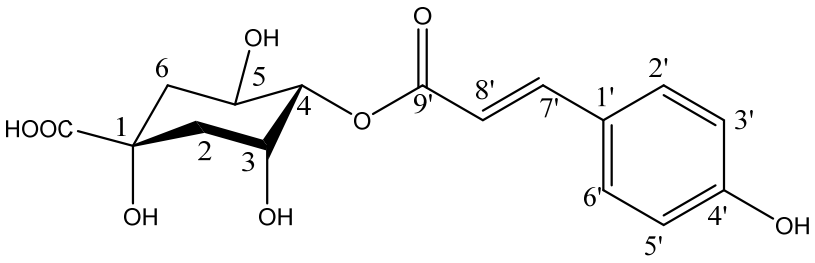
E. viminalis Compounds	Inhibition of NO production IC_{50} in µg/mL	Inhibition of TNF- α production IC_{50} in µg/mL	Cytotoxicity LC_{50} in µg/mL	% Cytotoxicity
1	43.44 ± 7.3	20.37 ± 10.5	>36	136.89
2	34.10 ± 4.2	34.10 ± 18.1	>36	96.9
3	21.85 ± 3.7	18.97 ± 7.1	> 36	101.4
4	10.22 ± 1.0	17.00 ± 5.0	>22.5	129.5



Chapter 3a_Supporting Information

3a.1.a ^{1}H NMR spectrum of compound **3a.1** (DMSO-d_6, 400 MHz)





4-Coumaroylquinic acid

3a.1.b NMR data (DMSO-d_6, 400 MHz) for compound **3a.1**

Position	δ_H, Mult (*J* in Hz)	δc *	COSY	HMBC
1		73.3		
2	1.84 *m*	35.6	3	1
3	3.74 *brs*	67.1	2, 4	
4	4.82 *d(4.0)*	76.5	5, 3	5
5	3.61 *brs*	70.3	4	4
6	1.83 *m*	37.4		1, 5
1’		125.7		
2’	[a]7.52 *d(8.5)*	[a]129.8	3’	1’, 3’, 4’, 6’, 7’
3’	[a]6.79 *d(8.5)*	116.1	2’	1’, 4’, 5’
4’		160.0		
5’	[a]6.79 *d(8.5)*	115.6	6’	1’, 3’, 4’
6’	[a]7.52 *d(8.5)*	[a]129.8	5’	1’, 2’, 4’, 5’, 7’
7’	7.55 *(16.0)*	144.1	8’	1’, 2’, 6’, 8’, 9’
8’	6.35 *d(16.0)*	115.0	7’	1’, 7’, 9’
9’		166.5		

*Assignments supported by HSQC and HMBC experiments. [a] Overlapping signals.

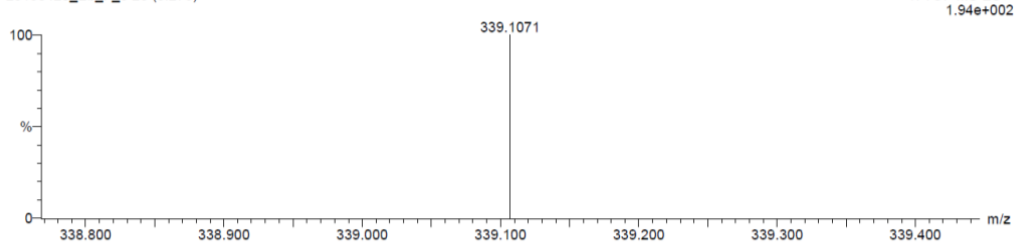
3a.1.c HR-ESI-MS of compound **3a.1**

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = 0.0, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
63 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-40 H: 0-50 O: 0-20
20180420_Eb_F_3 28 (0.276)

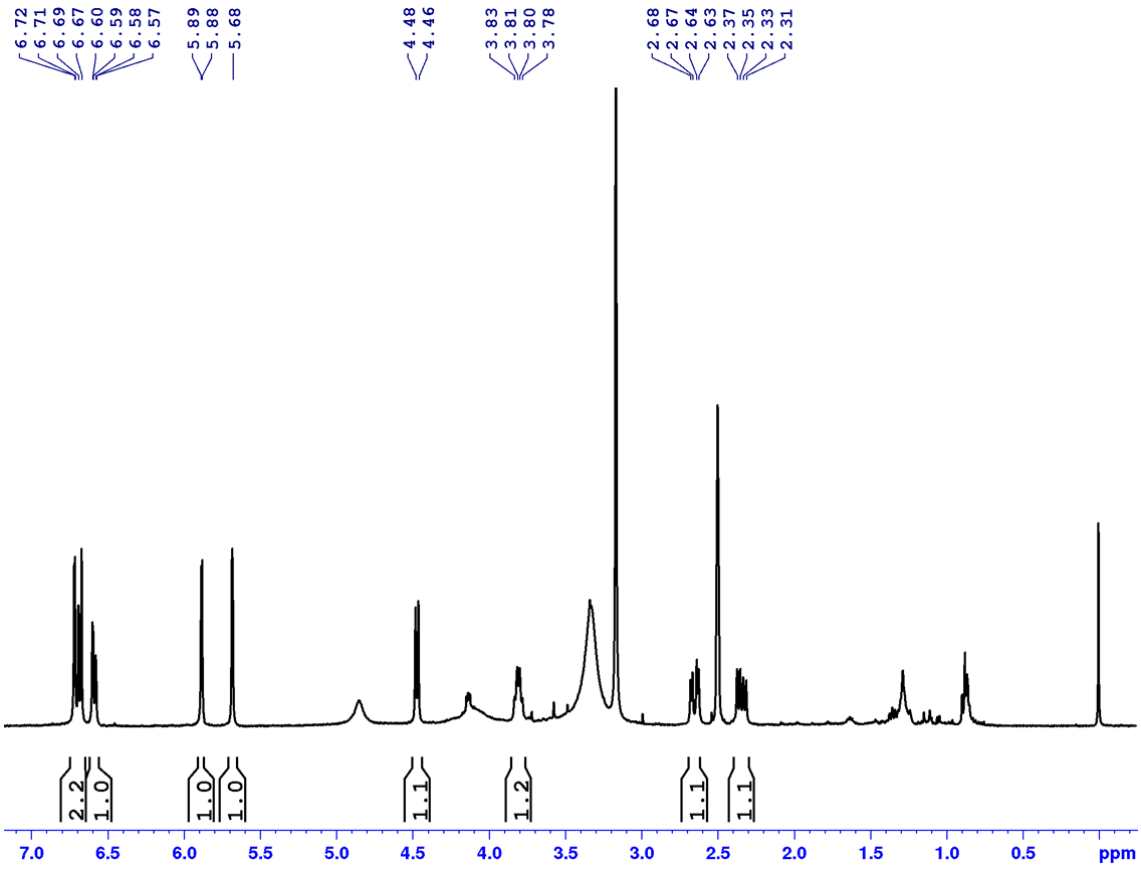
1: TOF MS ES+
1.94e+002

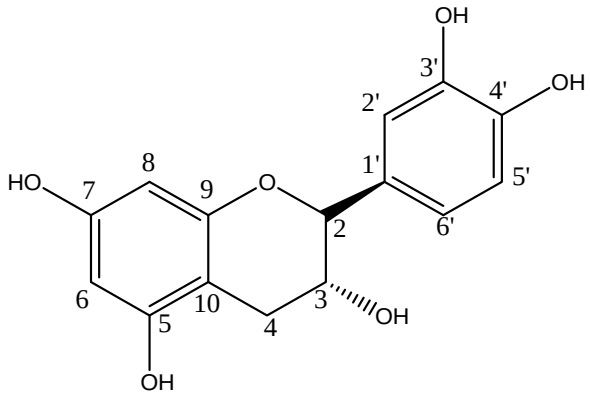


Minimum:		5.0	10.0	0.0			
Maximum:				50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
339.1071	339.1080	-0.9	-2.7	7.5	19.7	0.0	C16 H19 O8

Chapter 5_Supporting Information

5.1.a ^{1}H NMR spectrum of compound **5.1** (DMSO-d_6, 400 MHz)





3,3',4',5,7-Pentahydroxyflavan

5.1.b NMR data (DMSO-d_6, 400 MHz) for compound **5.1**

Position	δ_H, Mult (*J* in Hz)	δc *	COSY	HMBC
2	4.47 *d(7.4)*	81.6	3	3, 9, 1', 2', 5' 6',
3	3.81 *ddd(13.0, 7.3, 5.7)*	66.7	2, 4	2, 4, 10, 1'
4a 4b	2.65 *dd(16.0, 5.4*) 2.34 *dd(16.0, 7.4)*	28.2	3	2, 3, 5, 9, 10
5	-	156.0		
6	5.89 *d(2.0)*	95.7		5, 7, 8, 9, 10
7	-	157.0		
8	5.68 *d(2.0)*	94.4		5, 7, 6, 9, 10
9	-	155.9		
10	-	99.6		
1'	-	131.1		
2'	6.72 *d(1.8)*	115.1		2, 1', 3', 4', 6'
3'	-	145.0		
4'	-	145.0		
5'	6.68 *d(8.0)*	115.6	6'	2', 1', 3', 4', 6'
6'	6.58 *dd(8.0, 1.5)*	119.6	5'	2, 2', 3', 4', 5'

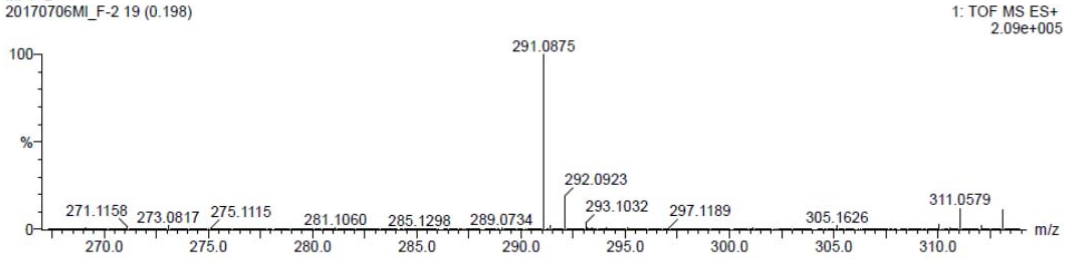
*Assignments supported by HSQC and HMBC experiments.

5.1.c HR-ESI-MS of compound **5.1**

Elemental Composition Report **Page 1**

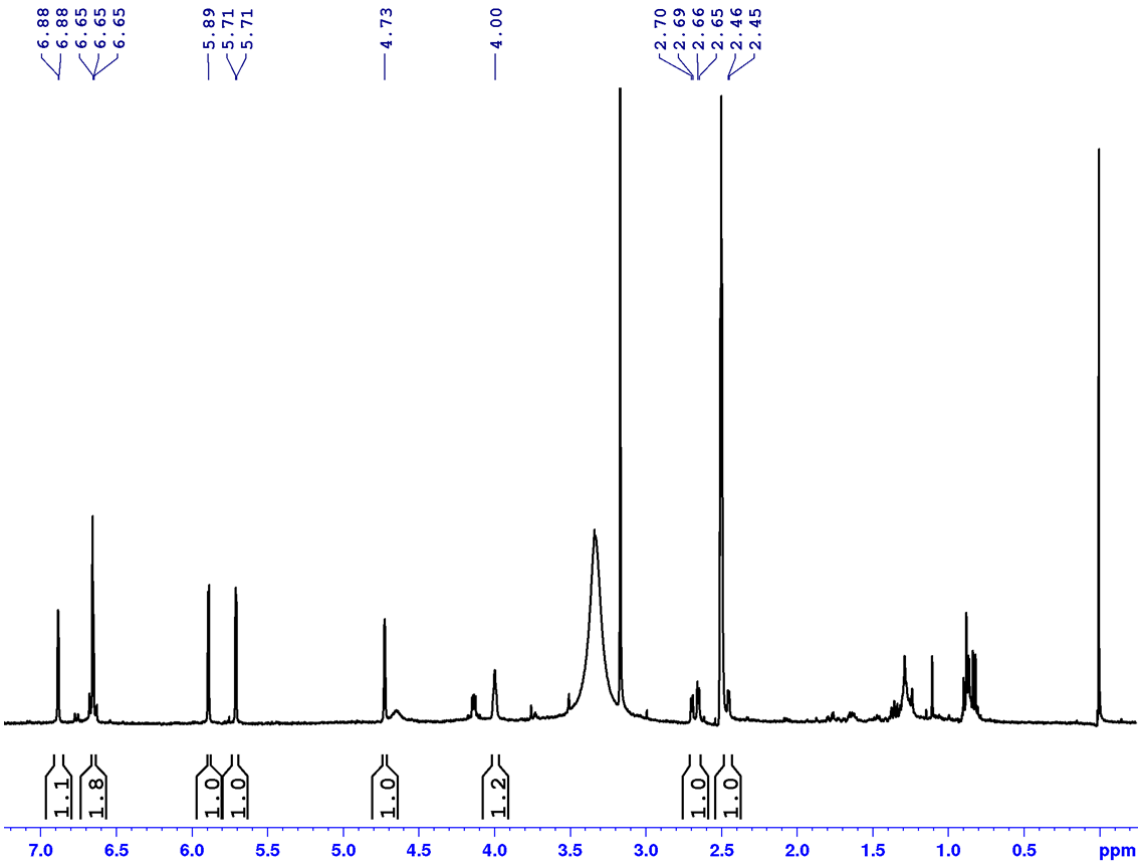
Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

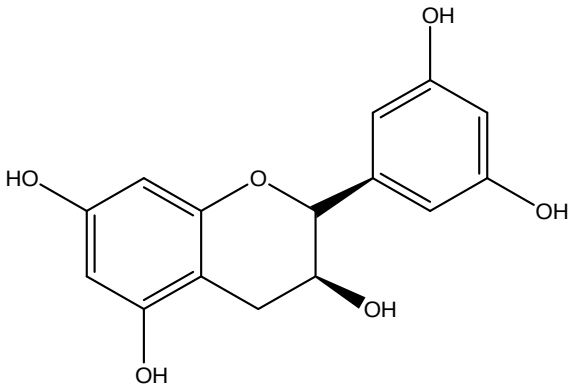
Monoisotopic Mass, Even Electron Ions
111 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 1-30 H: 1-100 O: 1-20 Na: 0-2
MI F-2
20170706MI_F-2 19 (0.198)



Minimum:				-1.5			
Maximum:		50.0	10.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
291.0875	291.0869	0.6	2.1	8.5	517.9	0.0	C15 H15 O6

5.2.a ^{1}H NMR spectrum of compound **5.2** (DMSO-d_6, 400 MHz)





3, 3', 5, 5', 7- Pentahydroxyflavan

5.2.b NMR data (DMSO-d_6, 400 MHz) for compound **5.2**

Position	δ_H, Mult (*J* in Hz)	δc *	COSY	HMBC
2	4.72 *s*	77.8	3	4, 9, 1', 2', 6'
3	4.0 *brs*	64.6	2, 4	2, 4, 9, 10
4a 4b	2.67 *dd(16.0, 5.1);* *2.45 d(3.6)*	28.0	3	2, 3, 10
5	-	156.4		
6	5.88 *d(2.4)*	95.0		5, 7, 8, 10
7	-	157.1		
8	5.71 *d(2.4)*	94.0		6, 7, 10
9	-	156.4		
10	-	99.0		
1'	-	131.5		
2'	[a]6.65 *d(2.7)*	115.1		2, 1', 3',, 6'
3'	-	144.8		
4'	6.88 *d(2.4)*	118.0		1', 3', 5', 6'
5'	-	144.8		
6'	[a]6.65 *d(2.7)*	115.1		2, 1', 2', 4', 5'

*Assignments supported by HSQC and HMBC experiments; [a] overlapping signals.

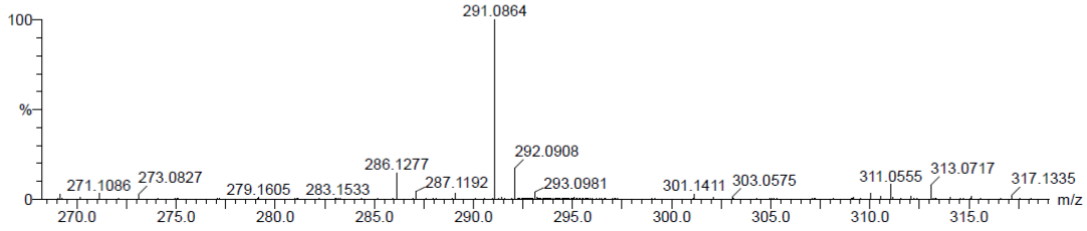
5.2.c HR-ESI-MS of compound **5.2**

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

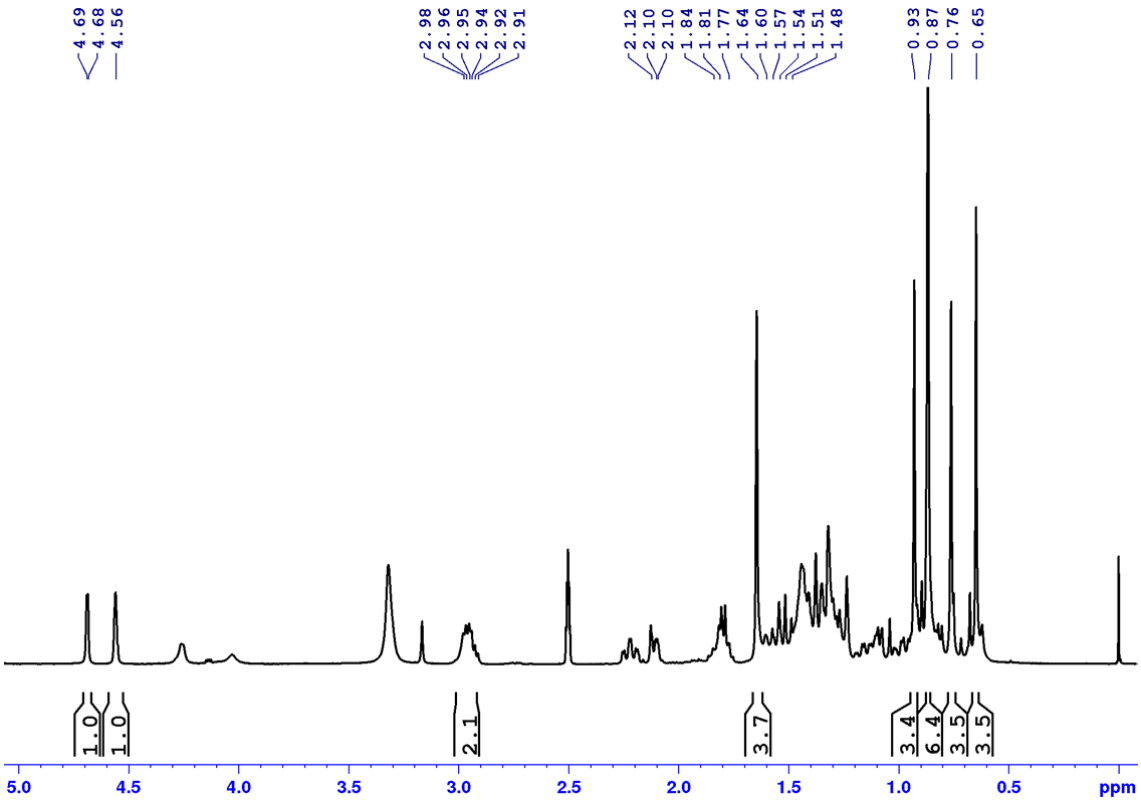
Monoisotopic Mass, Even Electron Ions
111 formula(e) evaluated with 2 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 1-30 H: 1-100 O: 1-20 Na: 0-2
MI_F-4
20170706MI_F-4 91 (0.854)

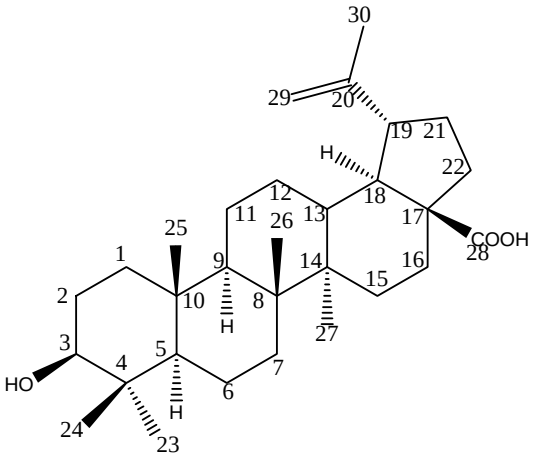
1: TOF MS ES+
2.34e+005



Minimum:				-1.5			
Maximum:		50.0	10.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
291.0864	291.0869	-0.5	-1.7	8.5	548.7	0.1	C15 H15 O6
	291.0845	1.9	6.5	5.5	550.6	2.0	C13 H16 O6 Na

5.3.a ^{1}H NMR spectrum of compound **5.3** (DMSO-d_6, 400 MHz)





Betulinic acid

5.3.b NMR data (DMSO-d_6, 400 MHz) for compound **5.3**

Position	δ_H, Mult (*J* in Hz)	δ_C*	COSY	HMBC
1	1.52 *m*	[a]37.7	2	3, 5, 9, 10
2	[a]1.80 *m*	29.0	1, 3	1, 3, 5, 9
3	[a]2.96 *m*	76.5	2	1, 2, 5, 23, 24
4	----	39.1	----	----
5	0.65 *m*	54.7	6	1, 3, 4, 6, 7, 10, 25
6	1.31 *m*	18.0	5, 7	5, 7
7	1.42 *m*	[a]37.7	6	5, 6, 8, 26
8	----	40.8	----	----
9	1.23 *m*	49.8	11	1, 8, 10, 11, 12, 25
10	----	37.4	----	----
11	1.38 *m*	20.7	9, 12	9, 12
12	1.08 *m*	28.0	11, 13	9, 11, 13, 14
13	1.22 *m*	37.5	12, 18	12, 14, 18, 26, 27
14	----	42.5	----	----
15	1.34 *m*	28.3	16	14, 16, 17, 18, 27, 28
16	2.1 *m*	31.5	15	14, 15, 17, 18, 28
17	----	55.4	----	----
18	1.52 *m*	48.8	13, 19	13, 15, 16, 17, 19, 20, 21, 28
19	[a]2.96 *m*	46.6	18, 21	13, 18, 20, 21, 22, 29, 30
20	----	150.7	----	----
21	[a]1.80 *m*	30.4	19, 22	17, 18, 19, 20, 22, 28, 30
22	1.54 *m*	37.7	21	17, 19, 20, 21, 28
23	[a]0.88 *s*	[a]28.0	----	3, 4, 5
24	[a]0.88 *s*	[a]15.7	----	3, 4, 5
25	0.65 *s*	[a]15.7	----	1, 5, 9, 10, 14
26	0.76 *s*	[a]15.7	----	7, 10, 13, 27
27	0.94 *s*	14.0	----	8, 13, 14, 15, 26
28	----	177.5	----	----
29	4.68, 4.56 *s*	110.2		19, 20, 30
30	1.64 *s*	18.6		19, 20, 29

*Assignments supported by HSQC and HMBC experiments; [a] overlapping signals.

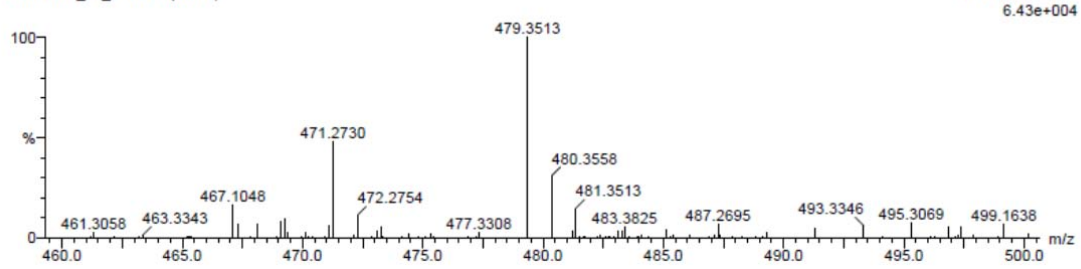
5.3.c HR-ESI-MS of compound **5.3**

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 50.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 6

Monoisotopic Mass, Even Electron Ions
25 formula(e) evaluated with 4 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 1-30 H: 5-50 O: 0-5 Na: 0-2

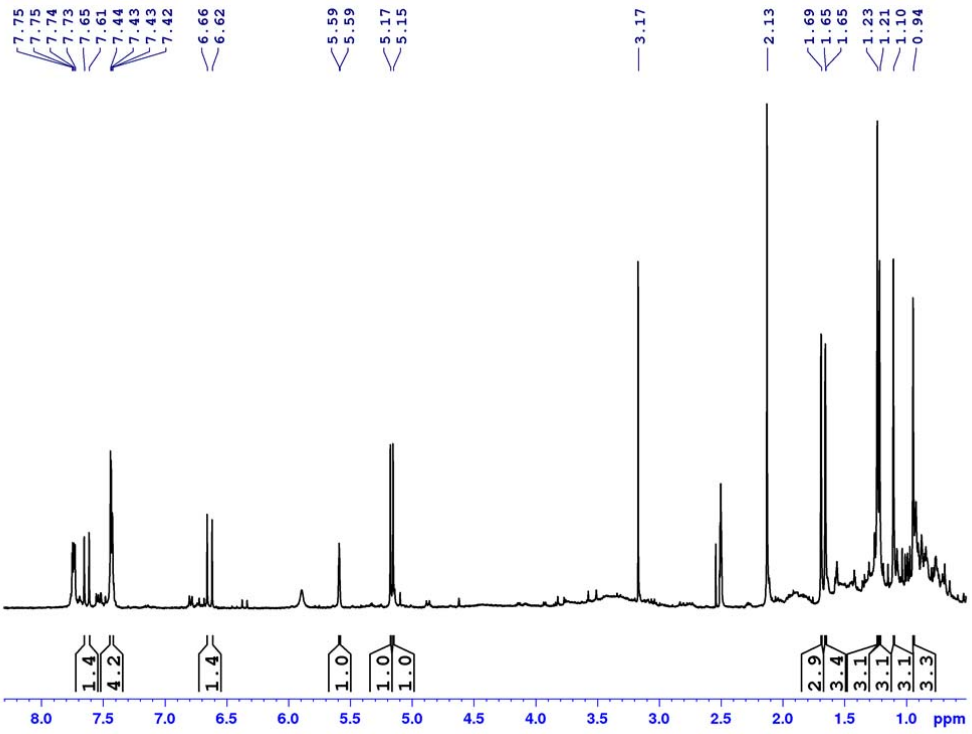
20171023_MI_F-19 79 (0.742) 1: TOF MS ES+ 6.43e+004

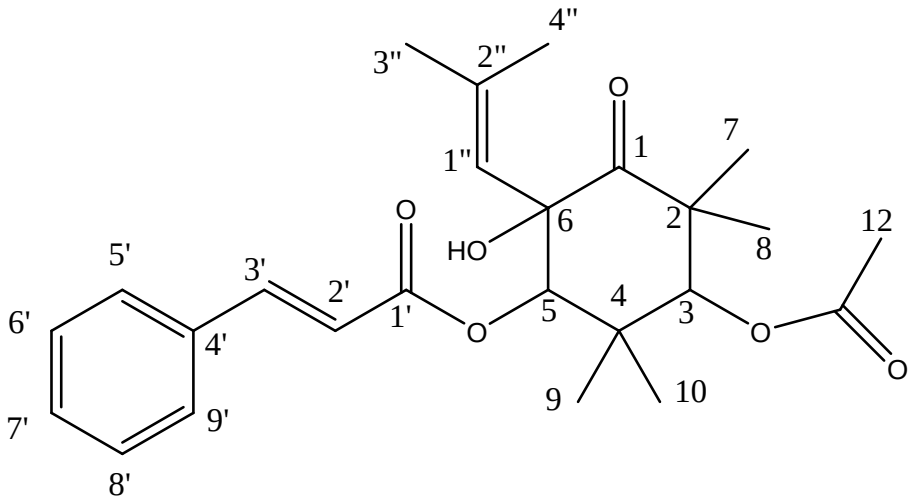


Minimum:				-1.5						
Maximum:		50.0	10.0	50.0						
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula			
479.3513	479.3501	1.2	2.5	6.5	748.8	1.3	C30	H48	O3	Na
	479.3137	37.6	78.4	7.5	748.8	1.3	C29	H44	O4	Na
	479.3477	3.6	7.5	3.5	749.0	1.5	C28	H49	O3	Na2
	479.3113	40.0	83.4	4.5	749.0	1.5	C27	H45	O4	Na2

Chapter 6_Supporting Information

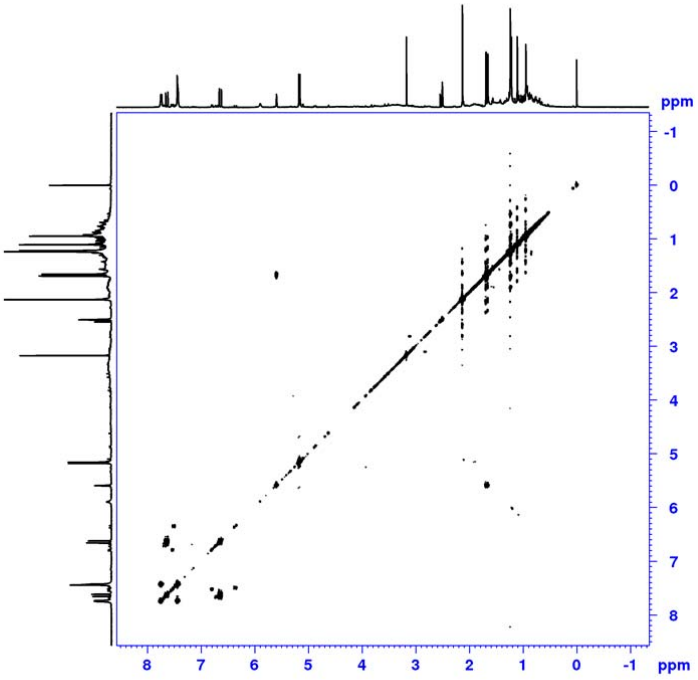
6.1.a ^{1}H NMR spectrum of compound **6.1** (DMSO-d_6, 400 MHz)



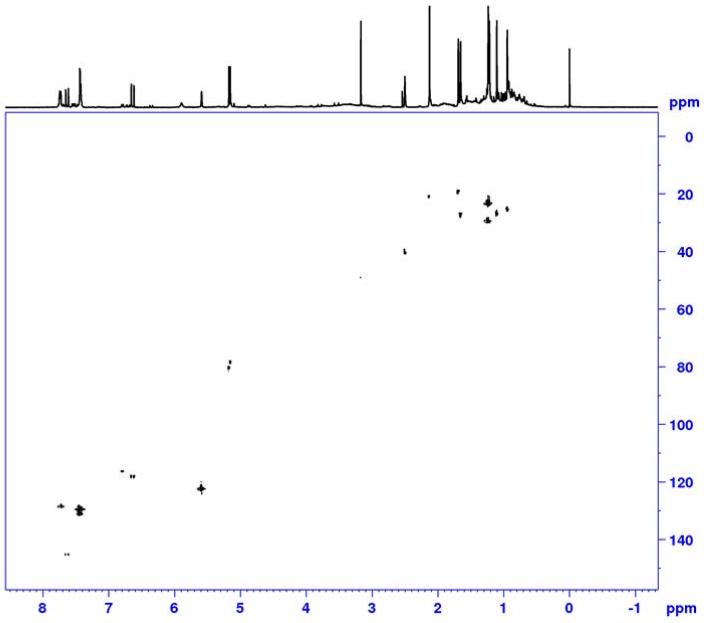


Compound **6.1**

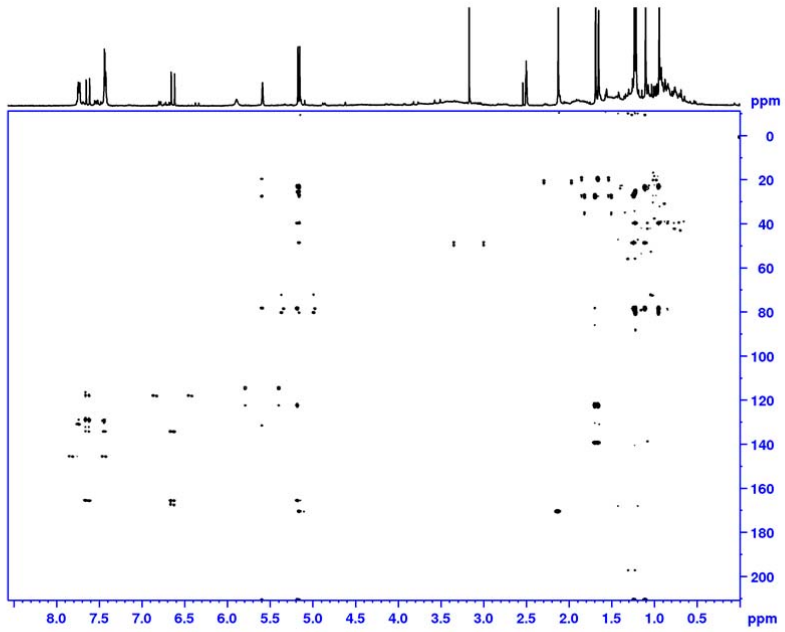
6.1.b COSY spectrum (DMSO-d_6, 400 MHz) for compound **6.1**



6.1.c HSQC spectrum (DMSO-d_6, 400 MHz) for compound **6.1**



6.1.d HMBC spectrum (DMSO-d_6, 400 MHz) for compound **6.1**



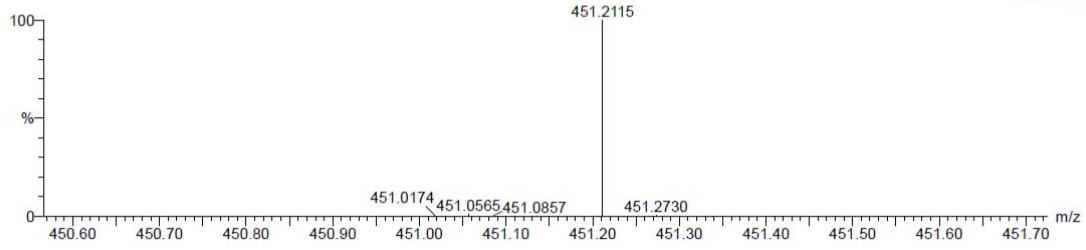
6.1.e HR-ESI-MS of compound **6.1**

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

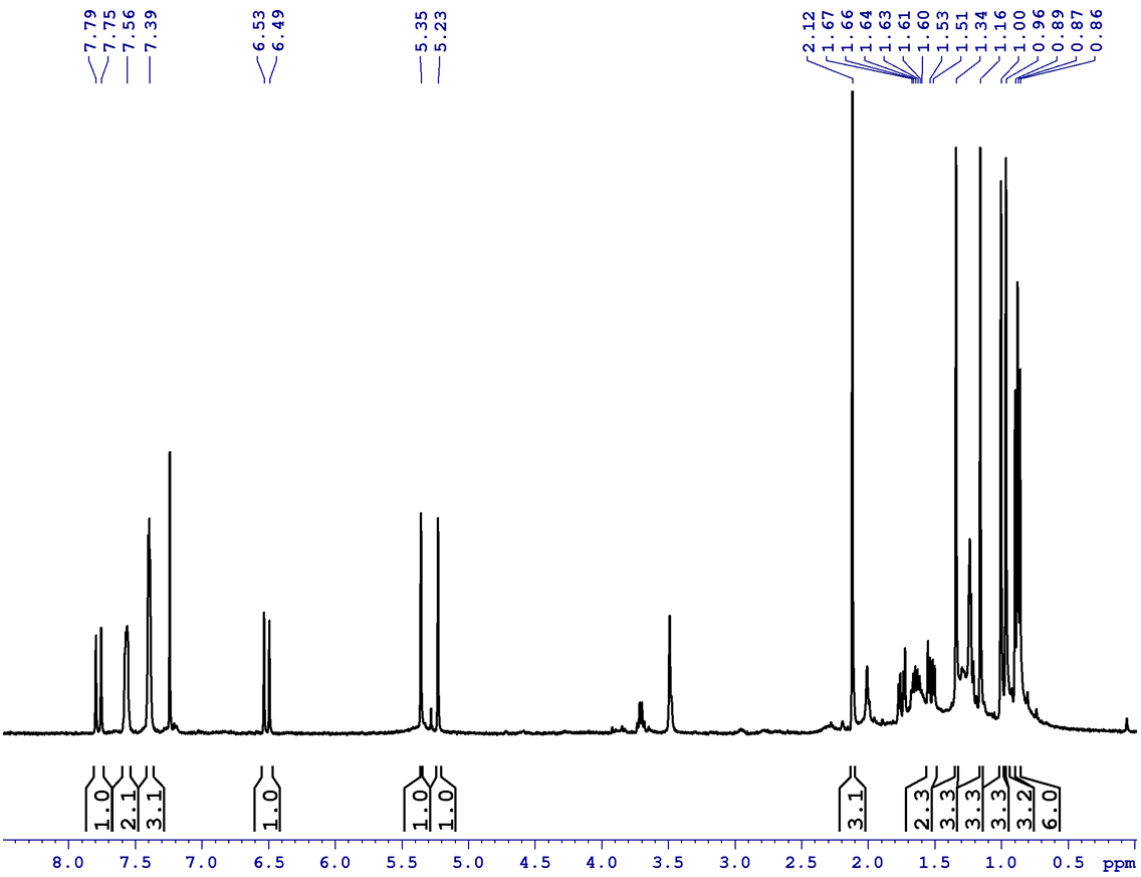
Monoisotopic Mass, Even Electron Ions
208 formula(e) evaluated with 3 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-26 H: 0-1000 O: 0-200 Na: 0-2
2017_12_05_Sgg_F-22a 51 (0.483)

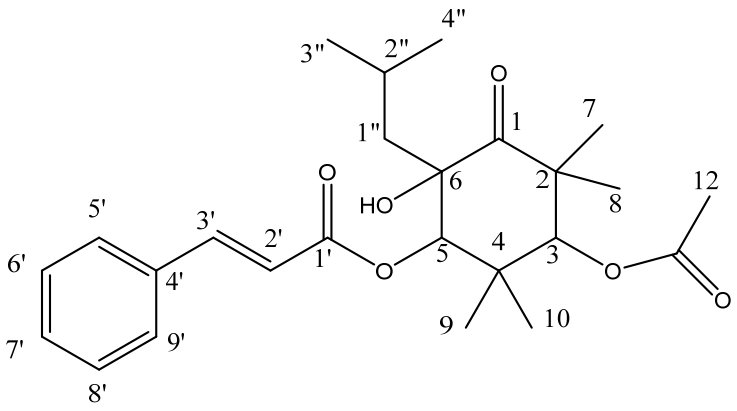
1: TOF MS ES+
2.20e+003



Minimum:				-1.5			
Maximum:		5.0	10.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
451.2115	451.2097	1.8	4.0	9.5	31.5	0.9	C25 H32 O6 Na
	451.2155	-4.0	-8.9	0.5	31.6	1.0	C18 H36 O11 Na
	451.2073	4.2	9.3	6.5	32.1	1.5	C23 H33 O6 Na2

6.2.a ^{1}H NMR spectrum of compound **6.2** ($CDCl_3$, 400 MHz)





Tetragocarbone B

6.2.b NMR data($CDCl_3$, 400 MHz) for compound **6.2**

Pos	δ_H, Mult (*J* in Hz)	δc *	COSY	HMBC
1	-	216.0		
2	-	48.0		
3	5.36 *s*	79.0		1, 2, 4, 5, 7, 8, 9, 10, 11
4	-	39.5		
5	5.22 *s*	79.7		1, 3, 4, 6, 9, 10, 1', 1"
6	-	79.5		
7	1.15 *s*	21.8		1, 2, 3, 8
8	1.33 *s*	27.3		1, 2, 3, 7
9	0.96 *s*	21.0		3, 4, 5, 10
10	1.00 *s*	24.0		3, 4, 5, 9
11	-	170.8		
12	2.11 *s*	20.6		3, 11
1'	-	165.9		
2'	6.52 *d(16.0)*	116.9	3'	1', 3', 4'
3'	7.78 *d(16.0)*	146.3	2'	1', 2', 4', 5', 9'
4'	-	134.2		
5'	[a]7.56 *m*	128.5	6'	3', 4', 6', 7', 9'
6'	[a]7.39 *m*	128.8	5', 7'	5', 7', 8',
7'	[a]7.56 *m*	130.6	6', 8'	5', 6', 8', 9'
8'	[a]7.39 *m*	128.8	7', 9'	4', 5', 6', 7', 9',
9'	[a]7.56 *m*	128.5	8'	3', 4', 5', 7', 8'
1"	1.64 *m*	23.6	2"	5, 6, 2", 3", 4"
2"	1.53, 1.75 *dd(6.0, 14.0)*	44.5	1", 3", 4"	6, 1", 3", 4"
3"	[a]0.88 *dd(6.8, 8.0)*	24.8	2"	1", 2", 4"
4"	[a]0.88 *dd(6.8, 8.0)*	24.8	2"	1", 2", 3"

*Assignments supported by HSQC and HMBC experiments; [a] overlapping signals.

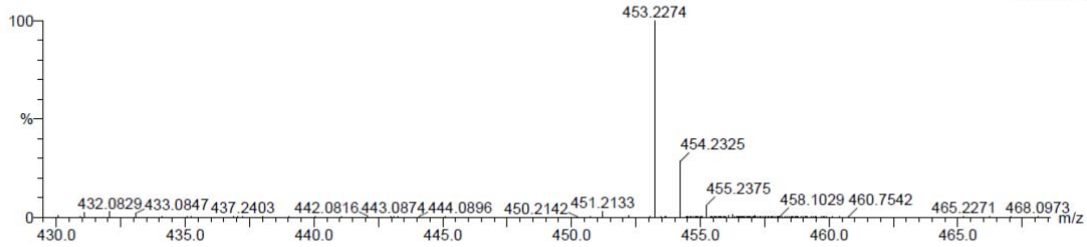
6.2.c HR-ESI-MS of compound **6.2**

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = 0.0, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

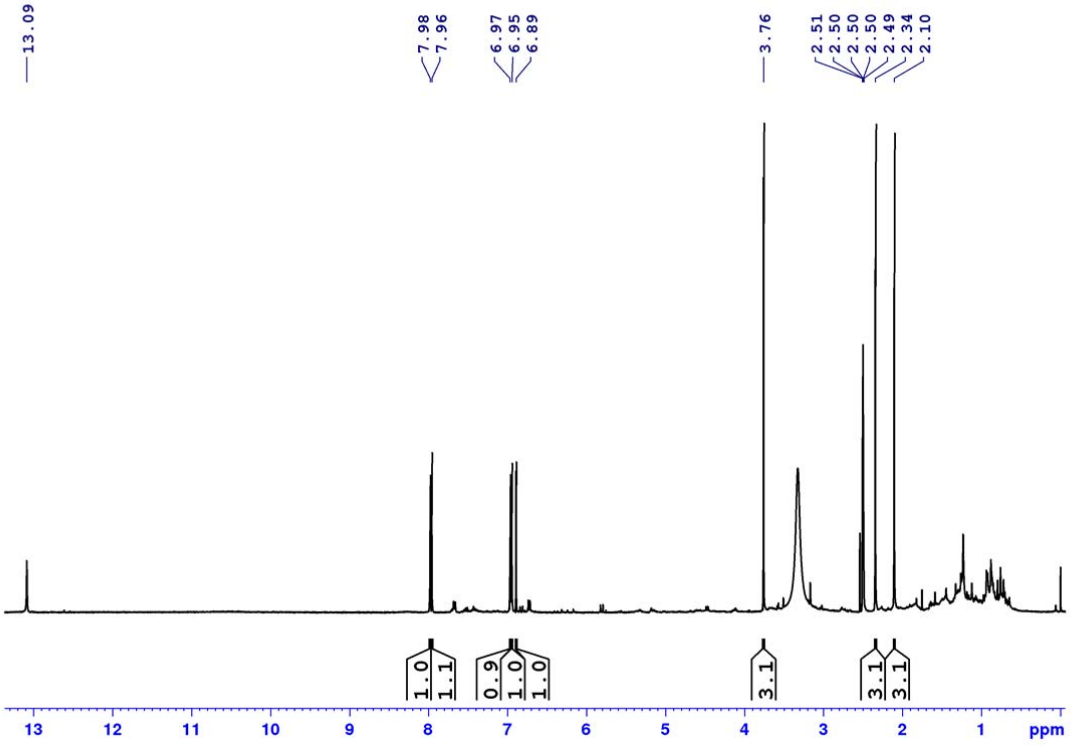
Monoisotopic Mass, Even Electron Ions
107 formula(e) evaluated with 3 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 20-40 H: 10-50 O: 0-10 Na: 0-2
2018_Sgg_K_10 84 (0.785)

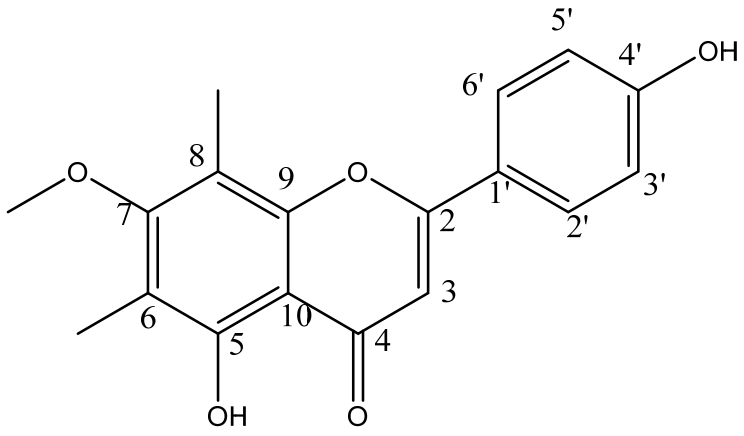
1: TOF MS ES+
1.58e+005



Minimum:				0.0						
Maximum:		5.0	10.0	50.0						
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula			
453.2274	453.2277	-0.3	-0.7	11.5	423.7	1.2	C27	H33	O6	
	453.2253	2.1	4.6	8.5	422.9	0.4	C25	H34	O6	Na
	453.2229	4.5	9.9	5.5	426.3	3.8	C23	H35	O6	Na2

6.3.a ^{1}H NMR spectrum of compound **6.3** (DMSO-d_6, 400 MHz)





Sideroxylin

6.3.b NMR data(DMSO-d_6, 400 MHz) for compound **6.3**

Position	δ_H, Mult (*J* in Hz)	δc *	COSY	HMBC
2		164.4		
3	6.89 *s*	102.6		2, 4, 10, 1’
4		183.1		
5		156.5		
6		113.7		
7		162.5		
8		109.6		
9		152.8		
10		107.3		
1’		121.6		
2’	7.97 *d(8.9)*	128.0	3’	2, 4’, 6’
3’	6.96 *d(8.9)*	116.0	2’	2, 1’, 4’
4’		161.9		
5’	6.96 *d(8.9)*	116.0	6’	2, 1’, 4’
6’	7.97 d(8.9)	128.0	5’	2, 2’, 4’
CH_3-6	2.11 *s*	8.0		5, 6, 7
CH_3-8	2.35 *s*	8.0		7, 8, 9
OCH_3-7	3.76 *s*	60.0		7
OH-5	13.08 *s*			

*Assignments supported by HSQC and HMBC experiments.

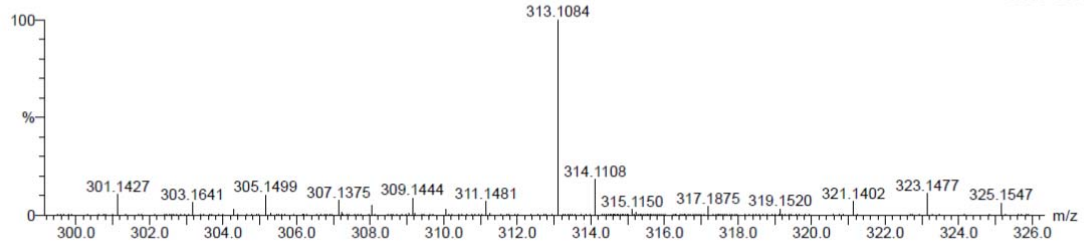
6.3.c HR-ESI-MS of compound **6.3**

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 50.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 6

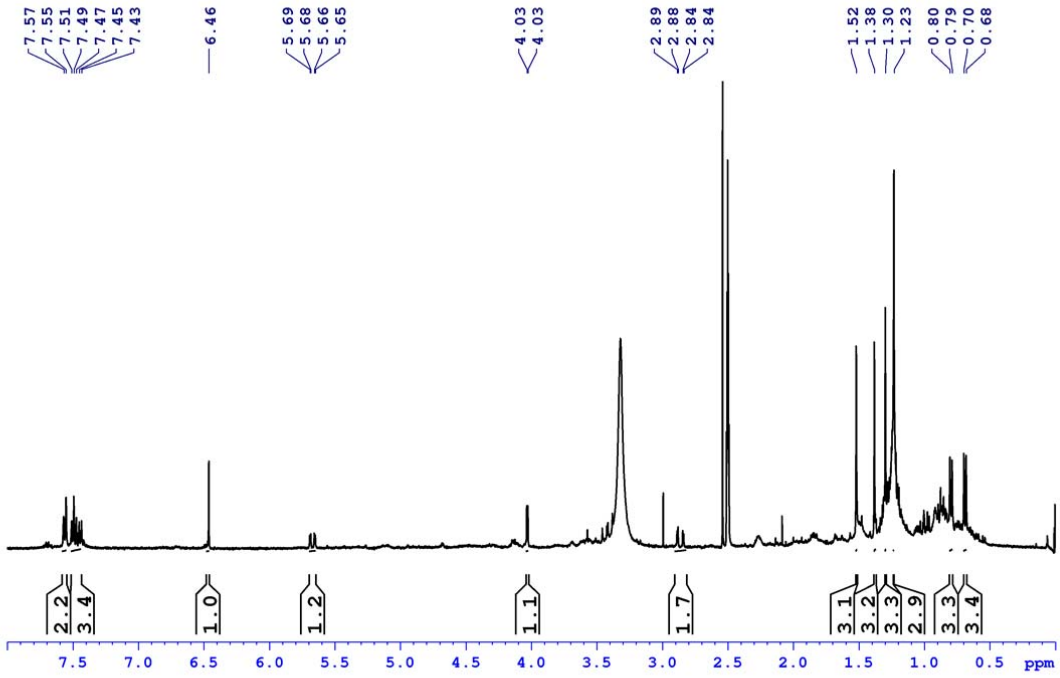
Monoisotopic Mass, Even Electron Ions
11 formula(e) evaluated with 3 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 1-20 H: 5-20 O: 0-10
20171023_Sgg_F-24a 44 (0.422)

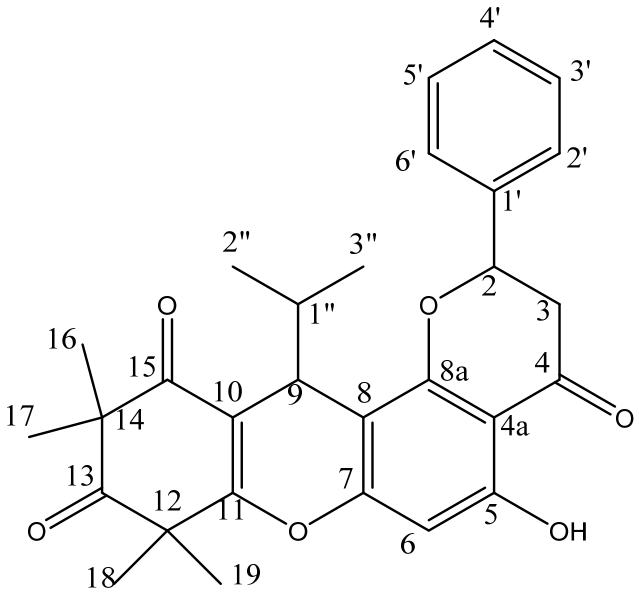
1: TOF MS ES+
3.50e+004



Minimum:				-1.5					
Maximum:		50.0	10.0	50.0					
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula		
313.1084	313.0923	16.1	51.4	6.5	829.0	0.3	C14	H17	O8
	313.0712	37.2	118.8	11.5	830.1	1.4	C17	H13	O6
	313.1076	0.8	2.6	10.5	837.2	8.5	C18	H17	O5

6.4.a ^{1}H NMR spectrum of compound **6.4** (DMSO-d_6, 400 MHz)





Compound **6.4**

6.4.b HR-ESI-MS of compound **6.4**

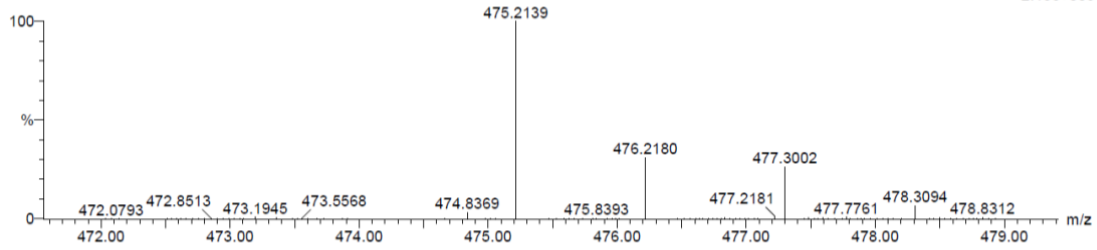
Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = 0.0, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
47 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-40 H: 0-50 O: 0-10

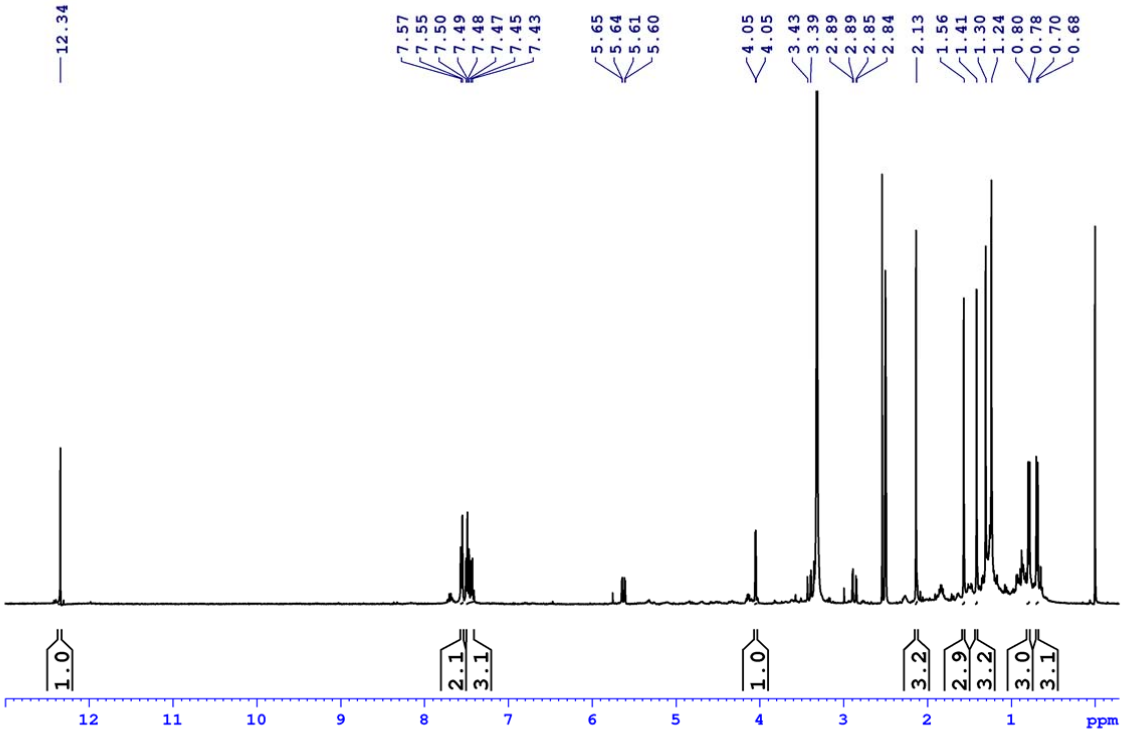
20180523_Sgg_26c_pos 20 (0.206)

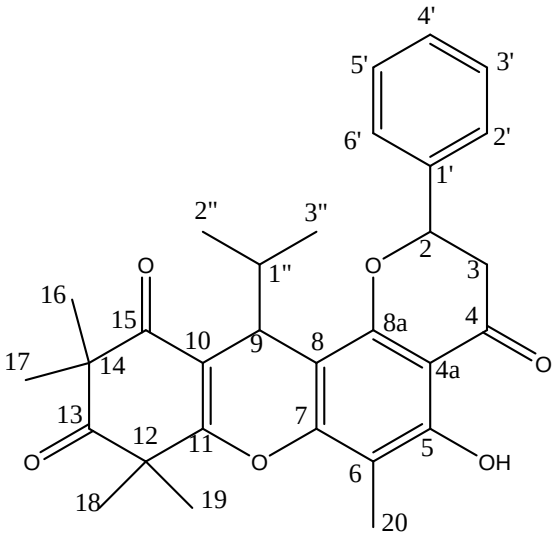
1: TOF MS ES+
2.18e+005



Minimum:				0.0			
Maximum:		5.0	10.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
475.2139	475.2121	1.8	3.8	14.5	334.4	0.0	C29 H31 O6

6.5.a ^{1}H NMR spectrum of compound **6.5** (DMSO-d_6, 400 MHz)





Lumaflavanone A

6.5.b NMR data(DMSO-d_6, 400 MHz) for compound **6.5**

Position	δ_H, Mult (*J* in Hz)	δ_C	COSY	HMBC
2	5.62 *dd(17.1, 2.7)*	78.6	3	3, 4, 1', 2', 6'
3	3.39 *d(3.7)*, 2.86 *dd(13.6, 2.7)*	42.6	2	2, 4, 1',
4	--	197.6		
4a	--	103.0		
5	--	158.8		
6	--	104.4		
7	--	155.8		
8	--	104.4		
8a	--	158.8		
9	4.05 *d(3.5)*	31.5	1"	7, 8, 10, 11, 15, 1", 2", 3"
10	--	110.9		
11	--	167.2		
12	--	46.6		
13	--	210.0		
14	--	55.4		
15	--	196.4		
16	1.31 *s*	23.3		13, 14, 15, 17
17	1.23 *s*	24.5		13, 14, 15, 16
18	1.56 *s*	24.8		11, 12, 13, 19
19	1.41 *s*	24.1		11, 12, 13, 18
20	2.14 *s*	6.6		5, 6, 7
1'	--	138.4		
2'	7.56 *dd(8.6, 2.0)*	126.0	3'	2, 6', 3'
3'	7.48 *m*	128.3	2', 4'	1', 2', 4', 5'
4'	7.44 *m*	128.3	3', 5'	2', 3', 5', 6'
5'	7.48 *m*	128.3	4', 6'	1', 2', 3', 4'
6'	7.56 *dd(8.6, 2.0)*	126.0	5'	2, 2', 5'
1"	1.84 *m*	34.3	2", 3", 9	9, 2", 3"
2"	0.69 *d(7.0)*	18.4	1"	9, 1", 3"
3"	0.79 *d(7.0)*	19.1	1"	9, 1", 2"
C-5-OH	12.3 *s*	--		

*Assignments supported by HSQC and HMBC experiments.

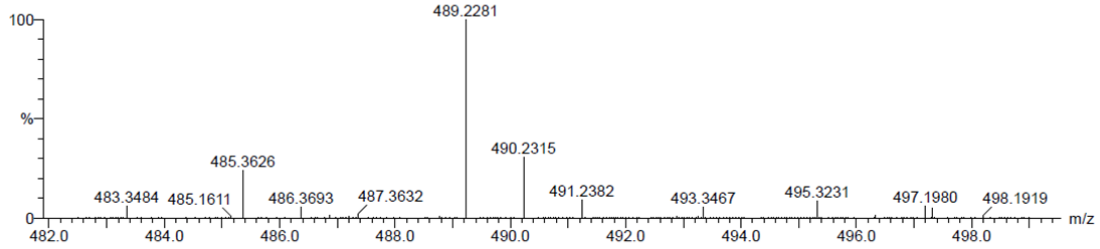
6.5.c HR-ESI-MS of compound **6.5**

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = 0.0, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

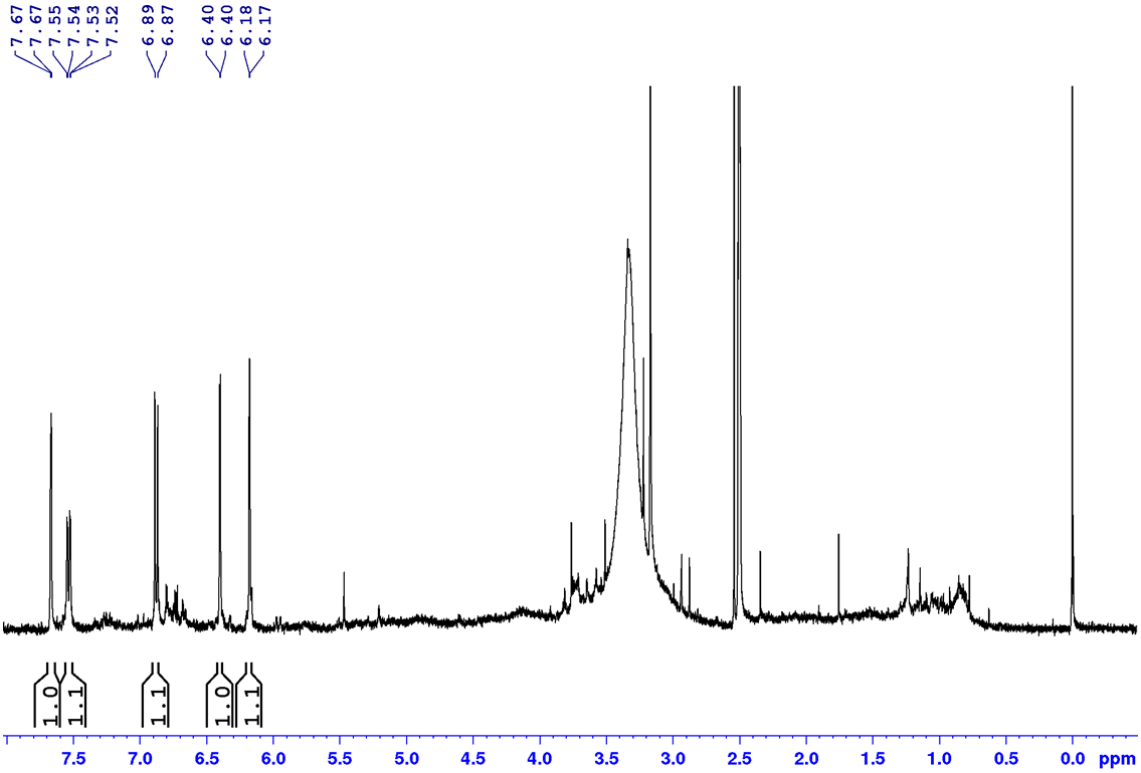
Monoisotopic Mass, Even Electron Ions
47 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-40 H: 0-50 O: 0-10

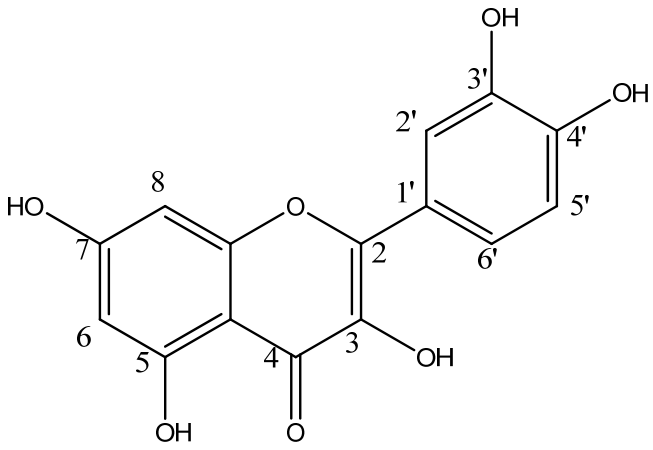
20180523_Sgg_27b_pos 28 (0.276) 1: TOF MS ES+ 3.88e+004



Minimum:				0.0			
Maximum:		5.0	10.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
489.2281	489.2277	0.4	0.8	14.5	344.4	0.0	C30 H33 O6

7.1.a ^{1}H NMR spectrum of compound **7.1** (DMSO-d_6, 400 MHz)





3,5,7,4',5'-Pentahydroxy flavone
or Quercetin

7.1.b ^{1}H NMR data(DMSO-d_6, 400 MHz) for compound **7.1**

Position	δ_H, Mult (*J* in Hz)
6	6.18 *d(2.0)*
8	6.40 *d(2.0)*
2’	7.67 *d(2.0)*
5’	6.88 *d(8.5)*
6’	7.53 *dd(8.5, 2.0)*

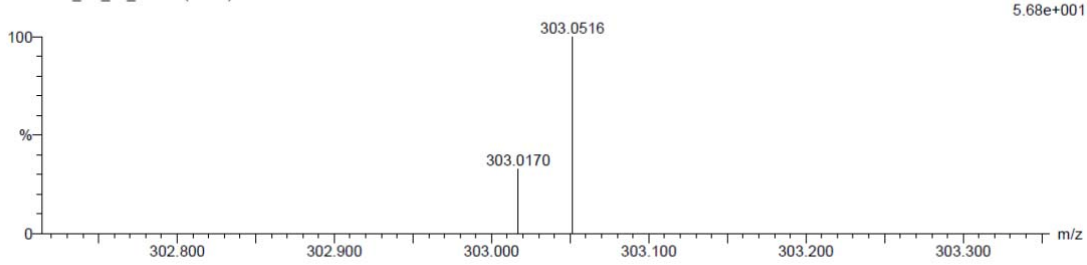
7.1.c HR-ESI-MS of compound **7.1**

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 20.0 PPM / DBE: min = 0.0, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 6

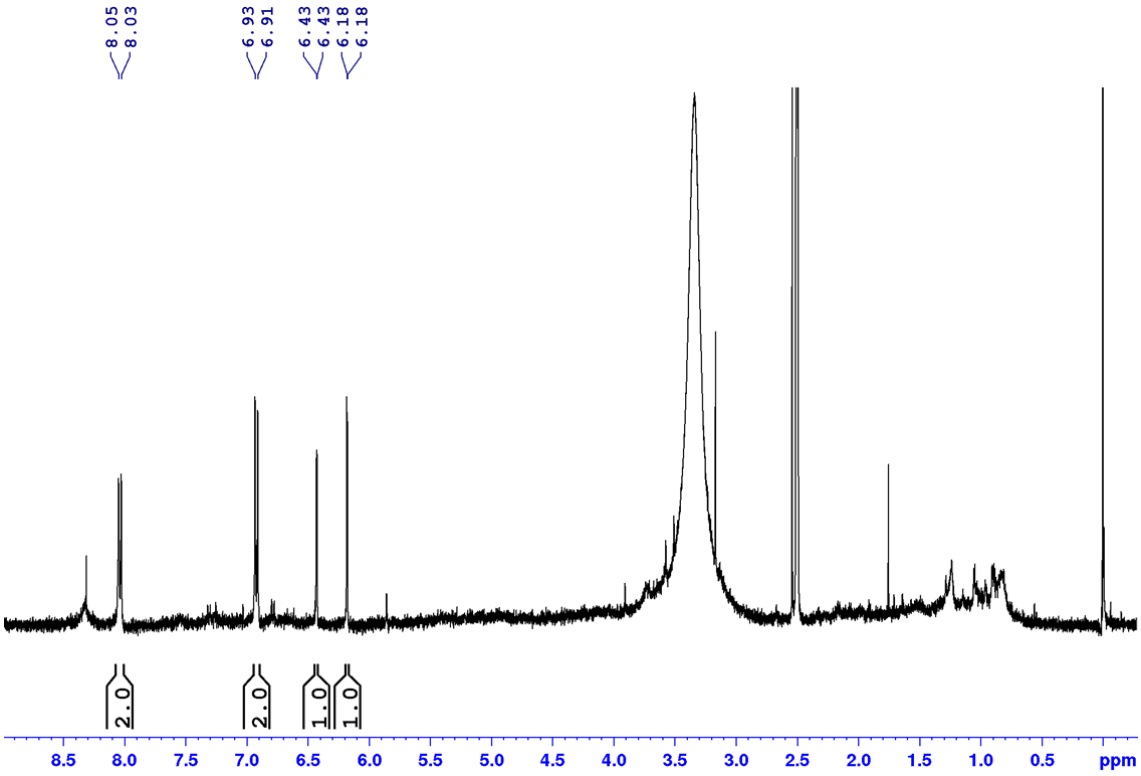
Monoisotopic Mass, Even Electron Ions
95 formula(e) evaluated with 3 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-40 O: 0-20 Na: 0-1
21092018_Brr_10_run3 6 (0.077)

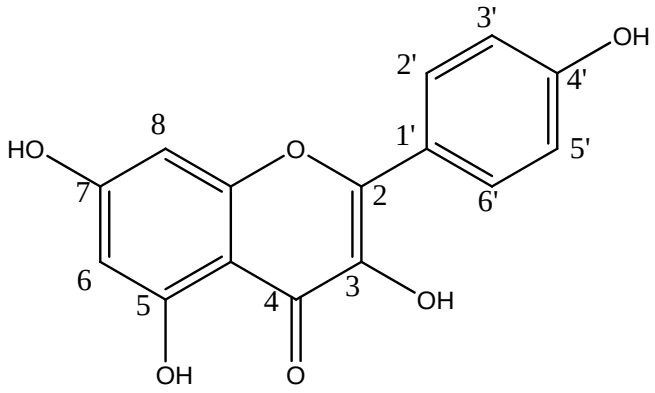
1: TOF MS ES+
5.68e+001



Minimum:				0.0						
Maximum:		5.0	20.0	50.0						
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula			
303.0516	303.0505	1.1	3.6	10.5	21.5	2.2	C15	H11	O7	
	303.0481	3.5	11.5	7.5	21.3	2.0	C13	H12	O7	Na
	303.0564	-4.8	-15.8	1.5	19.6	0.3	C8	H15	O12	

7.2.a ^{1}H NMR spectrum of compound **7.2** (DMSO-d_6, 400 MHz)





3,5,7,4’-Tetrahydroxy flavone
or Kaemferol

7.2.b ^{1}H NMR data(DMSO-d_6, 400 MHz) for compound **7.2**

Position	δ_H, Mult (*J* in Hz)
6	6.18 *d(2.0)*
8	6.43 *d(2.0)*
2’	8.04 *d(9.0)*
3’	6.92 *d(9.0)*
5’	6.92 *d(9.0)*
6’	8.04 *d(9.0)*

7.2.c HR-ESI-MS of compound **7.2**

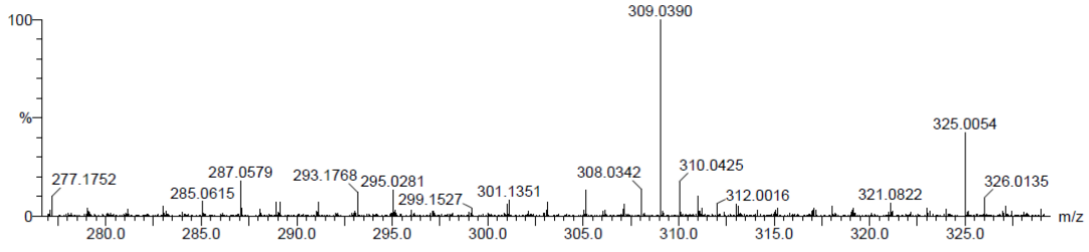
Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = 0.0, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 6

Monoisotopic Mass, Even Electron Ions
57 formula(e) evaluated with 2 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-20 O: 0-20 Na: 0-1

2018_07_27_Brr_12 31 (0.302)

1: TOF MS ES+
4.34e+002



Minimum:				0.0			
Maximum:		5.0	10.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
309.0390	309.0399	-0.9	-2.9	13.5	205.3	0.6	C17 H9 O6
	309.0375	1.5	4.9	10.5	205.5	0.8	C15 H10 O6 Na