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IN VITRO AND *IN VIVO* ANTI-INFLAMMATORY ACTIVITY OF EXTRACTS FROM A MARINE MOLLUSC

A thesis submitted to Southern Cross University

By:

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This thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Southern Cross University

School of Environment, Science and Engineering,

Lismore, New South Wales, Australia

2017

“Seek **medicine**, for God has not created a **disease** except that he has also created its **cure**.”

By Prophet Muhammad



Photos from one of the collection site in the Northern beaches of NSW, Australia showing the research species *Dicathais orbita*.

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I certify that the work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university.

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Dedication

To my parents **Belkasm AHMAD** and **Aisha MASOUD** and my wife
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Abstract

Inflammation is implicated in almost all animal and human diseases. The available treatments for inflammation include steroidal and non-steroidal anti-inflammatory drugs (NSAIDs), which have many common and sometimes severe side effects. Hence, new anti-inflammatory agents with different structures and/or mode of action are needed. In the last few decades, the search for new biologically active compounds has focused on marine natural products. This focus is driven by the diversity of marine organisms and their production of secondary metabolites with an extensive array of bioactivity. Molluscs in particular, are well-known for the production of valuable secondary metabolites and have been used in the traditional medicine systems from many cultures to treat inflammatory conditions. A review of literature in ethnomedicine indicates that there are in excess of 100 different anti-inflammatory preparations described, including 70 preparations from the well-documented traditional Chinese medicine. Despite this extensive traditional use, only a few studies have investigated the anti-inflammatory activity of marine mollusc in scientifically rigorous *in vitro* and *in vivo* experiments.

The main aim of this project was to assess the *in vitro* and *in vivo* anti-inflammatory activity of extracts and associated indole compounds from the marine Muricidae *Dicathais orbita*. The extracts were tested for their ability to inhibit the production of the recognised pro-inflammatory modulator nitric oxide (NO) and cytokines, such as tumour necrosis factor alpha (TNF α) and prostaglandin E2 (PGE2). Bioassay guided fractionation of the organic extracts was used to help identify the active compounds, along with liquid chromatography-mass spectrometry. In parallel, synthetic indole compounds were tested to compare the relationship between the activity and the chemical structure. Results of this study indicated that chloroform extracts from *D. orbita* hypobranchial glands exhibited promising anti-inflammatory activity as detected by inhibition of NO and TNF α in RAW264.7

macrophages, as well as PGE2 in 3T3 fibroblasts. The chloroform extract of the mollusc egg capsules showed similar activity along with some semi-purified fractions containing brominated indoles. Purified tyrindoleninone and tyriverdin, as well as synthetic 6-bromoisatin, 5-bromoisatin, 6-bromoindole and isatin all inhibited the production of NO in LPS-stimulated RAW 264.7 macrophages. A hypobranchial gland extract, 6-bromoindole and 6-bromoisatin significantly downregulated the production of PGE2 in 3T3 fibroblasts ($p < 0.0001$) and blocked the translocation of the NF κ B into the nucleus of LPS-stimulated RAW 264.7 ($p < 0.0001$), as demonstrated by confocal microscopy. The structure-activity relationship experiments revealed that the brominated indoles were more active than non-brominated indoles and that the position of the bromine atom on the indole ring affects the activity with C5 > C6 > C7. Simple indoles were more active than dimers and may reflect the fact that dimer molecules were significantly less soluble than monomers. These outcomes suggest that simple brominated indoles may be a valuable source of anti-inflammatory drug leads and support the further development of extracts from the Australian muricid *D. orbita*, as a new potential natural medicine for the treatment of inflammation.

The active hypobranchial gland (HBG) extract and 6-bromoistain were further tested for their anti-inflammatory activity *in vivo* using a murine LPS-induced acute lung inflammation model in C57 Black/6 mice. Mice were pre-treated orally with three doses of HBG extract or 6-bromoisatin using oral gavage prior to stimulation with LPS intranasally. The chloroform extract of the HBG along with 6-bromoisatin demonstrated significant anti-inflammatory activity ($p < 0.0001$) *in vivo* preventing the development of LPS-stimulated acute lung inflammation in mice. The anti-inflammatory activity was confirmed by the inhibition of alveolar neutrophil infiltration, reduction of TNF α , IL-1 β and proteins levels in bronchio alveolar lavage, as well as the preservation of the lung tissue morphology. These results confirm the *in vitro* anti-inflammatory activity of the HBG extract from *D. orbita* and the abundant brominated compound in this extract, 6-bromoisatin, in an *in vivo* model and provide more support for their potential for development as natural therapeutic agents for inflammation.

Finally, lipid extracts from the foot tissue and viscera of *D. orbita* were characterised and tested for anti-inflammatory activity using *in vitro* assays. The fatty acid profile of these lipid extracts was compared to lipid extracts derived from common seafoods including salmon, sardine, school prawn, octopus and squid using gas chromatography fatty acids methyl esters analysis. The anti-inflammatory activity was tested *in vitro* by investigating the ability of the lipid extracts to inhibit the production of NO and downregulate the levels of TNF α in LPS-stimulated RAW 264.7 macrophages. *D. orbita* was found to possess a unique lipid composition compared to the other species, as they were rich in docosapentanoic acid (DPA), an omega 3 polyunsaturated fatty acid (PUFA) that was found only in traces amounts or not at all in the other organisms tested. Lipid extracts from *D. orbita*, in particular the 7 % tartaric acid-stabilised foot lipid extract and viscera lipid extracts, were the most active, inhibiting NO production and down-regulating TNF α production at a significantly lower IC₅₀ than the lipid extracts from other seafood ($p < 0.05$). Nevertheless, all the extracts, including those from waste products such as prawn and fish heads and viscera from cephalopods, had high levels of PUFAs and showed significant inhibition of NO ($p < 0.05$). These results identify new sources of biologically active PUFAs from undervalued seafood processing waste. In addition, the results from this study demonstrate that the foot tissue and viscera, as well as the hypobranchial glands contain anti-inflammatory agents in the muricid *D. orbita*.

Overall, this work represents the first study assessing the *in vitro* and *in vivo* anti-inflammatory activity of extracts from the Muricidae marine mollusc *D. orbita*. Further testing of the biologically active extracts and the simple brominated indoles to characterise their modes of action could lead to promising outcomes. The data from this study demonstrated that the Australian muricid *D. orbita* is a source of naturally occurring anti-inflammatory agents that have potential for further development as pharmaceuticals and nutraceuticals.

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Abbreviations:

5-HETE	5- hydroxyeicosatetraenoic acid
5-HT	5-hydroxytryptamine
AA	arachidonic acid
ACP	acid phosphatase
AIA	adjuvant-induced arthritis
ALP	alkaline phosphatase
BALF	bronchio alveolar lavage fluid
CIA	collagen-induced arthritis
CINC 1	cytokine-induced neutrophil chemoattractant 1
COX	cyclooxygenase
DNFB	dinitrofluorobenzene
DTH	delayed type hypersensitivity reaction
ETA	eicosatetraenoic acid
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
FLH	<i>Fissurella latimarginata</i> hemocyanin
GLME	green lipped mussel extract
HFCM	hannai fermented with <i>C. militaris</i> mycelia
HMLE	hard-shelled mussel lipid extract
HpH,	<i>Helix pomatia</i> hemocyanin
HRBC	human red blood cell
i.p.	interaperitoneal
i.v.	intravenous
IFN-γ	interferon gamma

IL	interleukin
iNOS	induced nitric oxide synthase
LOX	lipoxygenase
LPS	lipopolysaccharide
LTB4	leukotriene B4
MMP	matrix metalloproteinase
NFκB	nuclear factor kappa B
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
OA	osteoarthritis
ORAC	oxygen radical absorbance capacity
OTC	over-the-counter
p.o.	<i>per os</i> = orally
PCT	placebo-controlled trial
PFC	plaque forming cell
PG	prostaglandin
PLA2	phospholipase A2
PT	partial thickness
PUFAs	polyunsaturated fatty acids
RA	rheumatoid arthritis
ROS	reactive oxygen species
TCC	total cell count
TCM	Traditional Chinese Medicine
TIMPs	tissue inhibitors of metalloproteinases
TNFα	tumour necrosis factor alpha

TRAP	tartrate-resistant acid phosphatase
TT	tetanus toxoid
TXB2	thromboxane B2
WoRMS	World Register of Marine Species
ZKC	Zhikang capsule

Chapter 1: General introduction

1. Chapter 1: general introduction

1.1. Inflammation:

Inflammation is the rapid response of the body to injury and infection. The inflammatory response is a spatially and temporally arranged episode in which cells and mediators collaborate to neutralise and eliminate the damaging stimuli, to allow the maintenance of homeostasis ([Medzhitov, 2010](#), [Alessandri et al., 2013](#)). The inflammatory reaction is recognised macroscopically by four basic signs (which were first described by Cornelius Celsus in the first century) of *calor* (heat), *rubor* (redness), *tumor* (swelling), *dolor* (pain) and loss of function ([Alessandri et al., 2013](#)). Vascular response with hyperaemia (increased blood flow) and increased permeability of vessel walls are the first steps in the inflammatory cascade ([Alessandri et al., 2013](#)). Local hyperaemia is formed initially by transient arteriolar vasoconstriction, encouraged by vascular smooth muscle contraction, which in turn gives rise to arteriolar vasodilation leading to increased blood flow into the damaged tissue ([Lawrence et al., 2002](#), [Alessandri et al., 2013](#)). At this early stage, modifications in the vascular endothelium are readily detectable leading consequently to exudation of plasma proteins and fluid from the blood into the tissue. This is followed by migration of the circulating leukocytes into tissue ([Alessandri et al., 2013](#), [Lawrence et al., 2002](#)).

In order for the body to repair the damaged tissue or fight back against a pathogen, it launches a complex series of immune responses. During the onset of the inflammation, many pro-inflammatory mediators such as cytokines including tumour necrosis factor alpha (TNF α) and interleukin-1 (IL-1), as well as pro-inflammatory molecules such as nitric oxide (NO) and reactive oxygen species (ROS) are released ([Baumann and Gauldie, 1994](#)). All play an essential role in eliciting the local inflammatory response. Failure to control the inflammatory reactions locally may lead to leakage of pro-inflammatory mediators into the circulation, causing a systemic inflammatory response ([Pavlov et al., 2003](#)). Systemic inflammation can progress to shock, multiple organ failure and ultimately to death.

Polymorphnuclear Leukocytes respond to foreign stimuli in a process mediated by glycogen adhesion molecules (selectins) and chemoattractants on the surface of endothelium ([Alessandri et al., 2013](#), [Kubes, 2002](#), [Petri et al., 2008](#)). Activation of the chemoattractant receptor in leukocytes results in strong binding to endothelial cells and allows leukocytes to migrate into the interstitium at the site of infection. Once in the tissue, leukocytes can be further triggered and become an important source of a variety of substances, which include cytokines, lipid mediators, chemokines, reactive oxygen species (ROS) and colony stimulating factors (Figure 1.1) ([Alessandri et al., 2013](#)). Although the inflammatory process promotes the elimination of damaging stimuli, the inflammatory process itself may contribute to damage of neighbouring tissues and can therefore increase the severity of symptoms ([Cara et al., 2000](#), [Alessandri et al., 2013](#)).

Macrophages are a crucial component of the innate cellular immune system, with a variety of functions prominently involved in host defence and immunity (Figure 1.1) against foreign microorganisms like viruses, bacteria, fungi and parasites ([Zhang and Wang, 2014](#)). In brief, after foreign pathogens invade the host, the inflammatory process of macrophages has four orderly and interrelated stages; 1) pattern recognition receptors (PRRs) expressed on/in macrophages recognise pathogen-associated molecular patterns (PAMPs) from the invading foreign pathogen, which in turn starts an anti-infection inflammatory response; 2) enrichment of the quantity of macrophages in the infected local tissue can occur by two means, including recruitment of circulating monocytes in response to CCL2 and IL-4-triggered *in situ* macrophage proliferation; 3) macrophage differentiation either into classically activated macrophages (M1) for intracellular virus or bacterial infection, or into alternatively activated macrophages (M2) for helminth infection. The activated macrophages can then express microbiocidal molecules, such as reactive oxygen species (ROS), nitric oxide (NO), matrix metalloproteinase-12 (MMP12) and defensins, and produce pro-inflammatory cytokines and anti-virus interferon-I (IFN-I) to clear invading pathogens; 4) after the infection stage, the macrophages convert to an anti-inflammatory phenotype to eliminate inflammation and endorse tissue repair ([Zhang and Wang, 2014](#)) (Figure. 1.1).

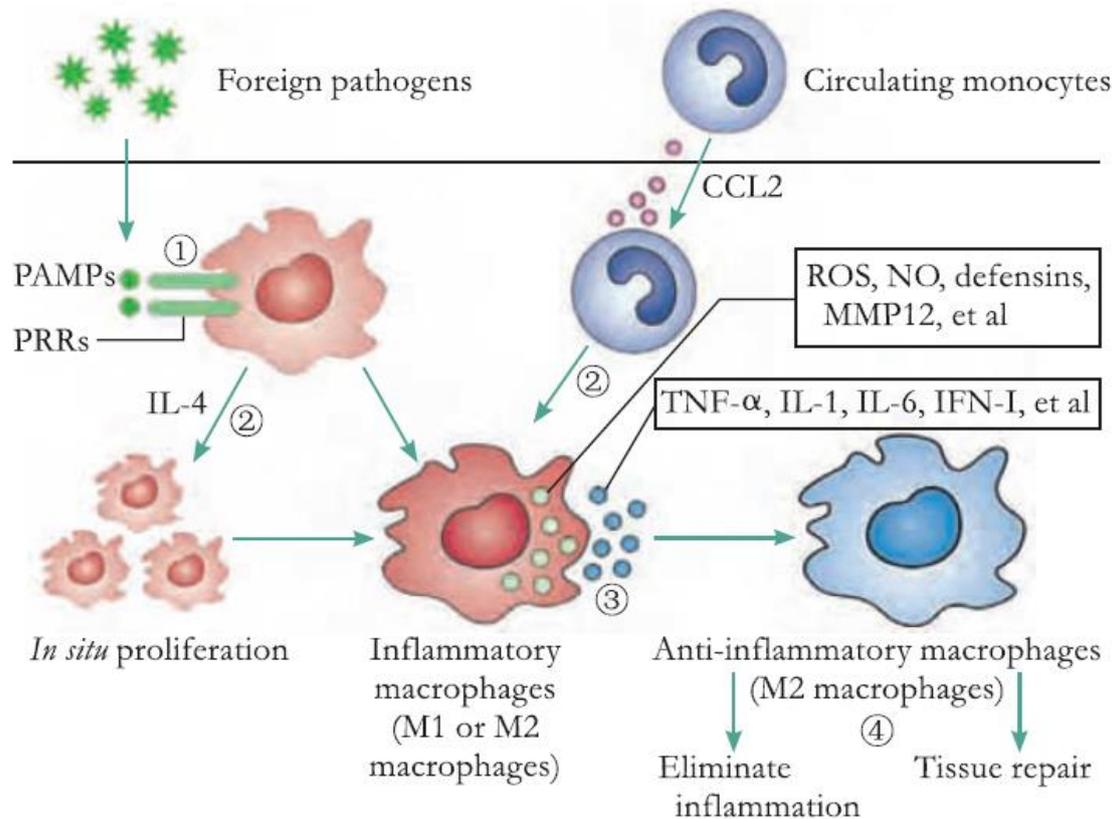


Figure. 1.1: The role of macrophages in the inflammatory reaction. Following the invasion of the host by foreign pathogen, the macrophage inflammatory response has four well-ordered and inter-related phases. 1) pattern-recognition receptors (PRRs) recognise the pathogen-associated molecular patterns (PAMPs) from the foreign pathogens, hence, starting the inflammatory response. 2) Enrichment of the macrophages in the infected local tissue by two means: *in situ* macrophage proliferation driven by interleukin 4 (IL-4) and recruitment of the circulating monocytes in response to C-C motif ligand 2 (CCL2). 3) The recruited macrophages then differentiate into classically activated macrophage (M1) for intracellular pathogens or alternatively activated macrophages (M2) for helminth infection. M2 macrophages are able to express microbicidal molecules such as reactive oxygen species (ROS), nitric oxide (NO), defensin, and matrix metalloproteinase 12 (MMP12) and produces pro-inflammatory cytokines (such as tumour necrosis factor alpha (TNF α), and interleukins IL-1 and IL-6), as well as anti-virus interferon (INF-I), which in turn clear the foreign pathogens. 4) Recruited macrophages transform to the anti-inflammatory phenotype to eliminate the inflammation and promote tissue repair. Sourced from (Zhang and Wang, 2014) .

Reactive oxygen species (ROS), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^\bullet) are normally produced during cellular energy production in aerobic cells and are removed by antioxidant enzymes, such as peroxiredoxins (Prxs), glutathione peroxidase (GPx), catalase and superoxide dismutase (SOD) (Alessandri et al., 2013). ROS has impacts on many physiological processes, including host defence against different pathogens, hormone biosynthesis and intracellular signalling (Bogdan et al., 2000, Droge, 2002, Alessandri et al., 2013). Even though ROSs are considered relevant to appropriate anti-microbial responses, ROS can also damage host

proteins, DNA and lipids. ROS activates many transcription factors and signalling pathways important for pro-inflammatory responses, such as nuclear factor kappa B (NF κ B), the activator protein 1 (AP-1), mitogen-activated protein kinases (MAPKs) and phosphoinositide-3-kinase (PI3K) ([Li et al., 2002](#), [Groeger et al., 2009](#), [Carreras and Poderoso, 2007](#), [Chen et al., 2004](#)). It has been suggested that ROS may contribute to the development of many inflammatory ailments, including arthritis, diabetes, cancer, atherosclerosis and degenerative diseases ([Quinlan et al., 1994](#), [Droge, 2002](#), [Riedl and Nel, 2008](#), [Phillips et al., 2010](#)). According to the mentioned findings, it has been recommended that anti-oxidative agents may be a beneficial strategy to reduce ROS levels and act as anti-inflammatory remedies ([Yasui and Baba, 2006](#), [Impellizzeri et al., 2011](#)).

Prostaglandins are short-lived localised hormones that can be produced by any cell in the body during chemical, tissue or traumatic injury ([Maroon et al., 2006](#)). Arachidonic acid-derived prostaglandins (PGs) are significant endogenous mediators which have a crucial role in many biological processes like inflammation, immune response and cell proliferation ([Mizumura et al., 2003](#), [Williams and Shacter, 1997](#), [Mitchell et al., 1995](#)). Prostaglandins also have different functions in the central nervous system, which include the perception of pain, development of fever and moderating the wake and sleep cycle ([Fiebich et al., 2002](#)). PGs are created as a result of the combined enzymatic activity of phospholipase A2 (PLA2) and cyclooxygenase (COX) ([Mizumura et al., 2003](#)). PLA2 releases arachidonic acid (AA) from the phospholipid layer in cell membranes ([Evans et al., 2003](#), [Mizumura et al., 2003](#)). There are two distinct isoforms of PLA2 according to their location in the cell; sPLA2 (secretory) and cPLA2 (cytosolic) ([Evans et al., 2003](#)). PLA2 is activated by different stimuli (via phosphorylation of PLA2) to liberate the arachidonic acid ([Fiebich et al., 2002](#)). Then, arachidonic acid is converted to the endoperoxide intermediate prostaglandin H2 (PGH2), which is then metabolised to different PGs like PGE2, PGD2, PGI2 and thromboxane A2 (TxA2) by COX enzymes ([Fiebich et al., 2002](#), [Mizumura et al., 2003](#)). Therefore, prostaglandins and COX 2 inhibition are important inflammatory indicators and used regularly in anti-inflammatory assays.

Despite the fact that inflammation is predominantly a physiological and advantageous process, non-resolving inflammatory processes may be involved in pathogenesis and the progression of many inflammatory-related diseases ([Alessandri et al., 2013](#)). Inflammation is a contributing factor to several age related chronic diseases such as musculoskeletal diseases, acute and chronic neurodegenerative diseases, cardiovascular diseases, asthma, inflammatory bowel disease and rheumatoid arthritis ([Alessandri et al., 2013](#), [Brod, 2000](#), [Gu et al., 2010](#)). Therefore, inflammation is now the focus of global scientific research, due to its implications in almost all animal and human diseases ([Phanse, 2012](#)).

1.2. Anti-inflammatory drugs

To date, control of inflammatory responses is primarily based on the use of steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) ([Gunawardena et al., 2014a](#)). The anti-inflammatory properties of NSAIDs are directed at the prevention of PGs by inhibiting COX enzymes which catalyse the conversion of arachidonic acid to PGs ([Auriel et al., 2014](#), [Seibert et al., 1997](#), [Vane and Botting, 1998](#)). However, NSAIDs have been linked to increased blood pressure, greatly increased risk of congestive heart failure, occurrence of thrombosis and they can also cause serious gastrointestinal toxicity ([McMurray and Hardy, 2002](#), [Aisen et al., 2003b](#)). These side effects are to some degree common to almost all NSAIDs ([Vane and Botting, 1998](#)). The main cause for these side effects is the mechanism of action of the NSAIDs (Figure 1.2), whereby conventional NSAIDs target both the enzymes COX-1 and COX-2, blocking the production of the homeostatic prostaglandin, as well as the inflammatory prostaglandins. By comparison, selective COX-2 inhibitors selectively inhibit COX-2 without interfering with COX-1 which reduces the interference with this significant constitutive enzyme ([Vane and Botting, 1998](#)) (Figure 1.2). Hence, the selective inhibition of COX-2 reduces the side effects that can result from the undesired inhibition of COX-1, such as gastrointestinal complications ([Grosser et al., 2006](#)). Nevertheless, COX-2 inhibitors can contribute to other side effects, as COX-2 is believed to be involved in maternal labour and ovulation processes ([Vane and Botting, 1998](#)). Furthermore, there is some clinical evidence that COX-2 inhibition could

increase hypertension and cardiovascular risks by interfering with the regulation of thrombogenesis and atherogenesis ([Grosser et al., 2006](#)).

Steroidal anti-inflammatory drugs such as corticosteroids are a form of anti-inflammatory drugs used to treat mostly the chronic inflammation, and their mode of action is to prevent the activation of multiple inflammatory genes, which are up-regulated during chronic inflammatory conditions ([Barnes, 2006a](#), [Barnes, 2006b](#)). Steroidal anti-inflammatory drugs have numerous side effects including metabolic disturbances, osteoporosis, steroid withdrawal syndrome, dermal thinning and skin capillary fragility ([Barnes, 2006a](#)). Consequently, there is a desperate need for the development of alternatives that address or minimise the side effects.

Anti-inflammatory drugs are among the most consumed pharmaceuticals with for example over 70 million prescriptions and 30 billion tablets of NSAID sold over the counter each year ([Maroon et al., 2006](#)). This demand increased after the discovery of the three most used selective anti-inflammatory drugs (Vioxx, Celebrox, and Bixtra), which exceeded \$9 billion sales in 2003 alone ([Maroon et al., 2006](#)). Nevertheless, the strong market value is not an indicator of the safety of these drugs. For example, Mark Research laboratories had to withdraw its selective COX-2 NSAID, Vioxx in 2004, because after 18 months of use, this drug was found to cause serious complications like myocardial infraction and thrombotic cerebrovascular and cardiovascular incidents ([Fosslien, 2005](#), [Maroon et al., 2006](#)). All this information indicates the importance of anti-inflammatory drugs and the real need for a new family of anti-inflammatory drugs to replace the NSAIDs. The new drugs should preferably be of a chemical structure that is different to the NSAIDs.

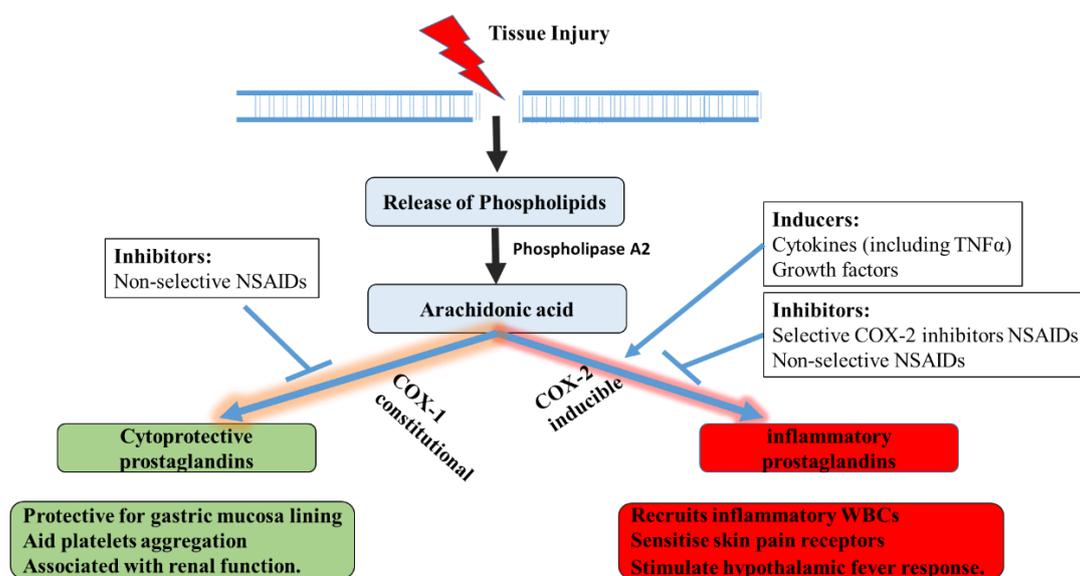


Figure 1.2: The production of prostaglandins from membrane phospholipids as a result of tissue injury and the mechanism of action of the traditional non-steroidal anti-inflammatory drugs (NSAIDs) compared to the selective COX-2 inhibitors. Modified from ([Namrata, 2014](#), [Maroon et al., 2006](#)).

1.3. Marine natural products as potential anti-inflammatory agents:

Many natural products are sourced from the marine biosphere due to its phenomenal biodiversity. Marine invertebrates are one of the major groups of biological organisms and include Porifera, Echinodermata, Cnidarian, Mollusca and Arthropoda. For a long time these organisms have been considered as a rich source of pigments, polyunsaturated fatty acids (PUFAs), polysaccharides, sterols, proteins, and antioxidants, some of which are used in the formulation of novel drugs ([Senthilkumar and Kim, 2013](#)). These natural products have an extensive array of therapeutic properties, including anticoagulant, antimicrobial, wound healing and immune modulating, antioxidant, anticancer, anti-inflammatory, anti-hypertensive, and other medicinal properties ([Senthilkumar and Kim, 2013](#), [Perdicaris, 2013](#)).

A preliminary systematic search using the Web of Science database (1960-2015) was performed to gain an insight into the scope of previous research on marine invertebrate anti-inflammatory compounds, using the key words “anti-inflammatory” and “marine natural products”. A total of 201 articles related to anti-inflammatory marine natural products were systematically reviewed. In total at least 90 distinct secondary metabolites were reported to have anti-inflammatory activity (Appendix

1) and these are distributed across five animal phyla. This search excludes plant and Protista natural products and marine anti-inflammatory natural products from a cyanobacterial or fungal sources from the search results. As seen in Figure 1.3, there has been relatively little focus on the anti-inflammatory properties of the phylum of Mollusca.

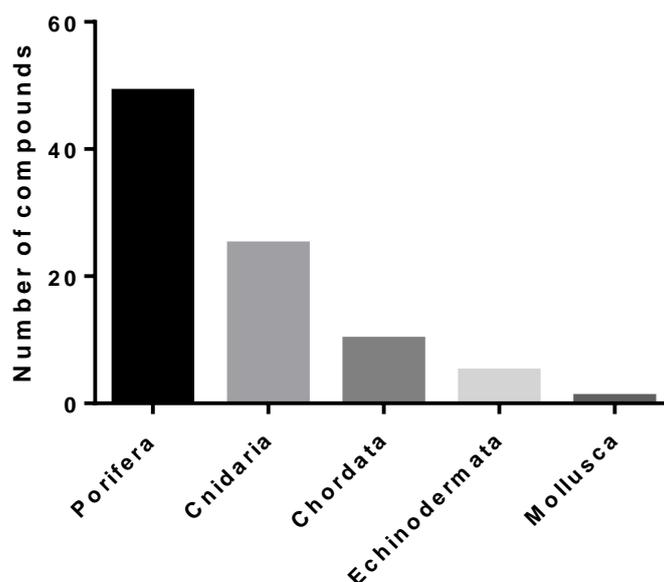


Figure. 1.3: Number of marine natural products with anti-inflammatory activity from various marine invertebrate phyla. The total natural products reviewed were 90 from 64 papers published from 1980-2014. Detailed information about this search and the raw data are shown in Appendix 1.

Alkaloids are one class of biologically active natural products commonly found in marine invertebrates. Alkaloids are biological amine and cyclic compounds with a nitrogen atom in the structure that naturally occur in terrestrial animals and plants, algae, microorganisms and marine invertebrate organisms ([Souto et al., 2011](#), [Franca et al., 2014](#)). Due to their pharmaceutical significance as bioactive compounds and as biological probes for physiological studies, both halogenated and non-halogenated forms of alkaloids have attracted substantial research interest ([Jaswir and Monsur, 2011](#)). Many indole alkaloids from marine invertebrates have been reported as potential anti-inflammatory agents (Table 1) ([Senthilkumar and Kim, 2013](#), [Souto et al., 2011](#)). Examples of anti-inflammatory marine alkaloids include carteramine A from sponges (Table 1)

([Kobayashi et al., 2007](#)), Lepadiformines A and B from ascidian *Clavelina moluccensis* ([Sauviat et al., 2006](#)), aplysinopsintype from the sponge *Hyrtios erecta* ([Aoki et al., 2001](#)), conicamin from tunicates ([Aiello et al., 2003](#)), manzamine from sponges ([Sayed et al., 2008](#)) and ascidiathiazones A and B ([Pearce et al., 2007a](#), [Pearce et al., 2007b](#)) from ascidians. All these studies indicate the value of the marine invertebrates as a beneficial source of new alkaloid compounds, which could provide new leads for anti-inflammatory agents.

A broad range of potential anti-inflammatory compounds from marine invertebrates have been tested for their ability to inhibit inflammatory markers using both *in vitro* and *in vivo* assays (Appendix 1). Tricyclic alkaloids ascidiathiazone isolated from the Ascidian *Aplidium* species have been shown to inhibit superoxide production by human neutrophils *in vitro* ($IC_{50} = 0.44\text{--}1.55 \mu\text{M}$), as well as by murine peritoneal neutrophils *in vivo* in a mouse model of gout ([Pearce et al., 2007a](#)). Frajunolides from the Taiwanese gorgonian *Junceella fragilis*, significantly inhibited superoxide anion and the generation of elastase from human neutrophils *in vitro* ($IC_{50} > 10 \mu\text{g/mL}$) ([Shen et al., 2007](#)). Hexahydro-8-hydroxymanzamine ($IC_{50} = 1.97 \mu\text{M}$), Manzamine A ($IC_{50} = 0.25 \mu\text{M}$) and (-)-8-hydroxymanzamine A ($IC_{50} < 0.1 \mu\text{M}$), significantly inhibited the generation of thromboxane (TXB₂) in brain microglia ([Sayed et al., 2008](#)). [Kossuga et al. \(2008\)](#) established that the polyketide plakortide P from the Brazilian sponge *P. angulospiculatus* strongly inhibited thromboxane B₂ release ($IC_{50} = 0.93 \mu\text{M}$) from activated rat brain microglia and this compound appears to be a novel anti-neuroinflammatory agent. A halogenated furanone rubrolide O isolated from a New Zealand ascidian *Synoicum* sp., inhibited superoxide anion production in human neutrophils ($IC_{50} = 35 \mu\text{M}$) *in vitro* with low toxicity (Table 1) ([Pearce et al., 2007b](#)). These marine natural products provide examples of significant activity against a range of targets and support the use of marine invertebrates as an important source of pharmaceutically active agents with anti-inflammatory activity. Some marine-derived natural products have entered the drug development stage of which some have been approved by Food and Drug administration (FDA-approved) (Table 1) ([Mayer et al., 2010](#), [Mayer, 2017](#)).

However, these are mostly for treatment of cancer, with no marine natural products currently in clinical trials as anti-inflammatory pharmaceuticals.

Even though no marine-derived anti-inflammatory pharmaceuticals have been approved for therapeutic use, there are a number of marine nutraceuticals available on the market for the treatment of inflammation ([Suleria et al., 2015](#)). These include fucoidans derived from algae and sea cucumbers (Echinodermata) ([Li et al., 2008a](#)), carotenoids from algae ([Voutilainen et al., 2006](#), [Ciccone et al., 2013](#)), glucosamine from crustacean exoskeletons ([Largo et al., 2003](#), [Chou et al., 2005](#), [Nakamura and Nishioka, 2002](#)), chondroitin from shark cartilage ([Iovu et al., 2008](#), [Xu et al., 2008](#)) and a range of fish, mussel and krill oils ([Kantor et al., 2012](#)). However, apart from the well-studied PUFAs, there is very limited evidence or scientific data on the proposed anti-inflammatory activity for these mentioned nutraceuticals ([Kantor et al., 2012](#)).

1.4. Polyunsaturated fatty acids (PUFAs)

Marine organisms are well known as a rich source of biologically active lipids. Marine lipid extracts contain a high percentage of PUFAs including ω -3 PUFAs and ω -6 PUFAs. Evidence supporting the beneficial effects of increased consumption of ω -3 fatty acids is growing. [Kalogeropoulos et al. \(2010\)](#) reported that an increase in dietary ω -3 fatty acids decreased the levels of the early response cytokine TNF α , as well as IL-6 and high sensitivity C-reactive protein (inflammatory marker) in the serum of 374 healthy men and women. The pro-inflammatory cytokine TNF α is considered the master controller of many cellular cascades that regulate a variety of processes related to gene expression, cell viability, synaptic integrity and ion homeostasis ([Frankola et al., 2011](#)). TNF α inhibition is the main priority target for inhibition of neuroinflammation and neurodegenerative disease development ([Frankola et al., 2011](#)). Omega-3 fatty acids are well known to have a beneficial impact on many inflammatory diseases including neurodegenerative diseases ([Dyall, 2015](#)).

The American Heart Association recommends having at least two meals of fish, in particular fatty fishes, per week due to their richness of ω -3 fatty acids ([Balzano et al., 2017](#), [American Heart](#)

[Association Nutrition et al., 2006](#)). However, controlling the ω -6/ ω -3 ratio seems to be an important factor in gaining the desired benefit. In Western diets, the ω -6/ ω -3 ratio is about 15/1-16.7/1 which is very high when compared to the normal recommended dietary ratio of \sim 1-4 ([Simopoulos, 2002a](#)). That is thought to be because Western demographics tend to consume high amounts of red meat and sausage products with high intake of vegetable oils (rich in ω -6 PUFAs especially, linoleic acid (C18:2 ω -6; LA)) ([Blasbalg et al., 2011](#), [Strobel et al., 2012](#)). The increase in the ω -6/ ω -3 fatty acid ratio stimulates the onset of numerous diseases, including inflammation, cancer, cardiovascular disease, and autoimmune diseases, while low ω -6/ ω -3 ratio (higher ω -3 values) exerts suppressive properties ([Simopoulos, 2002a](#), [Adkins and Kelley, 2010](#)). In particular, the increase of linoleic acid (C18:2n6c) in diet is responsible for the exacerbation of the inflammatory response ([Simopoulos, 2002a](#)). The ω -3/ ω -6 ratio is generally low in marine fish and shellfish due to the fact that marine animals accumulate various kinds of ω -3 fatty acids in their lipids, while ω -6 PUFAs form a majority in terrestrial animals lipids ([Joseph, 1989](#), [Ackman, 1994](#)). However, most of the world's fisheries are already at maximum capacity or over fished ([Mullon et al., 2005](#)). Therefore there is a strong need to identify new sources of marine lipids that may contribute to human health.

Table 1.1: Compounds derived from marine organisms, which are FDA-approved, or in phase III, phase II or phase I of drug development. The table modified from ([Mayer, 2017](#)).

Marine Organism	Compound Name	Trade mark	Chemical Class	Disease Area	Molecular Target	US - Active Clinical Trials	Europe – Active Clinical Trials	Company/ Institution
FDA-approved								
Fish	Omega-3-acid ethyl esters	Lovaza®	Omega-3 fatty acids	Hypertriglyceridemia	Trygliceride-synthesizing enzymes	[22+]	[1+]	[GlaxoSmith-Kline]
Mollusc	Ziconotide	Prialt®	Peptide	Pain: Severe Chronic Pain	N-Type Ca Channel	[3+]	[2+]	Jazz Pharmaceuticals
Mollusc/cyanobacterium	Brentuximab vedotin (SGN-35)	Adcetris®	ADC (MMAE)	Cancer	CD30 & microtubules	[43+]	[15+]	[Seattle Genetics]
Sponge	Cytarabine (Ara-C)	Cytosar-U®	Nucleoside	Cancer	DNA polmerase	[247+]	[111+]	[Bedford Laboratories]
Sponge	Eribulin Mesylate (E7389)	Halaven®	Macrolide	Cancer	Microtubules	[33+]	[8+]	[Eisai Inc.]
Sponge	Vidarabine (Ara-A)	Vira-A®	Nucleoside	Anti-Herpes Simplex Virus (HSV).	Viral DNA polymerase	[103+]	[2+]	No Link
Tunicate	Trabectedin (ET-743) (EU Registered only)	Yondelis®	Alkaloid	Cancer	Minor groove of DNA	[12+]	[22+]	[Pharmamar]
Tunicate	Plitidepsin	Aplidin®	Depsipeptide	Cancer	Rac1 & JNK activation	[2+]	[4+]	[Pharmamar]
Pufferfish	Tetrodotoxin	Tectin®	Guanidinium alkaloid	Pain: Chronic Pain	Sodium Channel	[2+]	None	[Wex Pharmaceutical Inc.]

Phase II trial								
Mollusc	PM00104	Zalypsis®	Alkaloid	Cancer	DNA-binding	None	[1+]	[Pharmamar]
cyanobacterium	PSMA-ADC	NA	ADC (MMAE)	Cancer	PSMA & microtubules	[1+]	None	[Progenics Pharmaceuticals]
Mollusc cyanobacterium	Glembatumumab Vedotin (CDX-011)	NA	ADC (MMAE)	Cancer	GPNMB & microtubules	[1+]	None	[Celldex Therapeutics]
Mollusc/cyanobacterium	DNIB0600A	NA	ADC (MMAE)	Cancer	NaPi2b & microtubules	[3+]	[1+]	[Genentech/Roche]
Mollusc/cyanobacterium	Pinatuzumab vedotin (DCDT-2980S)	NA	ADC (MMAE)	Cancer	CD22 & microtubules	None	[1+]	[Genentech/Roche]
Tunicate	PM01183	NA	Alkaloid	Cancer	Minor groove of DNA	[7+]	[4+]	[Pharmamar]
Worm	DMXBA (GTS-21)	NA	Alkaloid	<ul style="list-style-type: none"> • Schizophrenia. • Alzheimer Disease. • Attention Deficit Hyperactivity Disorder. • Endotoxemia, Sepsis. 	α 7 nicotinic acetylcholine receptor	[1+]	None	No Link

				<ul style="list-style-type: none"> Vagal Activity. 				
Phase I/II trial								
Mollusc/ cyanobacterium	Polatuzumab vedotin (DCDS-4501A)	NA	ADC (MMAE)	Cancer	CD79b & microtubules	[1+]	[1+]	[Genentech/Roche]
Mollusc/ cyanobacterium	AGS-16C3F	NA	ADC (MMAE)	Cancer	ENPP3 & microtubules	[1+]	None	[Agensys & Astellas Pharma]
Phase I trial								
Bacterium	Marizomib (Salinosporamide A; NPI-0052)	NA	Beta-lactone-gamma lactam	Cancer	20S proteasome	[2+]	None	[Triphase]
Bryozoan	Bryostatin	NA	Macrolide lactone	Cancer	Protein kinase C	None	None	[National Cancer Institute]
Mollusc/ cyanobacterium	ASG-15ME	NA	ADC (MMAE)	Cancer	SLITRK6 & microtubules	[1+]	None	[Seattle Genetics]
Mollusc/ cyanobacterium	ASG-22ME	NA	ADC (MMAE)	Cancer	Nectin-4 & microtubules	[2+]	None	[Seattle Genetics]
Mollusc/ cyanobacterium	DEDN6526A	NA	ADC (MMAE)	Cancer	ETBR & microtubules	[1+]	None	[Genentech/Roche]
Mollusc/ cyanobacterium	DMUC5754A	NA	ADC (MMAE)	Cancer	Mucin 16 & microtubules	None	None	[Genentech/Roche]

Mollusc/ cyanobacterium	DSTP3086S	NA	ADC (MMAE)	Cancer	STEAP1 & microtubules	None	None	[Genentech/ Roche]
Mollusc/ cyanobacterium	HuMax®-TF-ADC	NA	ADC (MMAE)	Cancer	Tissue Factor & microtubules	[1+]	None	[GenMab]
Mollusc/ cyanobacterium	MLN-0264	NA	ADC (MMAE)	Cancer	GCC & microtubules	[1+]	None	[Millennium]
Mollusc/cyanobac terium	SGN-CD19A	NA	ADC (MMAF)	Cancer	CD19 & microtubules	[2+]	None	[Seattle Genetics]
Mollusc/cyanobac terium	SGN-LIV1A	NA	ADC (MMAE)	Cancer	LIV-1 & microtubules	[1+]	None	[Seattle Genetics]
Sponge	PM060184	NA	Polyketide	Cancer	Minor groove of DNA	None	None	[Pharmamar]

1.5. Mollusca as a rich source of biologically active compounds:

Mollusca encompasses 7% of living animals on the planet making it the 2nd largest animal phylum with an estimated total of 150-200 thousand species, of which more than 52 thousand are described and named species ([Benkendorff, 2010](#), [Barco et al., 2010](#)). The molluscan phylum includes seven different classes: Gastropoda, Bivalvia, Scaphopoda, Cephalopoda, Polyplacophora, Monoplacophora, and Aplacophora (encompasses Caudofoveata and Solenogastres) ([Benkendorff, 2010](#)). Molluscs have been a major focus of scientific research in the search of metabolites and natural products with over 1050 natural products reported in over 1000 scientific papers in the last three decades (Table 5) ([Benkendorff, 2010](#), [Avila, 2006](#), [Benkendorff, 2014](#)). The most represented molluscan classes in natural product research are Gastropoda and Bivalvia respectively and that is not unexpected as these are the biggest classes with an estimated 75,000-150,000 gastropod species and 10,000-20,000 bivalve species ([Benkendorff, 2010](#), [Avila, 2006](#)). This clearly indicates the importance of the gastropods in the area of natural products investigation with over 948 compounds being investigated ([Benkendorff, 2010](#)). However, only 1% of the different Gastropoda species have been studied for their secondary metabolites, which provide a great motivation for further research into the natural products and bioactivity in this class of Mollusca.

1.6. Muricidae family

The Muricidae family is one of the most diverse families in the molluscs with more than 1600 well described predatory species distributed in many parts of the world ([Barco et al., 2010](#), [Vasconcelos et al., 2008](#)). Muricids are an economically important family in the Gastropoda class of molluscs. Some species of muricids are large-sized (e.g. > 20cm) and obtained commercially as food and/or for decorative shells ([Vasconcelos et al., 2008](#), [Benkendorff, 2013](#)). Muricidae species are regularly fished for their valuable meat, which is rich in protein, by Asians, Europeans, and Central and South Americans ([Vasconcelos et al., 2008](#), [Barco et al., 2010](#)). Muricidae are also well known for the production of the ancient dye Tyrian purple or shellfish purple. This dye pigment, 6,6-dibromoindigo,

was the first marine natural product to ever to be structurally elucidated and achievement described by Friedlander in 1909 ([Cooksey, 2001](#)). This dye does not occur in the live animals, but it is created after a several chemical reactions in the presence of light and oxygen ([Naegel, 2004](#), [Cooksey, 2001](#), [Benkendorff, 2013](#)).

The Australian species *Dicathais orbita* in the Muricidae family of predatory marine molluscs is currently the focus of significant research aimed at developing a new complementary medicine ([Benkendorff et al., 2015](#)). Some studies focusing on the biological properties of the brominated indole precursors of Tyrian purple (Figure 1.4) have shown promising results. These indole compounds can be extracted from the hypobranchial gland, egg capsules or reproductive organs by using a variety of solvents such as dichloromethane, chloroform, ethanol and dimethylsulfoxide ([Benkendorff et al., 2000](#), [Benkendorff et al., 2001](#), [Cooksey, 2001](#), [Westley and Benkendorff, 2008](#)). The coloured indole compounds can be purified according to their colour based on their polarity using silica gel chromatography ([Esmaelian et al., 2013](#), [Benkendorff et al., 2000](#), [Edwards et al., 2012](#)). The brominated indole precursor tyriverdin showed significant antimicrobial properties (Benkendorff et al 2000), whereas tyrindoleninone, 6-bromoisatin and 6,6'-dibromindirubin demonstrated significant anti-cancer activity ([Meijer et al., 2003](#), [Benkendorff et al., 2011](#), [Westley et al., 2010b](#), [Edwards et al., 2012](#), [Esmaelian et al., 2013](#), [Esmaelian et al., 2014](#)). These precursors of Tyrian purple have not yet been tested for anti-inflammatory activity.

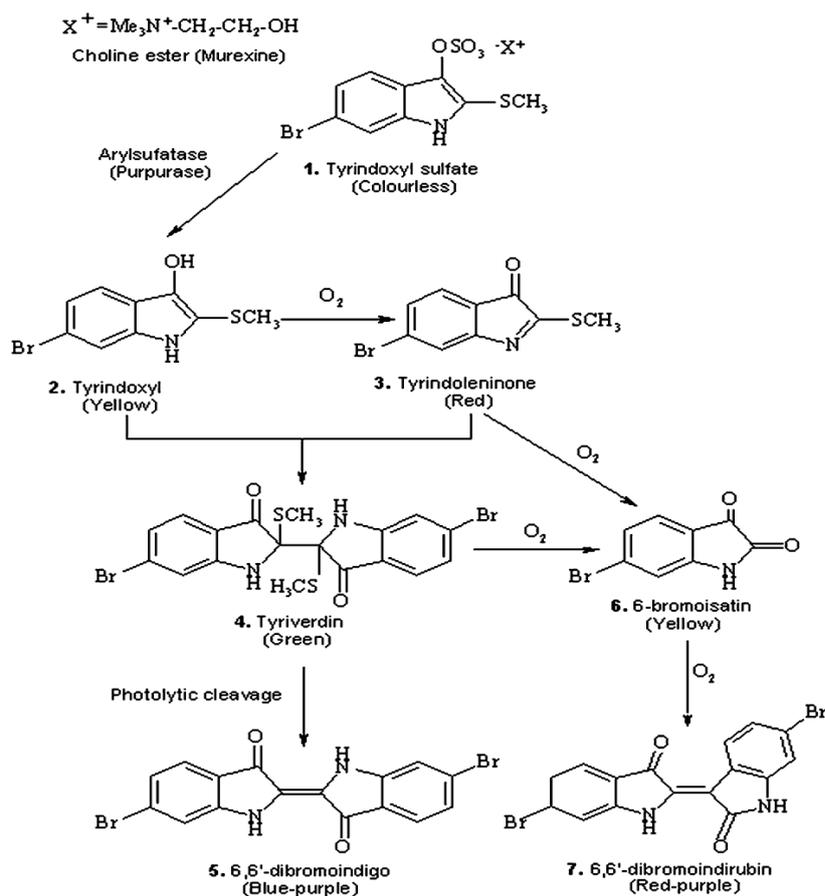


Figure 1.4: The production of the Tyrian purple from brominated indole precursors found in Muricidae. This figure was sourced from ([Westley and Benkendorff, 2008](#)).

1.7. Biologically active indole compounds

Although Muricidae compounds have not yet been tested for anti-inflammatory activity, other indole anti-cancer compounds have often shown promise for the treatment of a number of diseases including inflammation ([Gul and Hamann, 2005](#)). For example, the indole compounds 5,6-dibromotryptamine, 5,6-dibromo-*N*-methyltryptamine, 5,6-dibromo-*N*-methyltryptophan (dibromoabrine), 5,6-dibromo-*N,N*-dimethyltryptamine and 5,6-dibromo-*L*-hypaphorine isolated from different marine sources showed both anti-cancer and anti-inflammatory activity ([Mollica et al., 2012](#)). Indurubin (a non-brominated minor pigment in Tyrian purple), which shows phagocytosis attenuating activity by preventing the increase of ROS in macrophages ([Man et al., 2012a](#)). Derivatives of indirubin have also shown anti-inflammatory activity in RAW264.7 macrophages and rat microglia. For RAW264.7

cells in particular, the indirubins were shown to inhibit the release of IL-6 and IL-1 β ([Kim and Park, 2012](#)). In addition, isatin, another non brominated indole derivative, has anti-inflammatory activity, as demonstrated in LPS and INF γ - stimulated RAW264.7 cells assay in which inhibition of NO, PGE2, iNOS, COX-2 and TNF α was demonstrated ([Matheus et al., 2007](#)). This data indicates the potential for anti-inflammatory activity being associated with the indole class of molecules and suggests that the full scope of bioactivity associated with the brominated indoles from Muricidae should be tested in future drug screening research.

1.8. Thesis aims and objectives

The general aim of this PhD thesis is to assess the anti-inflammatory activity of the Australian Muricidae *Dicathais orbita* and assess the potential for developing anti-inflammatory nutraceuticals or pharmaceuticals. The specific objectives for each chapter of this study are:

1. To review the existing literature on the anti-inflammatory activity of molluscs and their traditional use in anti-inflammatory preparations (Chapter 2).
2. Extraction of hypobranchial gland and egg masses from *D. orbita* for comparative chemical analysis and bioactivity screening (Chapter 3).
3. To test extracts and isolated compounds from *Dicathais orbita* for their ability to inhibit inflammatory responses in macrophages: cytotoxicity testing against mouse RAW264.7 cells, inhibition of NO and inhibition of TNF α (Chapter 3).
4. To test the effects of the extracts and compounds in inflammatory pathways in fibroblast cells cytotoxicity against T3 fibroblasts, inhibition of PGE2 (Chapter 3).
5. To compare the anti-inflammatory effect of synthetic indole analogues to establish specific structure-activity relationships of brominated indoles (Chapter 3).
6. Investigation of the *in vivo* anti-inflammatory activity of the extract and 6-bromoistain in a mouse model for acute lung inflammation (Chapter 4)
7. Characterisation of lipid extracts from the foot tissue and viscera of *D. orbita* (Chapter 5).

8. To test the lipid extracts from *D. orbita* and other seafoods for their cytotoxic effect on RAW 264.7 and 3T3 ccl-92 fibroblasts, and their impact on the NO and TNF α production in LPS-stimulated RAW 264.7 (Chapter 5).

1.9. Thesis structure

This thesis was prepared in fulfilment of the requirements for the degree of Doctor of Philosophy. The research was undertaken in collaboration with supervisors and some other researchers from the Southern Cross University, Lismore, New South Wales, Australia and the Centre for Health Sciences Research, University of Southern Queensland, Queensland, Australia.

This thesis is comprised of six chapters. All chapters, including the literature review have been prepared as manuscripts for submission to refereed journals, therefore, there is some inevitable repetition of information, particularly in the introduction and methods sections. The manuscripts have been reformatted for consistency throughout the thesis and the references and supplementary material are compiled into one list at the end of the thesis. Statements of authorship for the published manuscripts are provided in Appendix 14.

Chapter 2. Review of anti-inflammatory, immune-modulatory and wound healing properties of molluscs: This chapter aims to review the available literature on the ethnomedicinal use of molluscs and the *in vitro*, *in vivo* and human clinical trials performed on extracts and/or compounds from molluscs as relevant background to this study. This chapter has been published in **Journal of Ethnopharmacology**.

AHMAD, T. B., LIU, L., KOTIW, M. & BENKENDORFF, K. 2018. Review of anti-inflammatory, immune-modulatory and wound healing properties of molluscs. *Journal of Ethnopharmacology*, 210, 156-178.

Chapter 3. Anti-Inflammatory Activity and Structure- Activity Relationships of Brominated Indoles from a Marine Mollusc: This chapter describes the anti-inflammatory activity of different extracts and compounds purified from the Muricidae mollusc *Dicathais orbita* and in parallel

describes the structure-activity relationships of purified and synthetic brominated indoles. This chapter has been published in **Marine Drugs**.

AHMAD, T., RUDD, D., SMITH, J., KOTIW, M., MOUATT, P., SEYMOUR, L., LIU, L. & BENKENDORFF, K. 2017. Anti-Inflammatory Activity and Structure-Activity Relationships of Brominated Indoles from a Marine Mollusc. *Marine Drugs*, 15, 133.

Chapter 4. Brominated indoles from a marine mollusc inhibit inflammation in a murine model of acute lung injury: This chapter investigates the *in vivo* anti-inflammatory activity of the hypobranchial extract from the *D. orbita* along with 6-bromoistain, an abundant brominated indole derivative in this extract, in a murine acute lung inflammation model. This chapter has been published in **PLoS One**.

AHMAD, T. B., RUDD, D., BENKENDORFF, K., MAHDI, L. K., PRATT, K. A., DOOLEY, L., WEI, C. & KOTIW, M. 2017. Brominated indoles from a marine mollusc inhibit inflammation in a murine model of acute lung injury. *PLoS One*, 12, e0186904.

Chapter 5. Quality and fatty acid profile of lipid extracts from an under-utilised species *Dicathais orbita* and other seafood waste products as potential nutraceuticals for management of inflammation: This chapter describes the lipid composition and anti-inflammatory activity of different lipid extracts from *D. orbita*, with comparison to lipid extracts from the edible parts and waste products of some common seafood organisms. This chapter is prepared for publication in **Journal of Functional food**.

Chapter 6. General discussion and conclusion: This chapter highlights the main findings from this research and provides suggestions for future research priorities.

Chapter 2: Review of anti-inflammatory, immune- modulatory and wound healing properties of molluscs

Chapter 2 of this thesis incorporates the following publication:

AHMAD, T. B., LIU, L., KOTIW, M. & BENKENDORFF, K. 2018. Review of anti-inflammatory, immune-modulatory and wound healing properties of molluscs. *Journal of Ethnopharmacology*, 210, 156-178.

2. Chapter 2: Review of anti-inflammatory, immune-modulatory and wound healing properties of molluscs

2.1. Abstract:

This review focuses on traditional and contemporary anti-inflammatory uses of mollusc-derived products summarising all the *in vitro*, *in vivo* and human clinical trials that have tested the anti-inflammatory activity of molluscan natural products. Inflammatory conditions, burns and wounds have been an ongoing concern for human health since the early era of civilisation. Many texts from ancient medicine have recorded the symptoms, signs and treatment of many inflammation, burns and wounds conditions. These are well-documented in traditional European medicine, Traditional Chinese Medicine (TCM), Siddha and ancient Mediterranean and African traditional medicine and include a surprisingly large number of molluscan species.

An extensive review of the *Materia Medica* and scientific literature was undertaken using key word searches for “mollusc” and “anti-inflammatory” or “immunomodulatory” or “wound healing”. Molluscs have been used in ethnomedicine by many traditional cultures to treat different aspects of inflammatory conditions. We found 104 different anti-inflammatory preparations from a variety of molluscan species, of which 70 were from the well-documented Traditional Chinese Medicine (TCM). This traditional use of molluscs has driven the testing for inflammatory activity in extracts from some species in the phylum Mollusca, with 20 *in vitro* studies, 40 *in vivo* animal studies and 14 human clinical trials performed to substantiate the anti-inflammatory and wound healing activity of molluscs. Some of these studies have led to the approval of mollusc-derived products to be used as over-the-counter (OTC) nutraceuticals, like Lyprinol® and Biolane™ from the New Zealand green lipped mussel *Perna canaliculus*. Natural products provided important leads for the development of pharmaceuticals, including anti-inflammatory agents. Only a small proportion of the molluscan traditional medicines have been tested to confirm their anti-inflammatory activity and most screening

studies have tested crude extracts from molluscs without any chemical- characterisation. This highlights the need for further research to strategically identify the anti-inflammatory compounds in molluscan medicines to provide leads for novel anti-inflammatory drugs in future.

2.2. Introduction

Many natural products are sourced from the marine environment due to its phenomenal biodiversity. Marine invertebrates (Porifera, Echinodermata, Cnidarian, Mollusca, Arthropoda) have to date provided a substantial diversity of natural products, including terpenes, alkaloids, aliphatic hydrocarbons, steroids, carbohydrates, amino acids and peptides ([Leal et al., 2012](#)). These marine-derived natural products have an extensive array of therapeutic properties, including anticoagulant, antimicrobial, wound healing and immune modulating, antioxidant, anticancer, anti-inflammatory, antihypertensive, and other medicinal properties ([Senthilkumar and Kim, 2013](#), [Perdicaris, 2013](#)). A number of marine natural products have provided important leads for drug development and many are now used in the formulation of novel drugs ([Senthilkumar and Kim, 2013](#), [Riguera, 1997](#), [Leal et al., 2012](#)). For example, Ziconotide, first isolated from the cone snail *Conus magus* (Linnaeus, 1758) venom effectively blocks N-type voltage gated calcium channels ([Schroeder et al., 2004](#)) and is effective for the treatment of chronic pain. The drug has now been Food and Drug Administration (FDA) approved and has been commercialised under the name of Prialt® ([Atanassoff et al., 2000](#), [Svenson, 2013](#)).

The Mollusca is a phylum of marine invertebrates that are of particular interest as a source of new potential drugs leads. Molluscs encompass 7% of living animals on the planet making them the second largest animal phylum with estimated 100-200 thousand species of which more than 52 thousand have been described and named ([Benkendorff, 2010](#), [Bouchet and Duarte, 2006](#)). The phylum Mollusca also includes eight different classes: Gastropoda, Bivalvia, Scaphopoda, Cephalopoda, Polyplacophora, Monoplacophora, Caudofoveata and Solenogastres ([Benkendorff, 2010](#), [2008](#)) which illustrates a significant evolutionary divergence over the past 500 million years.

Associated with this vast biological diversity is significant chemical diversity, as molluscs use secondary metabolites to communicate and defend themselves against predators and pathogenic invaders ([Benkendorff, 2014](#)). As marine invertebrates, molluscs lack acquired immunity and essentially depend on their innate immunity and bioactive compounds to protect against microbial pathogens ([Hooper et al., 2007](#), [Dang et al., 2015](#)) and heal wounds in the microbially-rich marine environment.

Molluscs have been a significant focus in the search for biologically active secondary metabolites, with > 1,145 natural products isolated from molluscan species in the last three decades ([Benkendorff, 2010](#), [Benkendorff, 2014](#)). Two molluscan derived natural products have been clinically tested and approved by the Food and Drugs Administration (FDA); ziconotide from cone shells for the treatment of severe pain and Brentuximab vedotin for treatment of lymphoma and Hodgkin's disease ([Mayer et al., 2010](#)). There are at least 18 other compounds originally found in molluscs and associated cyanobacteria that are currently in clinical trials ([Mayer, 2017](#)). However, ~ 52 % of the molluscan natural products that have been isolated to date have never been tested for any biological activity ([Benkendorff, 2014](#)). Furthermore, < 1 % of known molluscan species have been studied for their secondary metabolites, although a large number of molluscan species have been used as a source of traditional medicines (Table 2.1 & 2.2), which has provided the stimulus for further research into the therapeutic potential of natural products derived from this phylum.

One of the most common therapeutic applications for molluscs in traditional ethnomedical uses appear to be associated with inflammatory conditions (Table 2.1). Inflammation is associated with and may contribute significantly to the pathogenesis of acute and chronic diseases such as atherosclerosis, obesity, multiple sclerosis, chronic obstructive pulmonary disease, asthma, rheumatoid arthritis, neurodegenerative disease and inflammatory bowel disease ([Nathan and Ding, 2010](#)). Inflammation can be described as the rapid response of the body to insults such as injury and infection. The inflammatory reaction is recognised macroscopically by four cardinal signs (which were described by Cornelius Celsus in the first century), that of *calor* (heat), *rubor* (redness), *tumor*

(swelling), *dolor* (pain) and loss of function ([Alessandri et al., 2013](#)). The process of inflammation generally includes the isolation and removal of the injurious stimuli such as damaged cells, chemical irritants and infection, as well as the initiation of the healing process ([De Zoysa, 2012](#)). More specifically the response is a spatially and temporally arranged episode in which cells and mediators collaborate to neutralise and eliminate the damaging stimuli, to allow the restoration of homeostasis ([Medzhitov, 2010](#), [Alessandri et al., 2013](#)). Although the inflammatory process promotes the elimination of damaging stimuli, the inflammatory process itself may also contribute to damage of neighbouring tissues and can in some cases increase the severity of pathology ([Cara et al., 2000](#), [Alessandri et al., 2013](#)).

Current treatments for inflammatory diseases are primarily based on the use of steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) ([Gunawardena et al., 2014b](#)), which is often reflective of the severity and responsiveness of the inflammation to the particular therapeutic regime. NSAIDs modulate their effect by preventing the synthesis of prostaglandins (PGs) via the inhibition of cyclooxygenase (COX) enzymes, which catalyse the conversion of arachidonic acid to PGs ([Auriel et al., 2014](#), [Seibert et al., 1997](#), [Vane and Botting, 1998](#)). However, current NSAID options have been linked to increased blood pressure, greatly increased risk of congestive heart failure, occurrence of thrombosis and they also can predispose patients to serious gastrointestinal erosion ([McMurray and Hardy, 2002](#), [Aisen et al., 2003a](#)). These side effects are common to almost all NSAIDs to some degree ([Vane and Botting, 1998](#)). Because anti-inflammatory drugs are among the most consumed pharmaceuticals, with over 70 million prescriptions and 30 billion tablets of NSAIDs sold over the counter each year ([Maroon et al., 2006](#)), there is an urgent need to search for safer sources of anti-inflammatory drugs. Steroidal anti-inflammatory drugs also have many disadvantages including immunosuppressing effects, as well as the resistance of many diseases to steroidal anti-inflammatory drugs ([Barnes, 2010](#), [Barnes, 2006a](#)). Natural products have traditionally provided important leads for the development of pharmaceutical drugs and there is evidence that they could be a potential

source of anti-inflammatory agents that could provide the benefit of greater activity and less side effects.

The aim of this review is to explore the traditional use of molluscs as a means for controlling inflammatory conditions, as well as critically analysing evidence from *in vitro* and *in vivo* studies, and human clinical trials, supporting the further investigation of molluscan derived extracts and natural products for anti-inflammatory, immune-modulatory and wound healing properties. This timely review of anti-inflammatory properties of molluscan natural products should help identify priority targets for future bioassay guided isolation and development of novel potential anti-inflammatory agents.

2.3. Materials and methods

An extensive review of the scientific literature on molluscs with anti-inflammatory activities, immunomodulatory and wound healing activities was undertaken by searching bibliographic databases: MEDLINE/PubMed, Scopus, Web of Knowledge and Google Scholar from 1960-2017. The keywords used in the search were ‘anti-inflammatory’ or ‘immunomodulatory’ or wound healing’ AND mollusc. The word “mollusc” was replaced with “Gastropod”, “Bivalve” or “Cephalopod” to capture additional papers that only used lower taxonomic terms.. References lists of published research articles were also checked for relevant data. Research articles were selected for inclusion if they tested an extract/s or compound/s isolated from species in the phylum Mollusca anti-inflammatory or wound healing activity. Studies using *in vitro* assay for anti-inflammatory activity included inhibition of reactive oxygen species (nitric oxide), oxidative enzymes (nitric oxygen synthase, lipoxygenase, cyclooxygenase), cytokines (tumor necrosis factor alpha, interleukins, nuclear factor beta kappa), immunoglobulin G or prostaglandins. *In vitro* papers testing for immune-modulation included phagocytosis assays using macrophages or neutrophils, and haemolysis using red blood cells, but papers that just screened for antibacterial activity were not included.

In vivo preclinical trials for anti-inflammatory activity were included if they used an antigen or adjuvant to stimulate the immune system in animal models for delayed-type hypersensitivity, paw oedema, ear oedema, experimentally induced arthritis, colitis, pyrexia, as well as trials on animals suffering from arthritis. Immuno-modulatory animal models were included that investigated carbon clearance or utilised immunisation followed by investigation of the immune response. Animal models for wound healing included hot-plate latency assay, skin burn and surgical wounds. Additional investigation of pain response was only included if contained within the same paper as any of the experiments listed above (e.g. tail immersion, hot plate and tail flick reaction time, induced writhing model). Research articles using mollusc extracts in human clinical trials were included for any form of arthritis and asthma, muscle injury and wound healing in burns patients.

Books and monographs, including review articles and regionally specific ethnomedical research articles (published in English) were used as a source of information about the historical use of molluscs in anti-inflammatory, immunomodulatory and wound healing medicines. The Chinese Compendium of *Materia Medica* was also searched for applications of mollusc in TCM and the Chinese Marine *Materia Medica* ([Guan and Wang, 2009](#)) was translated to English by the second author. English translations of the Arabic and Medieval Eastern Mediterranean *Materia Medica* ([Lev and Amar, 2008](#)) were also screened for any use of molluscs for conditions that could be related to inflammation.

Nomenclature of the mollusc species included in this review is corrected according to the World Register of Marine Species ([WoRMS Editorial Board, 2017](#)). All unaccepted names of species found in the literature have been replaced with the accepted names with reference to the name used in the original publication in footnotes.

2.4. Traditional use of molluscs for inflammatory conditions and wound healing

2.4.1. Overview

Molluscs have been involved in the everyday life of humans in many cultures throughout history, providing a source of shells, food, dyes and medicine ([Benkendorff, 2010](#)), in addition to the use of molluscs for magical-religious purposes ([Leo Neto et al., 2012](#)). Shelled molluscs are considered as healthy food and some species could be value-added as functional or medicinal foods, as they are still an important element in a variety of traditional natural medicines ([Benkendorff et al., 2015](#), [China State Administration Traditional Chinese *Materia Medica* Editorial Board, 1999](#), [Lev and Amar, 2008](#)). Molluscan natural medicines have been used in an extensive array of therapeutic applications such as for control of inflammation and in wound healing (Table 2.1 & 2.2).

2.4.2. Historical use of Molluscan natural products in ethnomedicine

Molluscs were an essential part of the ethnomedicine of many cultures throughout history. For example they were used to treat inflammation in Medieval Eastern Mediterranean, Ancient Greco-Roman, European, Zimbabwean, Indian and ancient Latin American people (Table 2.1). There is also evidence that different parts of molluscs were used as remedies to treat a range of inflammatory diseases. For example, the operculum from Strombidae and Muricidae were used in the eastern Mediterranean medicine to treat a range of conditions including skin diseases, stomach ulceration and arthritis by smelling the aromatic substances and the smoke of the slowly burned opercula ([Benkendorff et al., 2015](#), [Lev and Amar, 2008](#)) (Table 2.1), whilst in other parts of Europe the ash of the opercula was used to heal severed veins rather than smelling the smoke of the opercula ([Rätsch and Müller-Ebeling, 2013](#)). According to the medieval *Materia Medica* of eastern Mediterranean ([Lev and Amar, 2008](#)) (Table 2.1) snail shells have also been used by various cultures throughout the world to treat wounds in the stomach, arthritis and skin diseases. Similarly, the shell of the mollusc *Monetaria moneta* (Linnaeus, 1758) (Table 2.1) has been used as a treatment for a number of diseases including asthma in India ([Krishna and Singh, 2012](#)), whilst in Zimbabwe snail shells were used to treat topical ulcers ([Galfand et al., 1993](#)). The fisher people in the Bihar region of India also used

preparations from different parts of molluscs as a remedies for inflammation. For example, this group prepared a soup from the foot of the freshwater snail *Bellamya* sp. to treat asthma, arthritis, joint pain and rheumatism ([Prabhakar and Roy, 2009](#)). Although the specific parts or preparation methods for the molluscs used in Latin American ethnomedicines are not listed for each species, there is some evidence for the use of shells and powdered preparations for the treatment of asthma, skin ulcers, influenza, stomach pain, osteoporosis, pneumonia, cancer, pain relief and tuberculosis ([Alves and Alves, 2011](#)). There are at least 34 documented species of molluscs that were used in the ethnomedicine from different cultures as remedies for inflammation (Table 2.1).

Traditional Chinese Medicine (TCM) is one of the oldest and best documented systems of ethnomedicine in the world. It still has a significant impact on the healthcare system of the people of China and Chinese communities outside China ([Seong, 2015](#)). Marine creatures and their products represent a crucial part in the TCM. Molluscs in particular, significantly contribute to the anti-inflammatory remedies available in TCM (Table 2.2). Molluscs in TCM are used to treat variety of inflammatory conditions including eczema, menstrual disorders, non-resolving ruptured abscesses, osteoarthritis, trachoma to asthma, as well as for healing burns and scalds (Table 2.2). Natural product use of molluscs in TCM ranges from the use of the whole mollusc, shells, flesh, operculum, egg masses and even the pearl (Table 2.2). The mollusc shells are the most used part to treat inflammation with more than 50 preparations documented as useful anti-inflammatory remedies (Table 2.2). Other parts of mollusc which are often used as anti-inflammatory in TCM include flesh with flesh from 8 different species used (Table 2.2).

The range of different mollusc species used in TCM include four different classes with two species of Polyplacophora, three Cephalopoda, 31 Bivalvia and 34 Gastropoda. Veneridae spp. is the most common used bivalve group within anti-inflammatory preparations (Table 2.2). Applications include ingesting the decocted shell or applying the ground shell, which is used to treat fever, phlegm and cough, scrofula, ecthyma, eczema, scald, ruptured abscess resistant to healing, gastric and duodenal ulcer. The most frequently used gastropod species used in the TCM for anti-inflammatory preparation

were the muricids, also with 9 anti-inflammatory remedies, which also included ingesting decocted shells and applying the ustulated powdered shells. Muricidae treatments are used for fever, carbuncle, furuncle, otitis media, and ulcers of the lower limb, stomach and duodenum. Some preparations containing Muricidae flesh and operculua were used to treat chest and abdomen infection, pain, swelling and skin ulcers (Table 2.2). The ethnobiological history provides a repository of medicinal efficacy and experience as a sound source for further investigation. It is possible that scientific and evidence-based studies will provide a basis for the anecdotal reports on the use of traditional mollusc medicines and may help to substantiate their use.

Table 2.1: Traditional use of molluscs in therapeutic preparations relevant to inflammation and wound healing summarising remedies used from around the world from ancient to current times. NA = Not available.

Country/Region	Species and family ¹	Part used	Preparation	Therapeutic application	Reference
Gastropoda					
Medieval Eastern Mediterranean	Chicoreus ramosus (Linnaeus, 1758) ² Lentigo lentiginosus (Linnaeus, 1758) ³	Operculum	NA	Skin diseases; wounds in the stomach; arthritis; eye and ear diseases; treatment of uterus; diseases,	(Lev and Amar, 2008, Lev, 2007)
		Snail shell		Skin diseases, wounds in the stomach, arthritis	(Lev and Amar, 2008, Lev, 2007)
	Muricidae such as Chicoreus virgineus (Röding, 1798) ⁴	Operculum	Smell the aromatic substance or smoke produced while placing the operculum on slowly burning charcoal	Rheumatism or arthritis; stomach problem (wounds in stomach); skin diseases; eye and ear diseases; tumors; treatment of uterus diseases	(Lev and Amar, 2008, Lev, 2007)
Islamic Nations (Middle ages)	Aplysia depilans (Gmelin, 1791) (Aplysiidae)	NA	NA	Dyspnoea (breathing difficulties), dry cough, haemoptysis (coughing up blood)	(Meyerhof and Sobhy, 1932)
Ancient Greco-Roman (Dioscorides, Oribasius and Galen)	Hexaplex trunculus (Linnaeus, 1758), Bolinus brandaris (Linnaeus, 1758) and Stramonita haemastoma (Linnaeus, 1767) ⁵ (Muricidae)	Flesh and ashes of burned shell	Burned flesh along with shell	Wound healing; treatment of cracked skin; healing parotid gland swelling; anti-inflammatory properties	(Voultsiadou, 2010)
Ancient Greece and early Byzantium	Hexaplex trunculus (Linnaeus, 1758), Bolinus brandaris (Linnaeus, 1758) and Stramonita haemastoma (Linnaeus, 1767) ⁵ (Muricidae)	Flesh and shell	Flesh and ashes of burned shell	Anti-inflammatory; sore healing; menstrual cycle abnormalities	(Voultsiadou, 2010)
	Charonia tritonis (Linnaeus, 1758) (Ranellidae)	Shell	Ashes of burned shell	strengthens body's immune system; sore and wound healing	(Voultsiadou, 2010)

		Shell	Burned shell with salt	Remedy for burns and scalds	(Voultsiadou, 2010)
		Shell	Shells burned with salt	Remedy for burns and scalds	(Voultsiadou, 2010)
India	Filopaludina sp. (Habe, 1964) (Viviparidae)	Foot	soup prepared from the snail's foot	Asthma; arthritis; joint pain; rheumatism	(Prabhakar and Roy, 2009)
	Filopaludina bengalensis (Lamarck, 1822) ⁶ (Viviparidae)	Whole snail	Snails are collected from pond and are kept in clean fresh water in an earthen pot for night and the water is used like eye drop.	Conjunctivitis	(Prabhakar and Roy, 2009)
	Monetaria moneta (Linnaeus, 1758) ⁷ (Cypraeidae)	Shell	Burned shell powder	Asthma	(Krishna and Singh, 2012)
Latin America	Pomacea lineata (Spix, 1827) (Ampullariidae)	NA	NA	Asthma, boils, ulcers	(Alves and Alves, 2011)
North east Brazil	Littoraria angulifera (Lamarck, 1822) ¹⁰ (periwinkle snail) (Littorinidae)	Flesh	Ingestion of the cooked flesh; ingestion of the crude flesh	Chesty cough	(Alves and Rosa, 2007a)
Jeju Island, Korea	Omphalius rusticus (Gmelin, 1791) (Tegulidae)	Shell	Topical application of shell powder	Knee pain	(Kim and Song, 2013)
Europe	Not specified	Operculum	Ashes of calcified operculum	Healing cut veins	(Rätsch and Müller-Ebeling, 2013)
Zimbabwe	Not specified	Hozhwa (Snail) shells	Powdered shell heated and applied directly	Treatment for tropical ulcers	(Galfand et al., 1993)
Bivalvia					
Ancient Greece	Chlamys (Röding, 1798) spp. and Pecten spp. (O. F. Müller, 1776) (Pectinidae)	Flesh	(fresh or preserved in salt)	Cystitis	(Voultsiadou, 2010)

Ancient Greece and early Byzantium	Mytilus galloprovincialis (Lamarck, 1819) (Mytilidae)	Shell	Ashes of burned shell	Sores and wounds	(Voultsiadou, 2010)
	Ostrea edulis (Linnaeus, 1758) (Ostreidae)	Shell	Ashes of burned and pulverized shell mixed with honey	Treatment of wounds and sores	(Voultsiadou, 2010)
India	Black-lip pearl Oyster (Pinctada margaritifera (Linnaeus, 1758)) (Pteriidae)	Pearl	Ashes of pearl with honey	Asthma and phthisis (tuberculosis)	(Gopal et al., 2008)
Latin America	Crassostrea rhizophorae (Guilding, 1828) (Ostreidae)	Shell	Powdered	Osteoporosis; pneumonia; stomach ache; flu; pain relief; tuberculosis	(Alves and Alves, 2011, Alves and Rosa, 2007b)
	Neoteredo reynei (Bartsch, 1920) (Teredinidae)	Shell	Powdered	tuberculosis	(Alves and Alves, 2011)
	Lyrodus pedicellatus (Quatrefages, 1849) ⁸	Shell	Powdered	Tuberculosis	(Alves and Alves, 2011)
	Anomalocardia flexuosa (Linnaeus, 1767) ⁹ (Veneridae)	Shell	Powdered	Asthma; flu; stomach ache	(Alves and Alves, 2011)
	Megalobulimus oblongus (Müller, 1774) (Strophocheilidae)	Shell	Powdered	Asthma	(Alves and Alves, 2011)
	Cassis tuberosa (Linnaeus, 1758) (Cassidae)	Shell	Powdered	Asthma	(Alves and Alves, 2011)
North east Brazil	Crassostrea rhizophorae (Guilding, 1828) (Ostreidae)	Flesh and shell	Ingestion of powdered shell with food; ingestion of the cooked flesh	Osteoporosis	(Alves and Rosa, 2007a)

	<i>Anomalocardia flexuosa</i> (Linnaeus, 1767) ⁹ (clam) (Veneridae)	Flesh and shell	Ingestion of powdered shell with food; ingestion of the cooked flesh	Asthma	(Alves and Rosa, 2007a)
	<i>Mytella guyanensis</i> (Lamarck, 1819) (Mytilidae)	Flesh and shell	Ingestion of powdered shell with food; ingestion of the cooked flesh	Osteoporosis	(Alves and Rosa, 2007b)
Central Chaco, Argentina	<i>Anodontites trapesialis</i> (Lamarck, 1819) (Mycetopodidae)	Shell	Shell ash applied externally	Skin wounds and injuries	(Martinez and Barboza, 2010)
Jeju Island, Korea	<i>Mytilus unguiculatus</i> (Valenciennes, 1858) ¹¹ (Mytilidae)	Whole animal	Ingestion of decocted whole animal	Fever	(Kim and Song, 2013)
Cephalopoda					
Ancient Greece and early Byzantium	<i>Octopus vulgaris</i> (Cuvier, 1797) (Octopodidae)	Flesh	Boiled or roasted flesh	many gynaecological diseases and conditions	(Voultsiadou, 2010)
North east Brazil	Cephalo. <i>Loligo</i> sp. (Lamarck, 1798) (squid) (Loliginidae)	Shell	Tea made from boiling the shell	Asthma	(Alves and Rosa, 2007a)

1. The species name is only listed when a single species is identified for use in the traditional medicine. All those only listed at the family level use multiple species within the family. Species are based on the accepted name in the World Register of Marine Species ([WoRMS Editorial Board, 2017](#)).
2. Listed in the publication as *Murex inflatus* (Lamarck, 1822)
3. Listed as *Strombus lentiginosus* (Linnaeus, 1758)
4. Listed as *Murex anguliferus* (Lamarck, 1822)
5. Listed as *Thais haemastoma* (Linnaeus, 1767)
6. Listed as *Bellamyia bengalensis* (Lamarck, 1822)
7. Listed as *Cypraea moneta* (Linnaeus, 1758)
8. Listed as *Teredo pedicellata* (Quatrefages, 1849)
9. Listed as *Anomalocardia brasiliiana* (Gmelin, 1791)
10. Listed as *Littorina angulifer* (Lamarck, 1822)
11. Listed as *Mytilus coruscus* (Gould, 1861)

Table 2.2: Traditional Chinese Medicines from molluscs as listed in the Chinese Marine Materia Medica (Guan and Wang, 2009) for use in conditions related to inflammation and/or wound healing.

Chinese Medicine	CLASS/ Family ¹	Part used	Preparation	Therapeutic application
Gastropoda				
<i>Bao Yu</i>	Haliotidae	flesh	Boil and eat the flesh or decoct and ingest (fresh: 6-9g each time or sun-dried: 15-50g each time)	Hectic fever; menstrual disorders
<i>Jia Qi</i>	Nacellidae	shell	Decoct and ingest (10-15g each time)	Conjunctive congestion with swelling and pain, scrofula (inflammation of lymph nodes)
<i>Jia Xiang</i>	Turbinidae	operculum	Decoct and ingest (5-15g each time), pulverised and homogenised with water for oral taking (3-9g),	Abdominal swelling and pain
<i>Zhui Luo Yan</i>	Turritellidae	operculum	Decoct and ingest (15-30g each time)	Conjunctive congestion with swelling and pain
<i>Shi She</i>	Vermetidae (<i>Thylacodes adamsii</i> (Mörch, 1859)) ²	shell and flesh	Decoct and ingest (5-15g each time), pulverised and homogenised with vinegar to apply externally	Carbuncle and swelling
<i>Xie Shou Luo</i>	Potamididae (<i>Pirenella cingulata</i> (Gmelin, 1791)) ³	shell and flesh	Decoct and ingest (5-15g each time)	Stomatitis (inflammation of the mouth); recurrent aphthous ulcer; gingivitis (swelling and aching of gums)
<i>Feng Luo Ke</i>	Strombidae	shell	Decoct and ingest (15-25g each time)	Tuberculosis of lymph node; gastric and duodenal ulcer; furuncle carbuncle
<i>Yu Luo Ke</i>	Naticidae	shell	Decoct and ingest (15-50g each time)	Tuberculosis of lymph nodes; gastric and duodenal ulcer; furuncle carbuncle
<i>Zi Bei</i>	Cypraeidae	shell	Decoct and ingest (15-25g each time)	Pyretic dysentery; children with fever; heat-toxicity ; conjunctive congestion with swelling and pain; acute and chronic sinusitis (inflammation of sinuses with pus and blood)
<i>Bai Bei</i>	Cypraeidae	shell	Decoct and ingest (5-15g each time)	Typhoid fever; heat-toxicity; pyretic dysentery; acute and chronic sinusitis; chancre (syphilus lesion) and vulval sore
<i>Rou Se Bao Bei</i>	Cypraeidae (<i>Lyncina carneola</i> (Linnaeus, 1758)) ⁴	shell	Ground into powder, decoct and ingest (5-15g each time)	Heat-toxicity; pyretic dysentery; acute and chronic sinusitis; high fever
<i>Yan Qiu Bei</i>	Cypraeidae (<i>Erosaris erosa</i> (Linnaeus, 1758))	shell	Ground into powder, decoct and ingest (5-15g each time)	Phlegm; tuberculosis of lymph nodes; conjunctive congestion with swelling and pain

<i>Guan Luo Ke</i>	Harpidae, Cassidae	shell	Ground into powder, decoct and ingest (15-50g each time)	Tuberculosis of lymph nodes; gastric and duodenal ulcer
<i>Chun Luo Ke</i>	Tonnidae	shell	Ground into powder or Ustulate (scorch) the shell, decoct and ingest (15-30g each time)	Tuberculosis of lymph nodes
<i>Pi Ba Luo Ke</i>	Ficidae	shell	Ground into powder, decoct and ingest (15-25g each time)	Children with fever; night sweating; chronic tracheitis
<i>Qian Xian Luo Ke</i>	Ranellidae, Personidae	shell	Ground into powder, decoct and ingest (15-50g each time)	Furuncle carbuncle; tuberculosis of lymph nodes; gastric and duodenal ulcer
<i>Wa Luo Ke</i>	Bursidae (<i>Bufo rana</i> Linnaeus, 1758) ⁵	shell	Decoct and ingest (15-50g each time)	Furuncle carbuncle; gastric and duodenal ulcer
<i>Gu Luo</i>	Muricidae (<i>Murex</i> spp., <i>Nassa francolina</i> Bruguière, 1789)	shell	Decoct the shell and ingest. Ustulate the shell, ground into powder and apply externally.	Clear heat; carbuncle; furuncle; otitis medium and ulcer of lower limb.
<i>Liao Luo</i>	Muricidae (<i>Mancinella</i> Link, 1807; <i>Thais</i> Röding, 1798; <i>Purpura</i> Bruguière, 1789) spp.)	shell	Decoct the shell (15–50 g) and ingest; used for making pills or medicinal powder; Ustulate the shell, ground into powder and apply externally.	Clear heat; swelling and ulcer on the body surface and scrofula
<i>Ji Luo</i>	Muricidae (<i>Chicoreus ramosus</i> Linnaeus, 1758)	shell	Decoct the crushed shell; Ustulate (scorch) the shell, ground into powder and apply externally.	Clear heat; scrofula (infection of the lymph nodes); Stomach and duodenal ulcer
<i>Hai Luo</i>	Muricidae (<i>Rapana</i> Schumacher, 1817) spp.)	flesh	Boil and eat the flesh	Chest and abdomen heat and pain
<i>Hai Luo Ke</i>	Muricidae (<i>Rapana</i> spp.)	shell	Decoct the shell and ingest, used as medicinal powder; Ustulate the shell, ground into powder, mixed with sesame oil and apply externally.	Stomach and duodenal ulcer; scrofula
<i>Hai Luo Yan</i>	Muricidae (<i>Rapana</i> spp.)	operculum	Decoct the operculum (10–20 g) and ingest; Ustulate the shell, ground into powder and apply externally.	Swelling and ulcer on the body surface
<i>He Ji Luo</i>	Muricidae (<i>Chicoreus brunneus</i> Link, 1807)	shell	Decoct the shell and ingest.	Scrofula
<i>Hong Luo</i>	Muricidae (<i>Rapana rapiformis</i> Born, 1778)	shell	Decoct the shell (15–25 g) and ingest.	Scrofula

<i>La Luo</i>	Muricidae (<i>Indothais gradate</i> (Jonas, 1846) ⁶ , <i>Reishia luteostoma</i> (Holten, 1803)) ⁷	shell	Decoct the crushed shell (15–25 g)	Clear heat; scrofula; phlegm and cough; swelling and ulcer on the body surface
<i>E Luo</i>	Buccinidae, Nassariidae	shell	Ground into powder and ingest, or decoct and ingest (15-25g each time); Ustulate (scorch) the shell and ground into powder and apply externally.	Gastric and duodenal ulcer; common skin infections; furuncle carbuncle
<i>Xiang Luo</i>	Buccinidae (<i>Neptunea cumingii</i> (Crosse, 1862)), Nassariidae (<i>Phos senticosus</i> (Linnaeus, 1758))	shell	Decoct and ingest (15-25g each time); apply externally	Tuberculosis of lymph nodes; gastric and duodenal ulcer; scald
<i>Jiao Luo Yan</i>	Melongenidae	operculum	Ground into powder, decoct and ingest (5-15g each time); apply externally	Aching and limp; ulcer of lower limb
<i>Jiao Luo Ke</i>	Melongenidae	shell	Ustulate (scorch) the shell, ground into powder and ingest	Gastric and duodenal ulcer
<i>Fei Luo Ke</i>	Olividae	shell	Decoct and ingest (20-50g each time)	Hectic fever
<i>Xi Lei Luo Ke</i>	Fasciolaridae	shell	Decoct and ingest (15-30g each time)	Phlegm; gastric and duodenal ulcer
<i>Yu Luo Ke</i>	Conidae	shell	Decoct and ingest (15-25g each time); Ustulate (scorch) the shell, ground into powder and ingest (3-6g)	Tuberculosis of lymph; gastric and duodenal ulcer
<i>Hai Fen</i>	Alpsysiidae	egg masses	Decoct and ingest (30-60g each time);	Tuberculosis of lymph nodes
Bivalvia				
<i>Wa Leng Zi</i>	Arcidae, Noetiidae, Limidae	shell	Decoct and ingest (9-15g each time); ground into powder for infusion (1.5-3g); Ustulate (scorch) the shell and ground into powder and apply externally.	Phlegm in hypochondrium; ulcerative gingivitis; bleeding wound; burn and scald
<i>Qing Han</i>	Arcidae (<i>Barbatia obliquata</i> (Wood, 1828))	shell	Decoct the crushed shell and ingest(15–50 g each time)	Menstrual disorders; gastric and duodenal ulcer
<i>Bi Na Han</i>	Arcidae (<i>Anadara inaequalis</i> (Bruguiere 1789)) ⁸	shell	Decoct the crushed shell and ingest (15–50 g each time)	Tuberculosis of lymph; gastric and duodenal ulcer
<i>Ban Niu Zhuan Han</i>	Arcidae (<i>Trisidos semitorta</i> (Lamarck, 1819))	shell	Decoct the crushed shell and ingest(15–50g each time)	Gastric and duodenal ulcer

<i>Jiang Yao Ke</i>	Pinnidae	shell	Decoct and ingest (15–25g each time)	Eczema
<i>Zhen Zhu</i>	Pteriidae	pearl	Ground into powder and ingest (0.3-1g each time) or apply externally.	Conjunctive congestion; ulcers in the mouth and on the tongue; sores and ulcers; ruptured abscess resistant to healing; burn; scald
<i>Zhen Zhu Mu</i>	Pteriidae	prismatic layer, hypostracum	Ground into powder and apply externally.	Ulcer and pyogenic infections, eczema and pruritus
<i>Ding Li</i>	Malleidae	shell	Ground into powder and apply externally (5-15g each time).	Eczema; furuncle and phyma
<i>Qian Ge</i>	Pteriidae (<i>Isognomon</i> spp.)	adductor muscle	Decoct and ingest (15–25g each time); or ground into powder and ingest	High fever; tuberculosis of lymph nodes; gastric and duodenal ulcer
<i>Hai Yu</i>	Placunidae, Anomiidae	flesh and shell	Boil flesh and ingest, ground shell into powder for infusion and ingest	Eczema; arthrosis like crane knees/osteoarthritis (flesh only)
<i>Mu Li</i>	Ostreidae, Grypaeidae	shell	Ground into powder and apply externally	Eczema and ulcer
<i>Mu Li</i>	Ostreidae, Grypaeidae	flesh	Smash flesh and apply externally	Tuberculosis of lymph nodes; swelling and aching of gum
<i>Man Yu Ge Ke</i>	Lucinidae, Carditidae, Semelidae, Glossidae	shell	Ground into powder and ingest, or decoct and ingest.	Tuberculosis of lymph nodes; gastric and duodenal ulcer; scald
<i>Ke</i>	Mactridae (<i>Maetra chinensis</i> (Philippi, 1846))	shell	Decoct the crushed shell and ingest(25–50g each time), ground into fine powder and apply externally	Gastric and duodenal ulcer; keratitis (inflammation of the cornea); skin ulcer
<i>Ge Li Fen</i>	Mactridae, Astartidae	Shell	Decoct the and ingest(50–100g each time), ground into powder and ingest or apply externally	Gastric and duodenal ulcer; retention of phlegm and asthmatic cough; carbuncle and swelling; scrofula; eczema; scald
<i>Xi Shi She</i>	Mactridae (<i>Maetra antiquata</i> (Spengler, 1802) ⁹ , Psammobiidae (<i>Haitula diphos</i> (Linnaeus, 1771) ¹⁰)	Shell	Ustulate (scorch) the shell, decoct and ingest.	Tuberculosis of lymph nodes
<i>Ying Ge Ke</i>	Tellinidae	shell	Ustulate (scorch) the shell, ground into powder and ingest	Tuberculosis of lymph nodes, gastric and duodenal ulcer
<i>Fu Ge Ke</i>	Donacidae	shell	Ustulate (scorch) the shell, decoct and ingest.	Tuberculosis of lymph nodes, gastric and duodenal ulcer

<i>Dou Fu Ge Ke</i>	Donacidae (<i>Donax faba</i> (Gmelin, 1791))	shell		Decoct and ingest (15-50g each time)	Tuberculosis of lymph nodes
<i>Zi Yun Ge</i>	Psammobiidae	shell		Decoct and ingest (15-50g each time)	Tuberculosis of lymph nodes, gastric and duodenal ulcer
<i>Ge Ke</i>	Veneridae	shell		Decoct the crushed shell and ingest(10–15g each time), ground into powder and apply externally	Phlegmatic heat and cough; scrofula; ecthyma; eczema; scald; ruptured abscess resistant to healing
<i>Fu Wen Ge Ke</i>	Veneridae (<i>Meretrix lamarckii</i> (Deshayes, 1853))	shell		Decoct and ingest(6–15g each time), ground into powder and apply externally	Scrofula; eczema; scald
<i>Jiang Hu Bu Mu Ge</i>	Veneridae (<i>Leukoma jodoensis</i> (Lischke, 1874)) ¹¹	shell		Decoct and ingest(10–15g each time), ground into powder and apply externally	Ecthyma
<i>Ju Chi Ba Fei Ge</i>	Veneridae (<i>Protapes gallus</i> (Gmelin, 1791)) ¹²	shell		Decoct and ingest(10–15g each time)	Scrofula
<i>Hai Ge Ke</i>	Veneridae	shell		Decoct and ingest(10–25g each time), ground into powder and apply externally	Scrofula; asthma; eczema; scald; furuncle
<i>Ge Zai</i>	Veneridae (<i>Ruditapes</i> spp.)	Flesh and shell		Boil and eat flesh, decoct and ingest, ustulate (scorch) the shell, ground into powder and apply externally	Ecthyma; asthmatic cough
<i>Ying Ke Ge</i>	Veneridae (<i>Mercenaria mercenaria</i> (Linnaeus, 1758))	Shell		Decoct and ingest (10–15g each time)	Dyspnea (breathlessness) with cough; scrofula; gastric and duodenal ulcer
<i>Ke Li Jia Fu Ge</i>	Veneridae (<i>Gafrarium dispar</i> (Holten, 1802))	shell		Decoct and ingest (5–15g each time)	Gastric and duodenal ulcer
<i>He Ai Ba Fei Ge</i>	Veneridae (<i>Paphia amabilis</i> (Philippi, 1847))	shell		Decoct and ingest (10–15g each time)	Phlegmatic heat and cough; tuberculosis of lymph nodes; gastric and duodenal ulcer
<i>Lv Lang Ke</i>	Glauconomidae (<i>Glauconome chinensis</i> (Gray, 1828))	shell		Decoct and ingest (15–50g each time)	Tuberculosis of lymph nodes; gastric and duodenal ulcer
<i>Sha Hai Lang</i>	Myidae (<i>Mya arenaria</i> (Linnaeus, 1758))	shell		Decoct and ingest (15–30g each time)	Tuberculosis of lymph nodes; gastric and duodenal ulcer
Cephalopoda					

<i>Qiang Wu Zei</i>	Sepiidae	flesh	Boil and eat flesh	Rheumatic lumbago; ulcer of lower limb; ulcerative carbuncle; furuncle and phyma
<i>Hai Piao Xiao</i>	Sepiidae	Cuttlebone	Decoct and ingest, ground into powder and apply externally	Stranguria with turbid discharge; eczema; skin ulcer with pus; ruptured abscess resistant to healing; trachoma (chronic inflammation of the mucous membranes of the eyes)
<i>Zhang Yu</i>	Octopodidae	flesh	Decoct and ingest, smash and apply externally	Sore and pyogenic infections; ruptured abscess resistant to healing
Polyplacophora				
<i>Hai Shi Bie</i>	Acanthochitonidae (<i>Acanthochiton rubrolineata</i> (Lischke, 1873))	whole	Ground into powder and infusion, or make capsule or tablet for oral taking (2-6g each time)	Asthma; pulmonary tuberculosis
<i>Cuo Shi Bie</i>	Ischnochitonidae	whole	Ground into powder and infusion for oral taking (1-3g each time)	Asthma; pulmonary tuberculosis

1. The species name is only listed when a single species is identified for use in the Chinese medicine. All those only listed at the family level use multiple species within the family. Species are based on the accepted name in the World Register of Marine Species ([WoRMS Editorial Board, 2017](#)).
2. The original genus quoted in China Marine Herbal is *Serpulorbis imbricatus* (Dunker, 1860).
3. Listed as *Cerithidea cingulate* (Gmelin, 1791)
4. Listed as *Cypraea carneola* (Linnaeus, 1758)
5. Listed as *Bursa rana* (Linnaeus, 1758)
6. Listed as *Thais gradata* (Jonas, 1846)
7. Listed as *Thais luteostoma* (Holten, 1803)
8. Listed as *Scapharaca binakayanensis* (*Arca binakayanensis* Faustino, 1932)
9. Listed as *Coelomactra antiquate* (Spengler, 1802)
10. Listed as *Sanguinolaria diphos* (Linnaeus, 1771)
11. Listed as *Prorothaca jedoensis* (Lischke, 1874)
12. Listed as *Paphia gallus* (Gmelin, 1791)

2.5. *In vitro* studies on the anti-inflammatory and immunomodulatory activity of molluscan natural products

2.5.1. Overview

The prevalent use of molluscan natural products in ethnomedicine has drawn the attention of scientists in the last few decades to test defined molluscan products in the laboratory to verify the activity. There have been 13 different studies (mostly in the last 10 years) on the *in vitro* anti-inflammatory activity of gastropod molluscs (Table 2.3). On the other hand, only seven studies have focused on just two different species of bivalve molluscs (Table 2.3).

2.5.2. Gastropoda

Muricidae feature in a number of records of traditional medicines (Table 2.1) and are also well known for their bioactive secondary metabolites (see recent review by [Benkendorff et al. \(2015\)](#), which include the brominated indole precursors to the dye Tyrian purple ([Cooksey, 2001](#), [Benkendorff, 2013](#)). This dye is dominated by 6,6 dibromoindigo, although the purple secretion from some muricids has been shown to contain a mixture of both brominated and non-brominated indigo and indirubin ([Cooksey, 2001](#)). Indirubin has been found to prevent the increase of reactive oxygen species (ROS) from macrophages ([Man et al., 2012b](#)). Brominated derivatives of indirubin have also shown anti-inflammatory activity in RAW264.7 and rat microglia cell culture ([Kim and Park, 2012](#)). With RAW264.7 cells in particular, indirubin was reported to inhibit the release of inflammatory cytokines interleukin (IL) IL-6 and IL-1 β ([Kim and Park, 2012](#)). In addition, isatin, an oxidation product produced during Tyrian purple formation and a precursor of indirubin, has also been shown to have anti-inflammatory activity, as demonstrated in a lipopolysaccharide (LPS) and interferon gamma (IFN γ)-stimulated RAW264.7 model by: inhibiting the production of nitric oxide (NO); prostaglandin 2 (PGE₂); inducible nitric oxide synthase (iNOS); cyclooxygenase 2 (COX-2) and tumor necrosis factor-alpha (TNF α) ([Matheus et al., 2007](#)). Recent studies have confirmed that extracts of the hypobranchial glands and egg masses of the Australian Muricidae *Dicathais orbita* (Gmelin, 1791), along with the brominated indole precursors of Tyrian purple effectively inhibit NO, PGE₂,

TNF α and the translocation of the prototypical proinflammatory signalling molecule nuclear factor kappa B (NF κ B) ([Ahmad et al., 2017b](#)). The oxidation product 6-bromoisatin is of particular interest as a bioavailable multifunctional compound with demonstrated efficacy and safety in animal models for colon cancer prevention ([Esmaeelian et al., 2014](#)), in addition to preventing acute lung inflammation in a recent mouse model after oral gavage (unpublished data). ([Benkendorff et al., 2015](#)) Indole anti-cancer drug leads have regularly shown promise for the treatment of a range of diseases including inflammation ([Gul and Hamann, 2005](#)). However, these indole compounds are only found in the hypobranchial glands and reproductive material of Muricidae ([Benkendorff et al., 2015](#)) and are present in trace amounts (if at all) in the operculum, flesh and the shells, which are the parts mostly used in traditional anti-inflammatory applications (Table 2.1).

2.5.3. Bivalvia

Bivalves provide a source of food in many cultures, as well as being a source of natural remedies. These molluscs are rich in polyunsaturated fatty acids (PUFAs) which are 'healthy' lipids and reportedly found to have anti-inflammatory activity. For example, New Zealand green lipped mussel (*Perna canaliculus*) extracts have been the focus of much scientific research on their anti-arthritic activity, following observation that the coastal Maori population who consumed this mussel regularly suffered less from arthritis than the inland population ([Halpern, 2000](#), [Sankaran and Mouly, 2007](#)). These observations lead to a series of formal studies that ultimately resulted in a range of lipid extracts from this mussel being commercialised and that are now available over the counter, including the clinically tested anti-inflammatory nutraceutical called Lyprinol®. An *in vitro* study was performed to assay the ability of Lyprinol to inhibit the production of leukotrienes and 5-hydroxyeicosatetraenoic acid (5-HETE) by human neutrophils ([Halpern, 2000](#)). In this study, human neutrophils were sourced from human blood and incubated with increasing concentrations of Lyprinol, followed by stimulation with arachidonic acid (AA) and calcium ionophore, to trigger the 5-lipoxygenase (5-LOX) inflammatory pathway. The results showed significant inhibition of leukotrienes and 5-HETE synthesis. The NZ green lipped mussel extract (GMLE) has been shown

to contain eicosatetraenoic acid (ETA), and omega-3 fatty acid that inhibits AA oxygenation by both the COX and LOX pathways ([Bierer and Bui, 2002](#)). A comparative study was also performed in Australia by [Cheras et al. \(2005\)](#) examining the activity of Biolane™ (another GMLE) and other well-known anti-arthritis agents such as chondroitin sulphate, glucosamine sulphate and Lyprinol. The study compared the ability of these anti-arthritis agents to inhibit different osteoarthritic mechanisms ranging from cholesterol biosynthesis inhibition, PGE inhibition, inhibition of COX-2 expression, TNF α inhibition, oxygen radical absorbance capacity-antioxidant (ORAC), anti-platelet aggregation activity and fibrinolytic activity. All the agents tested were initially digested with pepsin and pancreatic enzymes to simulate the *in vivo* digestive processes. Biolane showed broad activity with positive inhibitory results in all of the assays compared to the other tested anti-arthritis agents ([Cheras, 2005](#), [Cheras et al., 2005](#)). Lyprinol® was also found to inhibit PGE₂, cholesterol biosynthesis, phospholipase A₂ (PLA₂) and platelet aggregation ([Cheras, 2005](#), [Cheras et al., 2005](#)). These observations provided evidence supporting the oral use of the GMLE for control of some inflammatory conditions.

There are only few studies that have identified the bioactive compounds involved in anti-inflammatory activity from mollusc extracts and consequently there is a need for more bioassay guided fractionation and structure elucidation to determine the mode of action of these compounds. A large proportion of the *in vitro* studies listed in Table 2.3 have tested methanol, acetone or ether extracts although a few studies have tested fatty acids extracts and only one study has tested the anti-inflammatory activity of a polypeptide from *Anadara kagoshimensis* (Tokunaga, 1906) in lipopolysaccharide (LPS)-stimulated RAW264.7 ([Wu et al., 2014](#)). Consequently, there is need for further characterisation of methanol/ether extracts as they may lead to the identification of further active ingredients and provide lead molecules for development of novel anti-inflammatory drugs.

Table 2.3: *In vitro* anti-inflammatory, wound healing and immune-modulatory activity of extracts and compounds isolated from molluscs. The type of activity in specific assays is explained with the statistically significant concentrations relative to the negative controls (solvent or delivery agent).

Species	Geographic distribution	Extract or compound	Type of assay and concentrations used	Biological activity	Statistically significant concentrations	Reference
Gastropoda						
<i>Haliotis discus hannai</i> (Ino, 1953) (disk abalone)	Eastern Asia (including Japan)	Extract from <i>H. discus hannai</i> fermented with <i>C. militaris</i> mycelia (HFCM-5)	LPS- stimulated RAW264.7 macrophages treated with 0.05, 0.1 or 0.2 mg/mL	a) Inhibited the production of NO in RAW264.7 macrophages. b) Decreased TNF α and IL-6 in a dose-dependent manner.	0.05, 0.1 or 0.2 mg/mL	(Joung et al., 2014)
		Abalone intestine digest	LPS- stimulated RAW264.7 macrophages treated with 50, 100, 250 or 500 μ g/mL of the intestine digest	a) Supressed the production of NO via iNOS. b) Reduced the generation of TNF α , IL-6, IL1 β .	50, 100, 250 or 500 μ g/mL	(Qian et al., 2012)
<i>Haliotis diversicolor</i> (Reeve, 1846)	China	Shell powder	a) LPS-stimulated RAW264.7 treated with 1, 2, or 5 mg/mL of shell powder to evaluate the iNOS expression. b) Phagocytosis of FITC coated beads by RAW264 treated with treated with 1, 2, or 5 mg/mL of shell powder.	a) Decreased (iNOS) expression. b) Enhanced the functions of macrophages.	1, 2 and 5 mg/mL	(Chen et al., 2016)
<i>Neverita didyma</i> (Röding, 1798) ¹ (Röding, 1798) (the bladder moon snail)	Mozambique South Africa Madagascar	Acetone and methanol extracts of whole body	Human red blood cell (HRBC) membrane stabilisation method. 0.5 mL of 10 % v/v HRBC suspension added to 3 mL of saline or distilled water medium before treated with 0.2, 0.4, 0.6, 0.8, or 1.0 mL	Both acetone and methanol extracts decreased the percentage of haemolysis with the acetone extract exhibited higher activity than methanol extract.	0.2, 0.4, 0.6, 0.8, and 1.0 mL	(Ravi et al., 2012)
<i>Volegalea cochlidium</i> (Linnaeus, 1758) ²	Indo-West Pacific	Acetone and methanol extracts of whole body	Human red blood cell (HRBC) membrane stabilisation method. 0.5 mL of 10 % v/v HRBC suspension added to 3 mL of saline or distilled water medium before	Both acetone and methanol extracts decreased the percentage of haemolysis with the acetone extract exhibited higher activity than methanol extract.	0.2, 0.4, 0.6, 0.8, and 1.0 mL	(Ravi et al., 2012)

			treated with 0.2, 0.4, 0.6, 0.8, or 1.0 mL			
	Indo-West Pacific	The whole body ether extract	Tested for effects on <i>Candida albicans</i> phagocytosis by human neutrophils using slide method. The human neutrophils monolayer treated with 50, 100, 250, 500, 750, 1,000, 2,000, 5,000 or 10,000 ng of the ether body extract.	a) Stimulated the phagocytosis in low concentrations. b) Suppressed the phagocytosis in higher concentration <i>in vitro</i> .	NA	(Ponkshe and Indap, 2002)
<i>Euchelus asper</i> (Gmelin, 1791)	Indo-West Pacific	The whole body ether extract	Tested for effects on <i>Candida albicans</i> phagocytosis by human neutrophils using slide method. The human neutrophils monolayer treated with 50, 100, 250, 500, 750, 1,000, 2,000, 5,000 or 10,000 ng of the ether body extract.	a) Stimulated the phagocytosis in low concentrations. b) Suppressed the phagocytosis in higher concentration <i>in vitro</i> . c)	NA	(Ponkshe and Indap, 2002)
<i>Filopaludina bengalensis</i> (Lamarck, 1822) ³	India	Footpad lipid extract	LPS-stimulated macrophage treated with 20 or 40 µg/mL	a) Inhibited reactive oxygen species (ROS), TNFα, and NO production. b) Inhibited the NFκB p65 translocation.	20 and 40 µg/mL	(Bhattacharya et al., 2014)
<i>Aplysia fasciata</i> (Poiret, 1789)	Gulf of Mexico Mediterranean Sea North Atlantic Ocean Red sea	Fatty Acids	a) LPS- Stimulated RAW 264.7 treated with 15.6 -250 µg/mL of fatty acids from <i>A. fasciata</i> . b) Inhibition of lipoxigenase measured by non-cellular enzymatic assay based on linoleic acid. Concentrations of fatty acids used were: 2-4 mg/mL.	a) Decreased NO levels. EC ₅₀ 77 ± 7 µg/mL b) Inhibited the lipoxigenase. EC ₅₀ 3.06 ± 0.22 µg/mL	a) EC ₅₀ 77 ± 7 µg/mL b) EC ₅₀ 3.06 ± 0.22 µg/mL	(Pereira et al., 2015)
<i>Aplysia punctata</i> (Cuvier, 1803)	Mediterranean Sea North Atlantic Ocean Red sea	Fatty Acids	a) LPS- Stimulated RAW 264.7 treated with 15.6 -250 µg/mL of fatty acids from <i>A. punctata</i> . b) Inhibition of lipoxigenase measured by non-cellular	a) Decreased NO levels. EC ₅₀ 72 ± 12 µg/mL b) Inhibited the lipoxigenase EC ₅₀ 1.75 ± 0.09 mg/mL	a) EC ₅₀ 72 ± 12 µg/mL b) EC ₅₀ 1.75 ± 0.09 mg/mL	(Pereira et al., 2015)

			enzymatic assay based on linoleic acid. Concentrations of fatty acids used were: 2-4 mg/mL.			
<i>Dicathais orbita</i> (Gmelin, 1791)	Australia	Chloroform extract of the hypobranchial gland	<ul style="list-style-type: none"> a) LPS-stimulated RAW264.7 treated with 0.08, 0.4, 2, 10 or 50 µg/mL b) Calcium ionophore stimulated 3T3 ccl-92 fibroblast treated with 0.08, 0.4, 2, 10 or 50 µg/mL 	<ul style="list-style-type: none"> a) Inhibited the production of NO EC₅₀: 30.8 µg/mL. b) Downregulated the production of TNFα in RAW264.7 EC₅₀: 43 µg/mL. c) Inhibited the translocation of NFκB in RAW264.7. d) Downregulated the production of PGE2 in 3T3 fibroblasts EC₅₀: 34.2 µg/mL. 	<ul style="list-style-type: none"> a) 10 and 50 µg/mL for NO assay. b) 0.4, 2, 10 and 50 µg/mL for TNFα assay. c) 0.08, 0.4, 2, 10 and 50 µg/mL for PGE2 assay. d) 40 µg/mL in NFκB translocation assay 	(Ahmad et al., 2017b)
		6-bromoisatin	<ul style="list-style-type: none"> a) LPS-stimulated RAW264.7 treated with 0.08, 0.4, 2, 10 or 50 µg/mL b) Calcium ionophore stimulated 3T3 ccl-92 fibroblast treated with 0.08, 0.4, 2, 10 or 50 µg/mL 	<ul style="list-style-type: none"> a) Inhibited the production of NO EC₅₀: 30.8 µg/mL. b) Downregulated the production of TNFα in RAW264.7 EC₅₀: 43 µg/mL. c) Inhibited the translocation of NFκB in RAW264.7. d) Downregulated the production of PGE2 in 3T3 fibroblasts EC₅₀: 34.2 µg/mL. 	<ul style="list-style-type: none"> a) 0.4, 2, 10 and 50 µg/mL in NO assay. b) 0.08, 0.4, 2, 10 and 50 µg/mL in TNFα assay. c) 0.08, 0.4, 2, 10 and 50 µg/mL in PGE2 assay. d) 40 µg/mL in NFκB translocation assay. 	(Ahmad et al., 2017b)

<i>Muricidae</i>		indirubin, a minor pigment in Tyrian purple and indirubin derivatives	<p>a) LPS- Stimulated RAW 264.7 treated with.</p> <p>b) LPS-stimulated primary rat brain microglia 0.5, 1, 2, or 4 μM of Indirubiω-3'-oxime.</p>	<p>a) Indirubin suppressed the effect of extracellular ATP on macrophages</p> <p>b) Indirubiω-3'-oxime derivatives inhibited the release of IL-1β, TNFα and IL-6.</p> <p>c) Indirubiω-3'-oxime inhibits inflammatory activation of rat brain microglia.</p>	<p>Indirubin : NA.</p> <p>Indirubiω-3'-oxime :</p> <p>a) 4 μM for NO.</p> <p>b) 2 μM for TNFα, IL-1β, IL-6 and PGE2.</p> <p>c) 2 and 4 μM for NFκB.</p>	(Man et al., 2012b , Kim and Park, 2012 , Jung et al., 2011)
Bivalvia						
<i>Perna canaliculus</i> (Gmelin, 1791) (New Zealand green-lipped mussel)	New Zealand	Lyprinol (stabilised supercritical fluid fraction)	<p>a) Arachidonic acid and calcium ionophore-stimulated human polymorphonuclear (PMN) leukocytes.</p> <p>b) LPS-stimulated human macrophages.</p>	<p>a) Inhibited the production of leukotrien B-4 by human PMN.</p> <p>b) Unfractionated lipid extract inhibited the production of PGE2 by human macrophages EC₅₀ 1.2 μg/mL.</p>	NA	(Whitehouse et al., 1997)
		an homologous series of novel omega 3 polyunsaturated fatty acids (omega-3 PUFA)	<p>a) Arachidonic acid and calcium ionophore-stimulated human neutrophils treated with three dilutions of HPLC fractions 1:100, 1:1,000 or 1:10,000.</p>	Inhibited lipoxygenase products of the Arachidonic acid pathway.	Three fractions showed apparent inhibition at: 1:100, 1:1,000 and 1:10,000.	(Treschow et al., 2007)
		Biolane™ GLME	Biolane GLME digested with pepsin and pancreatic enzyme tested for its activity to prevent different Osteoarthritic mechanism	<p>a) Inhibited the biosynthesis of cholesterol, COX2, TNFα and PGE.</p> <p>b) Antioxidant activity.</p> <p>c) Anti-platelet aggregation activity.</p> <p>d) Fibrinolytic activity.</p>	NA	(Cheras, 2005 , Cheras et al., 2005)
		freeze dried green-lipped mussel powder	<p>a) LPS-stimulated human THP-1 monocytes treated with increasing concentrations of</p>	<p>a) Inhibited TNFα and IL-12p40 production in THP-1.</p>	<p>a) 0.1 and 1 mg/mL for TNFα and IL-12p40.</p>	(Lawson et al., 2007)

		(Perna®)	<p><i>Perna</i> extract: 0.0001, 0.001, 0.01, 0.1 or 1 mg/mL.</p> <p>b) Treated the peripheral blood neutrophils with increasing (100, 200 or 400 µg/mL) concentrations of <i>Perna</i> extract to measure superoxide burst.</p>	b) Reduced neutrophil superoxide burst activity <i>in vitro</i> .	b) 100, 200 and 400 µg/mL for superoxide inhibition.	
		hydrochloric acid extract of the freeze-dried powder of <i>Perna canaliculus</i>	<p>a) IgG antibody modulation V2E9 hybridoma cells treated with containing 0 µg, 5 µg, 10 µg, 15 µg, 20 µg, and 25 µg protein).</p> <p>b) Cytokine bioassays using V2E9, THP-1, L-929, U-937, A375.S2, Jurkat E6-1, EL-4, CTLL-2, LS174T, and 7TD1 cell lines.</p>	Decreased IgG production.	<p>a) For IgG suppression: 10 µg, 15 µg and 20 µg</p> <p>b) For cytokines bioassays: 5 µg, 10 µg, 15 µg,</p>	(Mani and Lawson, 2006)
		Tween-20 extract of the freeze-dried powder of <i>Perna canaliculus</i>	<p>a) IgG antibody modulation V2E9 hybridoma cells treated with containing 0 µg, 5 µg, 10 µg, 15 µg, 20 µg, and 25 µg protein).</p> <p>b) cytokine bioassays using V2E9, THP-1, L-929, U-937, A375.S2, Jurkat E6-1, EL-4, CTLL-2, LS174T, and 7TD1 cytokine bioassays cell lines</p>	<p>a) Decreased IgG production</p> <p>b) Decreased the production of IL-2 and IL-6</p> <p>c) Inhibited COX-1 and COX-2 cyclooxygenase activity</p>	5 µg, 10 µg, 15 µg, 20 µg and 25 µg	(Mani and Lawson, 2006)
<i>Anadara kagoshimensis</i> (Tokunaga, 1906) ⁴	north-western Pacific	a novel polypeptide fraction (P2)	LPS-induced RAW264.7 treated with 15, 50 or 150 µg/mL of P2 fraction for NO inhibition assay	a) Inhibited the production of NO in LPS-stimulated RAW264.7 macrophage.	<p>a) NO: 150 µg/mL.</p> <p>b) IL-6: 4 and 12 µg/mL.</p>	(Wu et al., 2014)

			and 1.33, 4 or 12 µg/mL for cytokines assays.	b) Inhibited the secretion of IL-6 and TNFα in human cervical cancer HeLa cells. c) Downregulated the IL-8 d) Inhibited the COX-2 and iNOS-related pathways.	c) TNFα: 1.33, 4 and 12 µg/mL. d) 12 µg/mL for IL-8, COX-2 and iNOS.	
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1. Listed in the publication as *Natica didyma* (Röding, 1798)
2. Listed as *Hemifusus pugilinus* (Born, 1778)
3. Listed as *Bellamyia bengalensis* (Lamarck 1822)
4. Listed as *Arca subcrenata* (Lischke, 1869) (Ark shell)

2.6. *In vivo* animal models using molluscan extracts for wound healing and inflammatory diseases

2.6.1. Overview

The promising anti-inflammatory results of molluscs are not only demonstrated in *in vitro* assays, but in fact the *in vivo* results are even more promising. Many studies in the last few decades have focused on *in vivo* anti-inflammatory activity of mollusc extracts in different animal models (Table 2.4). Out of the 40 *in vivo* studies on anti-inflammatory activity of the molluscs listed, there were 18 studies focused on gastropods, 18 on 8 different species of bivalves, and only 4 studies on cephalopods (Table 2.4). Extracts from the NZ green lipped mussel (*Perna canaliculus*) were the most examined using *in vivo* assays (Table 2.4).

2.6.2. *In vivo* anti-inflammatory models

Carrageenan is an inflammatory agonist standard as it induces paw oedema and is the most commonly used technique to screen the anti-inflammatory responses in animal models ([Winter et al., 1962](#)). This inflammatory agent is commonly used in experimental pharmacology because it causes inflammation by inducing histamine, 5-hydroxytryptamine (5-HT) and prostaglandin (PG). This model has been used to test anti-inflammatory agents *in vivo* on 10 different mollusc extracts and all showed positive anti-inflammatory activity (Table 2.4). Some other animal models have also been used to test the anti-inflammatory activity of mollusc extracts and compounds e.g. rodent models for arthritis including adjuvant-induced and collagen-induced arthritis. These models allow study of arthritis *in vivo* by measuring many arthritis-specific factors as well as general inflammatory factors. Wound healing from skin burns and cuts models were also used to assess the wound-healing activity where a defined diameter temperature controlled skin heater or sterilised blades are used to induce local cutaneous injuries on the dorsum skin. In addition, delayed hypersensitivity models where rodents are injected with an antigen-adjuvant have been used to induce delayed-type hypersensitivity demonstrating many measurable inflammatory factors. The immunomodulation is also measured using the carbon clearing model in rat/mice. In this model rat/mice are injected with a carbon particle suspension days after the

administration of test compound/extract. Small aliquots of venous blood drawn periodically are lysed with acetic acid before the amount of carbon in the blood is measured colourimetrically at 660 nm which indicates the phagocytic activity (Table 2.4) ([Ponkshe and Indap, 2002](#)). Furthermore, animal models have been used to test the anti-pyretic activity of mollusc extracts. In this model, rodents are injected with a certain amount of yeast mixture to stimulate inflammation and consequently high temperature and the anti-pyretic activity is assessed by measuring the temperature of the animal. Analgesic activity has also been tested *in vivo* in a well-established animal model where the animal is triggered by a thermal or physical stimulus to show sensitivity of animals to the stimulus used, indicating the degree of pain. All these are well-established models to study anti-inflammatory activity and have been used to investigate the *in vivo* activity of mollusc extracts across a wide range of different doses (Table 2.4).

Following on from the promising *in vitro* anti-inflammatory results with use of *Perna canaliculus* extracts, *in vivo* studies have been conducted to examine if these findings could be replicated in animal models using green-lipped mussel extract (GLME) (Table 2.4). [Miller and Ormrod \(1980\)](#) reported that intraperitoneal (i.p.) injection of a crude GLME significantly reduced the foot pad oedema in a carrageenan-induced oedema model in rats. Years later, in 1993, the same research group tested a glycogen extract from the same mollusc using the rat model, although but this time they used intravenous (i.v.) infusion of the extract and reported a significant reduction in the size of the foot pad oedema and suppression of neutrophil sequestration to the site of inflammation ([Miller et al., 1993](#)). In another study, [Lawson et al. \(2007\)](#) found that prophylactically treating Wistar rats with freeze dried GLM powder (*Perna*) effectively reduced the incidence, onset, and severity of collagen-induced arthritis. In addition, furan fatty acids derived from the GLM were also found to inhibit adjuvant-induced arthritis in Wistar rats in a dose dependent manner ([Wakimoto et al., 2011](#)). In another study to assess the effect of stabilisation of the lipid extracts on their activity, rats were subjected to experimentally-induced inflammatory swelling before feeding with stabilised or non-stabilised GLME for 4 days (the days 10-13). The rats fed GLME stabilised with tartaric acid showed complete

inhibition (100%) of swelling compared to use of non-stabilised GLME, which inhibited the swelling by 14%. This study demonstrated the importance of tartaric acid stabilisation of the lipid extracts which was observed to extend, as well as enhance the anti-inflammatory activity of the GLME ([Halpern, 2000](#)).

In vivo studies on a lipid extract from the Indian green mussel *Perna viridis* (Linnaeus, 1758) have shown that this related freshwater species, also contains anti-inflammatory activity, with the lipid extract significantly reducing carrageenan-induced oedema in rats. The extract was subsequently commercialised as anti-inflammatory product under the name of CadalminTM GMe (contains powder of freeze dried *P. viridis* as an active ingredient) ([Chakraborty, 2012](#), [Chakraborty et al., 2013b](#), [Chakraborty et al., 2013a](#)). Aqueous extract from the footpad of the Indian fresh water bivalve *Lamellidens marginalis* (Lamarck, 1819) also showed a significant healing effect when fed to male albino rats with adjuvant-induced arthritis (AIA). The treated rats showed reduced paw oedema, paw weight and ankle diameter, and the bivalve extract significantly restored the levels of serum IL1 β , IL6, CINC1, TNF α , IL10 and lysosomal enzyme levels, as well as reducing the levels of neutrophil infiltration ([Chakraborty et al., 2010](#)).

The anti-inflammatory properties or bioactive extracts from fresh water snails are also of interest to natural product researchers. The supernatant of homogenised tissue derived from the fresh water snail *Filopaludina bengalensis* (Lamarck, 1822) was used in an *in vivo* study to examine anti-osteoporotic and anti-osteoarthritic activity ([Sarkar et al., 2013](#)). Osteoarthritic and osteoporotic Wistar male/female albino rats received the *F. bengalensis* extract orally for 15 days. The results suggested a significant improvement in urinary (hydroxyproline/glucosamine/calcium/phosphate/creatinine) and serum parameter (serum acid phosphatase/alkaline phosphatase/Tartrate Resistant Acid Phosphatase (TRAP), calcium/creatinine, and cytokines (TNF α /IL-1 β /Cytokine-Induced Neutrophil Chemoattractant-1 (CINC-1) in rats that received VBE ([Sarkar et al., 2013](#)), supporting the anti-osteoarthritis activity of the fresh water molluscs. Further purification of the high molecular weight protein fraction (VB-P4) from the flesh extract of this fresh water gastropod also demonstrated

significant anti-osteoporosis activity in an experimentally induced osteoporotic model in rats. The results showed significant decrease in the calcium, creatinine and phosphate in urine and decreased levels of calcium and creatinine in serum, in addition to significantly decreased levels of TNF α , IL-12, IL-6 and PGE2 ([Sarkar et al., 2015b](#)). Another protein fraction of the aqueous flesh extract from the same mollusc (VB-P5) demonstrated anti-osteoarthritic, anti-nociception, anti-inflammatory activity in Wistar albino rat models. Feeding the rats on the purified fraction (VB-P5) prevented ankle/knee swellings, decreased the urinary glucosamine, calcium, phosphorous, and creatinine levels, suppressed the serum ACP, ALP and TRAP enzymes, as well as serum calcium and creatinine, decreased the levels of TNF α , IL-12, IL-12 IL-1 β and PGE2, reduced the size of paw and ear oedema and increased the reaction time of hot plate and tail flick model ([Sarkar et al., 2015a](#)). All these results support the anti-inflammatory activity of this Indian fresh water snail.

Cephalopods are not an exception when it comes to the anti-inflammatory activity of molluscs. Several studies have been carried out to support the anti-inflammatory properties of cephalopod products (Table 2.4). For example, assays of ink extract from *Sepia officinalis* (the common cuttlefish) demonstrated anti-inflammatory activity in Swiss mice by inhibiting the acetic acid-induced writhing and increasing the latency period of mice on the hot-plate, with the LD₅₀ above 2000mg/Kg i.p. ([Soliman and Fahmy, 2013](#)). Another study using *Sepia pharaonis* (Pharaoh Cuttlefish)-derived liver oil showed a major reduction in both carrageenan-induced and formalin-induced paw oedema ([Joseph et al., 2005](#)). *Ommastrephes bartramii* (Lesueur, 1821) Neon flying squid) also suppressed the enhanced capillary permeability and inhibited both leukocyte emigration and protein exudation into the pouch fluid in granuloma pouch model in rats ([Mimura et al., 1987](#)) (Table 2.4). The results of these studies provide evidence to support the anti-inflammatory activity of cephalopods, in addition to gastropods and bivalves.

2.6.3. *In vivo* wound healing assays

The *in vivo* wound healing properties of mollusc extracts have also been studied following evidence associated with the traditional use of some species. Lipid and amino acid extracts from mussels in the genus *Mytilus* and Muricidae gastropods in the genus *Rapana* (Schumacher, 1817) have been reported to enhance wound healing in rodent skin burn models ([Badiu et al., 2008](#), [Badiu et al., 2010](#)) (Table 2.4). The anti-pyretic and wound healing properties of the processed shell of *Monetaria moneta* (Linnaeus, 1758) were confirmed in a study undertaken by [Immanuel et al. \(2012\)](#). The processed shell was administered orally to albino rats injected with a yeast mixture to stimulate inflammation. The rats showed rapid recovery to normal body temperature (36°C) 3h post treatment compared to untreated controls which remained at 38.5°C for up to 5h. To test wound healing activity, the powder of the shell was used as an ointment over the 2 cm wound in thigh region and the scar formed in 8 days, compared to the control rats which showed very minor improvement in the wound (from 2cm to 1.6cm) even after 9 days ([Immanuel et al., 2012](#)). The results were highly indicative of the effective wound healing properties associated with the shell of a gastropod commonly used in traditional medicines (Table 2.1). Lipid extracts from the muricid *Rapana venosa* (Valenciennes, 1846) have been also demonstrated to significantly improve the healing of induced skin burns in Wistar rats, by reducing the healing time by 10 days compared to the control untreated rats ([Badiu et al., 2008](#)). The *in vivo* data revealed improved neoformation of the hypodermal, epidermal and dermal layers of the skin in a rat skin burns model ([Badiu et al., 2008](#)). The lipid extract used in the assay contained a mixture of polyunsaturated fatty acids, vitamin E, sterol and aromatic compounds ([Badiu et al., 2008](#)), although the most active compound/s that improved healing was not identified. Amino acid extracts from *Rapana venosa* were also reported to accelerate healing time in a burned skin in Wistar rats by improving the neoformation of dermal and epidermal tissue layers ([Badiu et al., 2010](#)). The bivalve *Mytilus galloprovincialis* (Lamarck, 1819) also demonstrated significant wound healing properties in the same model at the same effective dose ([Badiu et al., 2010](#)). These studies support the wound-healing properties associated with the flesh of Muricidae, although in traditional use, the flesh was

typically burned prior to use and it remains uncertain whether that burnt flesh or shells would retain the bioactive compounds.

Table 2.4: *In vivo* anti-inflammatory, wound healing and immune-modulatory activity of extracts and compounds isolated from molluscs in animal models. The active dose is used to describe the doses at which a statistically significant effect was observed relative to the negative controls.

Species	Geographic distribution	Extract compound or	Type of animal model	Biological activity	Active dose	Reference
Gastropoda						
<i>Helix pomatia</i> (Linnaeus, 1758) (terrestrial snail)	Local to Central and Southeast Europe but now moved by human to Asia and the Americas	hemocyanin (HpH)	Female 8-week old Balb/c mice immunised with 16 µg, 40 µg, and 100 µg/mouse HpH combined with tetanus toxoid (TT) (i.p.)	a) Increased number of anti-TT IgG producing plasmocytes b) Induced a significant increase of B and T cell proliferation.	a) 16 µg, 40 µg and 100 µg/mouse b) 100 µg/mouse	(Gesheva et al., 2015)
<i>Filopaludina bengalensis</i> (Lamarck, 1822) ¹	India	Footpad lipid extract	Antigen-adjuvant induced delayed-type hypersensitivity	a) Decreased paw oedema. b) Decreased NO level, serum TNFα level and CINC 1 level c) Decreased splenic CD4 ⁺ /CD8 ⁺ ratios. d) Increased the level of T _{reg} cells.	20 and 40 µg/mL	(Bhattacharya et al., 2014)
		Extra-pallial fluid	a) Carrageenan (s.c.) – induced Paw Oedema in male Wistar rats (150-160 g body weight) received 1.8, 3.6 or 7.2 µg/g, body weight, of extract orally. b) Freund’s adjuvant (into the sub plantar pad)-induced Poly-arthritis in Wistar rats received the same doses above. c) Eddy’s Hot Plate Method on male Swiss mice (25-30 g body weight) received oral dose of 3.6, 7.2 or 14.4 µg/mL of the extract.	a) Lowered paw inflammation in carrageenan-induced rats and in Freund’s adjuvant-induced rats. b) Showed analgesic dependency in Eddy’s hot plate and tail immersion in mice.	a) 1.8, 3.6 and 7.2 µg/g in. b) 1.8, 3.6, 7.2 and 14.4 µg/g.	(Adhikari et al., 2015)

			d) Tail Immersion Method on Swiss mice received oral dose of 3.6, 7.2 or 14.4 µg/mL of the extract.			
<i>Haliotis diversicolor</i> (Reeve, 1846)	China	Shell powder	full-thickness burn injury in male Wistar rat treated topically with 0.2 mL of 600 mg/mL or 200 mg/mL daily	a) Decreased neutrophil infiltration. b) Promoted wound healing noticeably after 14 days and was mostly healed on day 28. The wound	600 mg/mL and 200 mg/mL	(Chen et al., 2016)
<i>Filopaludina bengalensis</i> (Lamarck, 1822) ²	India	10% aqueous flesh homogenate (VBE)	a) Bilateral ovariectomy-induced osteoporotic and bacterial collagenase injection-induced osteoarthritic Wistar rats were fed orally with 1g/kg or 2 g/kg VBE for 15 days.	a) Prevented ankle/knee swellings. b) Decreased the urinary markers. c) Suppressed the serum enzymes and cytokines (TNFα and IL-1β).	1g/kg and 2 g/kg	(Sarkar et al., 2013)
		Purified protein fraction VB-P4 from the aqueous flesh extract	Experimentally induced-osteoporotic model in Wistar female albino rats treated with an i.p. dose of 400 µg/100g or 200 µg/100g VB-P4 for 15 days.	a) Restored the bone structural architecture. b) Decreased the urinary glucosamine, calcium, phosphorous, and creatinine levels. c) Suppressed the serum ACP, ALP and TRAP enzymes as well as serum calcium and creatinine. d) Decreased the levels of TNFα, IL-12, IL-1β and PGE2.	400 µg/100g and 200 µg/100g	(Sarkar et al., 2015b)

		Purified protein fractions VB-P4/VB-P5 from the aqueous flesh extract	<ul style="list-style-type: none"> a) Bacterial collagenase induced- osteoarthritic model in Wistar male albino rats. b) Hot plate and tail flick model in male Swiss mice received 400 µg/100g of VB-P4 or VB-P5i.p.. c) Xylene induced ear oedema in male Swiss mice received 400 µg/100g of VB-P4 or VB-P5 i.p.. d) Carrageenan-induced paw oedema in male Swiss mice received 400 µg/100g of VB-P4 or VB-P5 i.p.. 	<ul style="list-style-type: none"> a) Prevented ankle/knee swellings. b) Decreased the urinary glucosamine, calcium, phosphorous, and creatinine levels. c) Suppressed the serum ACP, ALP and TRAP enzymes as well as serum calcium and creatinine. d) Decreased the levels of TNFα, IL-12, IL-1β and PGE2. e) Reduced the size of paw and ear oedema. f) Increased the reaction time of hot plate and tail flick model. 	Both fractions VB-4 and VB-5 were 400 µg/100g.	(Sarkar et al., 2015a)
<i>Tectus tentorium</i> (Gmelin, 1791) ³	India	100% acetone fraction of crude extract purified by silica gel column	<ul style="list-style-type: none"> a) Analgesic effect in Swiss mice hot plate model and acetic acid-induced writhing model treated with 25 or 50 mg/kg body weight orally (p.o) of the extract. b) Carrageenan-induced rat paw oedema on albino rats treated with 25 or 50 mg/kg (p.o) of the extract. 	<ul style="list-style-type: none"> a) Inhibited acetic acid-induced abdominal constrictions. b) Inhibited the writhing. c) Decreased paw thickness. 	25 and 50 mg/kg	(Chellaram et al. 2012)
		100% acetone fraction of crude extract purified by silica gel column	Carrageenan-induced paw oedema in rats received 100 and 200mg/kg (p.o.).	Decrease the paw thickness.	100 and 200mg/kg	(Chellaram and Edward, 2009b)

<i>Drupella margariticola</i> (Broderip, 1833) ⁴	Indian ocean; Japan; Madagascar; Seychelles; Tanzania	100% acetone column purified extract	Carrageenan-induced paw oedema in rats received (p.o.) dose of 50 or 100 mg/kg of the extract.	Decreased in the paw thickness.	50 and 100 mg/kg	(Chellaram and Edward, 2009a)
<i>Babylonia zeylanica</i> (Bruguère, 1789)	India	Benzene:methanol extracts	a) Carrageenan induced paw oedema in Wistar albino rats received orally 100 or 200 mg/kg of the extract. b) Tail-immersion method for estimation of pain. c) Yeast induced Pyrexia.	a) Reduced the paw oedema in rats. b) Decreased the pain in analgesic activity. c) Reduced the Yeast induced Pyrexia raised body temperature.	a) 100 and 200 mg/kg. b) 200 mg/kg. c) 100 and 200 mg/kg.	(Santhi et al., 2012)
<i>Rapana venosa</i> (Valenciennes, 1846) ⁵ (veined rapa whelk)	Western Pacific Ocean (and invasive in some other regions in the northern hemisphere)	Hemocyanin (RtH)	Mice immunised multiple times with RtH incorporated with influenza A hemagglutinin (i.p.). each mice received 250, 100 or 40 µg of RtH.	Induce strong humoral immune response and could be used as adjuvant.	40, 100 and 250 µg/mouse	(Tchorbanov et al., 2008)
	Western Pacific Ocean (and invasive in some other regions in the northern hemisphere)	amino acids	Skin burns in Wistar rats treated with amino acids at a concentration of 0.3 mg/kg twice a day.	Accelerate skin wounds healing via enhancement of dermal and epidermal neoformation	0.3 mg/kg	(Badiu et al., 2010)
		Lipid extract	Skin burns in Wistar rats treated topically with 0.2 mg/kg lipid extract twice a day.	very efficient in healing induced skin burns	0.2 mg/kg	(Badiu et al., 2008)
<i>Fissurella latimarginata</i> (G. B. Sowerby I, 1835)	Pacific ocean	Hemocyanin (FLH)	C57BL/6 female mice were immunized with 200 µg of FLH and then with FLH coupled with 2, 4-dinitrofluorobenzene (DNFB) subcutaneously (s.c.).	Produced high robust specific humoral immune response: a) Increased IFN-γ and higher numbers of tumour-infiltrating CD4+ lymphocytes.	200 µg/mice	(Arancibia et al., 2014)

				b) Rapidly increased the generation of IL-6, IL-12, IL-23 and TNF α by dendritic cells.		
<i>Purpura persica</i> (Linnaeus, 1758)	India	100% chloroform purified extracts (in 0.5% (W/v) suspension of Sodium Hydroxide)	a) Carrageenan-induced oedema in Wistar albino rats treated with 100 and 200mg/kg, p.o. purified chloroform extract. b) Tail-immersion method. c) Brewer's yeast-induced pyrexia in albino rats received 100 and 200mg/kg, (p.o.)	a) Reduced carrageenan induced oedema. b) Reduced the pain in analgesic activity. c) Reduced the high temperature of the yeast-infected rats. d) No toxicity up to 800mg/Kg dose.	a) 100 and 200mg/kg. b) 100 and 200mg/kg. c) 100 and 200mg/kg.	(Santhi et al., 2011)
<i>Euchelus asper</i> (Gmelin, 1791)	Indo-West Pacific	The whole body ether extract	a) Carbon clearance methods in Swiss albino mice. b) Delayed type Hypersensitivity (DTH) reaction. c) Plaque Forming Cell (PFC) assay.	a) Immunosuppressive activity <i>in vivo</i> . b) Stimulated DTH. c) Reduced the number of plaque forming cells.	40, 80 and 160 mg/kg	(Ponkshe and Indap 2002 , Akerkar et al. 2009)
<i>Volegalea cochlidium</i> (Linnaeus, 1758) ⁶	Indo-West Pacific	The whole body ether extract	a) Carbon clearance methods in Swiss albino mice received 40, 80 or 160 mg/kg of the extract (i.p.). b) Delayed type Hypersensitivity (DTH) reaction. c) Plaque Forming Cell (PFC) assay.	a) Immunosuppressive activity <i>in vivo</i> . b) Increased the plaque forming cells.	40 and 80 mg/kg in carbon clearance method.	(Ponkshe and Indap 2002 , Akerkar et al. 2009)
Bivalvia						

<i>Perna canaliculus</i> (Gmelin, 1791) (New Zealand green-lipped mussel)	New Zealand	Furan fatty acid (F6) (active component of Lyprinol)	Adjuvant-induced arthritis in Wistar rats received 1, 5 or 10 mg/kg F6 for 5 days.	Showed dose dependent inhibition of adjuvant-induced arthritis in Wistar rats	10 mg/kg	(Wakimoto et al. 2011)
		A crude fraction of the New Zealand GLM	A carrageenan-induced inflammatory oedema of the rat hind footpad. These rats received 500 mg/kg of the crude preparation (i.p.) or (p.o.).	Effectively reduced the footpad oedema but only if injected (i.p.).	500 mg/kg	(Miller and Ormrod 1980)
		Lyophilized <i>Perna canaliculus</i> powder (Perna®)	Collagen-induced arthritis (CIA) in female Wistar rats received 100 mg/kg/day of Perna.	Significant reductions in disease incidence, onset, and severity of CIA in rats.	100 mg/kg/day	(Lawson et al., 2007)
		Freeze-dried, stabilized GLM powder	Dogs suffering from arthritis were fed GLM powder (>34 kg weighed dogs received 1000 mg/day 34–25 kg received 750 mg/d; <25 kg received 450 mg/d).	Significantly reduced : a) Total arthritic scores. b) Scores for joint pain and joint swelling.	a) For >34 kg weighed dogs = 1000 mg/day b) 34–25 kg = 750 mg/d; c) <25 kg = 450 mg/d.	(Bierer and Bui 2002)
		Glycogen extracts	Carrageenan induced foot pad oedema in female Dark Agouti rats received 5, 10, 15, 20 or 25 mg (i.v.) injection of the glycogen extract.	a) Decreased foot pad oedema. b) Reduced the infiltration of neutrophils.	5, 10, 15, 20 and 25 mg/rat	(Miller et al., 1993)
		Super fluid extracted, lipid-rich extract of Lyprinol®	Adjuvant-induced polyarthritis in Wistar and Dark Agouti rats.	Inhibited arthritis development.	5 mg/rat	(Whitehouse et al. 1997)

			Collagen (II)-induced autoallergic arthritis in Wistar and Dark Agouti rats. The rats received 5 mg/rat Lyprinol (p.o.).			
		GLM lipid extract (Seatone)	Adjuvant-induced Arthritis in female Wistar rats received 300 mg/kg of Seatonone.	a) Reduced the thickness of rear paw b) Reduced the inflammatory score in the fore paw	300 mg/kg	(Whitehouse et al. 1999)
		Lyprinol®	Dextran sulfate sodium-induced colitis model of male C57BL/6 mice 5 mg/mouse of Lyprinol via oral gavage for 13 days.	Reduced the loss of body weight. Reduced disease activity index. Reduced crypt area losses. Reduced cecum and colon weight.	5 mg/mouse	(Tenikoff et al., 2005)
		Lipid extract (GMLE)	Adjuvant-induced (AIA) and collagen-induced arthritis (CIA) in rats received 100 mg/kg of HMLE for 30 days.	a) Reduced the swelling of paw oedema. b) Suppressed the inflammatory mediators (LTB4, PGE 2, and TXB2). c) Suppressed pro-inflammatory cytokines (IL-1, IL-6, INF γ -, and TNF α) and MMPs (MMP1, MMP13) promoted anti-inflammatory cytokines (IL-4, IL-10) and TIMPs (TIMP1) productions.	100 mg/kg	(Li et al., 2014)
<i>Perna viridis</i> (Linnaeus, 1758)	Indian ocean, South pacific ocean, Coral sea, and Caribbean Sea	Cadalmin™ GMe	Histamine-induced paw oedema.	Showed significant decrease in histamine-induced oedema.	NA	(Chakraborty, 2012)

		Methanol and aqueous/ethanol extract of the whole body tissue.	<ul style="list-style-type: none"> a) Carrageenan induced paw oedema. b) Formalin-induced chronic paw oedema. c) Dextran-induced acute paw oedema. <p>Male Swiss Albino mice received (100, 500 or 1,000 mg/kg) of the extracts (p.o.) for 7 days in all assays listed above.</p>	<ul style="list-style-type: none"> a) Showed significant decrease in carrageenan-induced oedema. b) Inhibited the formalin-induced inflammation. c) Methanol extract inhibited the dextran-induced paw oedema. 	<ul style="list-style-type: none"> a) And b) 100, 500 and 1,000 mg/kg. b) 500 and 1,000 mg/kg MeOH extract only. 	(Sreejamole et al. 2011)
<i>Lamellidens marginalis</i> (Lamarck, 1819)	India	Aqueous extract of footpad	Adjuvant-induced Arthritis in male Albino rats received 500 mg/kg/day and or 1 g/kg/day of extract for 13 days.	<ul style="list-style-type: none"> a) Reduced paw diameter, ankle diameter and paw weight. b) Restored serum IL1_β, IL6, CINC1, TNFα, IL10 and lysosomal enzyme levels. c) Restored the level of neutrophils infiltration. 	500 and 1000 mg/kg	(Chakraborty et al. 2010)
<i>Parreysia cylindrical</i> (Annandale & Prashad, 1919)	India	processed shell powder	<ul style="list-style-type: none"> a) Carrageenan induced paw oedema in Wistar albino rats received 100 or 200 mg/L (p.o.). b) Analgesic activity model (sensitivity to warm water) in Wistar albino rats received an oral dose of 100 mg/mL or 200mg/ml. c) Wound healing properties in albino rats by inducing 2 cm wound using sterilised blades. Rats then treated topically with ointment prepared from 15 % 	<ul style="list-style-type: none"> a) Reduced the carrageenan paw oedema. b) Decreased the pain in an analgesic activity. c) Scar formed on 2cm wound in thigh region in 8 days. d) No toxicity up to 800mg/Kg dose. 	<ul style="list-style-type: none"> a) 100 and 200 mg/mL. b) 100 and 200 mg/mL c) 15 % of shell powder in egg albumin. 	(Swapna, 2015)

			shell powder in egg albumin.			
<i>Mytilus unguiculatus</i> (Valenciennes, 1858) ⁷ (Hard-shelled mussel)	Subtropical Western Pacific Ocean	lipid extract (HMLE)	Adjuvant-induced (AIA) and collagen-induced arthritis (CIA) in rats received 100 mg/kg of HMLE for 30 days.	<p>a) Reduced the swelling of paw oedema.</p> <p>b) Suppressed the inflammatory mediators (LTB₄, PGE (2), and TXB₂).</p> <p>c) Suppressed pro-inflammatory cytokines (IL-1, IL-6, INFγ-, and TNFα) and MMPs (MMP1, MMP13) promoted anti-inflammatory cytokines (IL-4, IL-10) and TIMPs (TIMP1) productions.</p>	100 mg/kg	(Li et al., 2014)
<i>Mytilus galloprovincialis</i> (Lamarck, 1819) (Mediterranean mussel)	Global in temperate to polar waters (Northern and Southern Hemisphere)	amino acids	Skin wound healing in Wistar rats topically treated with 0.3 mg/kg of amino acids.	Accelerated skin wound healing via enhancement of dermal and epidermal neoformation.	0.3 mg/kg	(Soliman and Fahmy 2013 , Badiu et al. 2010)
		Lipid extract	Skin burns in Wistar rats treated topically with 0.2 mg/kg lipid extracts 2 time/day for 22 days.	Very efficient in healing induced skin burns.	0.2 mg/kg	(Badiu et al., 2008)
<i>Coelatura aegyptiaca</i> (Cailliaud, 1827) (Egyptian freshwater mussel)	Northeastern Africa Northern Africa Western Africa	Fresh mussel extract	<p>a) Hot plate latency assay.</p> <p>b) Acetic acid-induced writhing test and formalin induced paw licking in mice.</p> <p>Mice treated with 200 mg/kg of the extract (i.p.).</p>	<p>a) Reduced the paw licking times.</p> <p>b) Slightly increased the latency time of mice in the hot plate.</p> <p>The LD₅₀ was above 2000mg/Kg i. p.</p>	200 mg/kg	(Soliman and Fahmy 2013)

<i>Pinctada imbricate</i> (Röding, 1798) ⁸	China	Pearl used (Zhikang Capsule (ZKC) component)	Dextran Sodium Sulfate-induced colitis in C57BL/6 mice fed on 170, 340 or 680 mg/kg ZKC.	<ul style="list-style-type: none"> • Suppressed TNFα, IFN-γ, IL-1β, and IL-12. • Promoted anti-inflammatory mediators (IL-4 and IL-10). 	680 mg/kg	(Fei and Xu, 2016)
Cephalopoda						
<i>Sepia officinalis</i> (Linnaeus, 1758) (the common cuttlefish)	Mediterranean Sea, North Sea, and Baltic Sea.	ink extract	<p>a) Hot plate latency assay.</p> <p>b) Acetic acid-induced writhing test and formalin induced paw licking in mice.</p> <p>Mice treated with 200 mg/kg of the extract (i.p.).</p>	<p>a) Inhibited the acetic acid-induced writhing.</p> <p>b) Increased the latency period of mice in the hot-plate.</p>	200 mg/kg	(Soliman and Fahmy, 2013)
<i>Sepia pharaonic</i> (Ehrenberg, 1831) (Pharaoh cuttlefish)	Indian Ocean eastern; Indian Ocean western; Pacific northwest; Pacific western central	Liver oil	Carrageenan and formalin induced paw oedema in male albino Sprague Dawley rats fed on 1% cuttlefish liver oil for 45 days	Decreased both carrageenan-induced and formalin-induced paw oedema.	1% in the diet for 45 days	(Joseph et al., 2005)
<i>Ommastrephes bartramii</i> (Lesueur, 1821) ⁹ (Neon flying squid)	subtropical and temperate waters of the Pacific, Atlantic, and Indian Oceans	low-molecular-weight melanoprotein from <i>Ommastrephes bartramii</i> (Fr.SM II)	<p>Carrageenan-induced rat paw oedema.</p> <p>Inducing mouse ear with xylene.</p> <p>Carboxymethyl cellulose pouch method.</p> <p>Mice/rats were injected (i.v.) with 10 or 20 mg/kg of (Fr.SM II).</p>	<p>Reduced the paw oedema.</p> <p>Suppressed the enhanced capillary permeability caused by xylene.</p> <p>Inhibit both leucocyte emigration and protein exudation into the pouch fluid.</p>	<p>10 and 20 mg/kg in the paw oedema assay</p> <p>20 mg/kg in enhanced capillary permeability induced by xylene assay in mice and pouch method in rats</p>	(Mimura et al., 1987)

<i>Sepiella inermis</i> (Van Hasselt [in Férussac & d'Orbigny], 1835) ¹⁰	China	Inner shell (Zhikang Capsule (ZKC) component)	<ul style="list-style-type: none"> Dextran Sodium Sulfate-induced colitis in C57BL/6 mice fed on 170, 340 or 680 mg/kg ZKC. 	<ul style="list-style-type: none"> Suppressed TNFα, IFN-γ, IL-1β, and IL-12. Promoted anti-inflammatory mediators (IL-4 and IL-10). Preserved the colon appearance. 	Only 680 mg/kg dose was showed significant effect in all assays.	(Fei and Xu, 2016)
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1. Listed in the publication as *Bellamyia bengalensis* (Lamarck 1822)
2. Listed as *Viviparous bengalensis* (Lamarck, 1822) (fresh water snail)
3. Listed as *Trochus tentorium* (Gmelin, 1791)
4. Listed as *Drupa margariticola* (Broderip in Broderip & Sowerby, 1833)
5. Listed as *Rapana thomasi* (Crosse, 1861)
6. Listed as: (*Hemifusus pugilinus* (Born, 1778))
7. Listed in the publication as *Mytilus coruscus*
8. Listed in the publication as *Pinctada martensii* (Dunker, 1880)
9. Listed as *Ommastrephes bartrami* (Lesueur, 1821)
10. Listed in the publication as *Sepiella maindroni* (Rochebrune, 1884)

2.7. Human clinical studies

Only a small number of human clinical trials (14) have been undertaken to test the anti-inflammatory efficacy of molluscan products (Table 2.5). Most of these (13 clinical trials) have focused on NZ green lipped mussel extracts (GLME) including Lyprinol® and Biolane™/Seatone® following on from the promising anti-inflammatory activity *in vitro* and in animal models (Table 2.3 & 2.4). These clinical studies have been previously subject to comprehensive critical review {Cobb, 2006 #835}{Ulbricht, 2009 #1155} and overall support the efficacy and the safety of GMLE for use in humans ([Lyprinol, 2017](#), [Halpern, 2000](#), [Gibson, 2000](#), [Whitehouse et al., 1997](#)). One of the earliest studies was a double-blind parallel control trial undertaken in Glasgow's Homeopathic hospital involving 66 outpatients who were scheduled to undergo surgery due to osteoarthritis and/or rheumatoid arthritis that was unresponsive to the conventional therapeutic regimes such as with the use of NSAIDs ([Gibson et al., 1980](#)). In brief, a total of 38 patients with osteoarthritis and 28 with rheumatoid arthritis were divided into two groups. Group 1 received a daily dose of GMLE and group 2 received a daily dose of powdered dry fish meal as a placebo. The trial was conducted for 3 months with regular checks for pain, grip strength, joint stiffness, functional efficiency, limbering up time and time taken to walk 15 meters. Overall 68% of rheumatoid arthritis and 39% of osteoarthritis patients treated with mussel extract showed improvement as indicated by a significant reduction in pain and joint stiffness and consequently improving the ability of patients to cope with life and the enhancement of general health. However, 10% of patients given the extract demonstrated a transient worsening of these symptoms, although no other side effects were detected in this study. This study showed the potential of the GLME as a life style nutraceutical supplement or as a possible alternative to other therapies for the treatment of rheumatoid arthritis and osteoarthritis. Nevertheless there may be a need to refine the dosage or treatment regimen to address the deterioration in a smaller number of patients. In another double blind study involving 53 patients with gonarthrosis (the arthritis of knee) patients, the treatment group received 2,100 mg of GLME/day for six months, which was compared to a control group which received a matching dose of placebo ([Audeval and Bouchacourt,](#)

[1986](#)). According to the results of this study, GLME was similarly effective in preventing the deterioration and enhancing the repair mechanisms, as well as providing both analgesic and anti-inflammatory benefits ([Audeval and Bouchacourt, 1986](#)). In several other double blind randomised control trials for rheumatoid arthritis using lower doses of GLME, positive effects were sometimes recorded, but there were not significant differences when compared to the placebo control ([Caughey et al., 1983](#), [Highton and McArthur, 1975](#), [Huskisson et al., 1981](#), [Larkin et al., 1985](#)). This suggests that the efficacy of GMLE for treatment of arthritic may depend on the dose, duration and the specific formulation or batch.

In a further year-long pilot study performed in USA involving 120 patients in the 60-70 age group also with gonarthrosis, the patients were given a dose of 3 capsules of GLME (500 mg GLME) daily with food ([Kendall et al., 2000](#)). At the end of the study the results were subjectively assessed by patients and separately by medical practitioner. In brief, patient assessments showed that 95 patients reported excellent symptom improvement, whilst 16 reported good improvement and 9 reported no change to the symptoms. However, practitioner assessments suggested that 63 of patients had demonstrated excellent improvement, 38 good improvement and 19 with no change ([Kendall et al., 2000](#)). Once again the study reported a low incidence of side effects and when this observation is added to the overall positive outcomes from these clinical trials it would seem that with an optimised treatment regime and formulation, GLME could be of significant value for at least some patients suffering from chronic and disabling inflammatory disorders such as arthritis ([Gibson et al., 1980](#), [Halpern, 2000](#)).

Lyprinol® has also been investigated for its potential efficacy for the treatment of asthma (Table 2.5). A double-blind randomised, placebo controlled clinical trial performed by [Emelyanov et al. \(2002\)](#) used the lipid extract to treat patients with atopic asthma. The study involved 46 patients who were given 2 capsules of Lyprinol® per day or a placebo containing olive oil ([Emelyanov et al., 2002](#)). In brief, Lyprinol® was found to relieve the symptoms of asthma compared to the placebo control. However, the difference between the treatment with Lyprinol and the placebo was not statistically

significant for the treatment of the atopic asthma. However, in a separate double blind placebo controlled study in 73 children with asthma for 16 weeks, the refined GLME product PCSO-524™ did results in statistically significant improvements relative to the controls ([Lello et al., 2012](#)) Another form of GMLE, (Biolane®) was also tested for its ability to heal the injury of soft tissues in otherwise healthy people ([Lambert et al., 1998](#)). This study was carried out on well trained cyclists who were given Biolane GLME 3 weeks prior to the induction of muscle injury with outcomes compared to a control group of cyclists who were given a placebo. The treatment group showed increased peak power and accelerated recovery of peak power after injury compared to the control group. This was an unprecedented study to reveal the effect of Biolane® on restoring damaged tissues.

Despite some conflicting results, all the clinical studies mentioned above have led to acceptance of the GLME as an effective anti-inflammatory agent. It is available now as an over the counter drug in different formulations. There are two main formulations of NZ GLME that have been optimised for stability and demonstrate more reliable *in vivo* efficacy for relieving inflammatory disorders, 1) Lyprinol® which contains 50 mg of PCSO-524® (lipid extract from *P. canaliculus*), 100 mg of a proprietary oleic acid blend and 0.225 mg of vitamin E, and 2) Biolane® which contains the *P. canaliculus* extract powder (Table 2.5). However, less refined GML products, such as those based on the freeze-dried mussel powder have less statistical support for differences between the GLME treated group and the placebo treated group in clinical trials, although health improvement was noticed among some of the participants (Table 2.5).

The only other human clinical trials that have been undertaken with mollusc extracts examined the effect of a formulated snail mucus product on wound healing in burn victims (Table 2.5). Elicina® (Locafar, Chile) is a cosmetic skin repair cream which is composed of 80% of the mucus secretions from the brown snail *Cornu (Helix) aspersum* (O.F. Muller, 1774) (common garden snail). In a clinical study, the cream was applied twice daily on adult patients with deep partial thickness facial burns for a maximum period of 14 days or until full epithelialisation ([Tsoutsos et al., 2009](#)). This cream healed the facial burns in around 11 days, significantly faster than the well-known burn

ointment MEBO which took around 15 days for healing. The snail cream not only accelerated the healing process, but also reduced the pain caused by the burns. The results suggest that the cream maybe an effective treatment of the management of open wounds caused by partial thickness burns ([Tsoutsos et al., 2009](#)). However, further double blind randomized control trials are required to test the suite of different snail mucus formulations that are currently available on the open market and promoted for their skin healing properties.

Table 2.5: Trials on anti-inflammatory and wound healing molluscan natural products.

Species	Geographic distribution	Extract compound or	Type of clinical trial and study design	Outcome and results	Reference
Gastropoda					
<i>Cornu aspersum</i> (O. F. Müller, 1774) (Common garden snail)	native to the Mediterranean region	Elicina® a cream contains 80% of mucus from the snail <i>Cornu aspersum</i>	Applied on 27 patients with deep partial thickness (PT) facial burns and its effect compared to 16 patients treated using a well-known burn ointment MEBO The treatments were applied twice daily for 14 days. Patients were randomly divided into these two groups and clinically evaluated by two residents who were blinded to the treatment.	Accelerated the healing process significantly compared to MEBO and reduced the pain triggered by the burns.	(Tsoutsos et al., 2009)
Bivalvia					
<i>Perna canaliculus</i> (Gmelin, 1791) (GML)	New Zealand	Powdered <i>Perna canaliculus</i> or Lyprinol®	54 osteoarthritis (OA) patients consumed 2 capsules of Lyprinol twice a day for 8 weeks.	<ul style="list-style-type: none"> a) 80 % of patients reported significant pain relief. b) 39 % showed improvement in joint function c) 87% of patients and 90% of doctors reported improvement in global assessment. 	(Cho et al., 2003)
		Powdered <i>Perna canaliculus</i> or Lyprinol®	Double-blind parallel comparison study included 30 patients with classical rheumatoid arthritis (RA) and 30 patients with osteoarthritis. Each category divided into two groups group A (received 1,150 mg/day mussel powder) and group B (received 210 mg/day Lyprinol®) for 90 days. Then, all patients received the lipid extract (Lyprinol) for another three months.	76 % of RA and 70 % of OA demonstrated significant improvement in the prospective of pain, swelling, stiffness and improving functional index in both RA and OA as compared to the baseline assessments. There was NSD between the GMLE and Lyprinol treatment.	(Gibson and Gibson, 1998)
		(Lyprinol®)	Placebo controlled study involved 46 patients with atopic asthma consumed 2 capsules of Lyprinol® (each capsule contains 50 mg of ω -3 PUFAs (eicosapentaenoic acid and docosahexaenoic acid) and 100 mg plive oil)) or placebo containing only 150 mg olive oil for 8 weeks.	Lyprinol® was suggested to relief the symptoms of asthma compared to the placebo control but there was no statistical difference (NSD).	(Emelyanov et al., 2002)

	Seatone® (freeze dried GLM powder-same as Biolane™)	Double-blind crossover randomised PCT involved 6 patients with rheumatoid arthritis consumed Seatone (no dosage information) or placebo for 6 weeks and then patients crossover to the other treatment for further 6 weeks.	NSD between Seatone and placebo treatments in all the observed parameters of RA.	(Highton and McArthur, 1975)
		Double-blind crossover randomised, placebo controlled trial involved 30 patients with rheumatoid arthritis consumed 900 mg/day Seatone or dried fish placebo for 8 weeks.	NSD between Seatone and placebo treatments in all the observed parameters of RA despite some improvements in some parameters.	(Huskisson et al., 1981)
		Double-blind randomised PCT involved 35 patients with rheumatoid arthritis consumed 920 mg/day Seatone or dried fish placebo for 3 months followed by 1,180 mg/day for another 3 months.	NSD between Seatone and placebo treatments in all measures.	(Larkin et al., 1985)
		Double-blind randomised PCT involved 53 Gonarthrosis (arthritis of the knee) patients received Biolane (2,100 mg/day) or placebo for 6 months.	<p>a) SD in most of the measures including visual analogue of pain score, global assessment, and the functional state of arthritis condition.</p> <p>b) NSD in the other measures (articular mobility; use of walking stick; time to walk set distance or maximum walking distance; pain intensity) between Seatone and placebo treatment.</p>	(Audeval and Bouchacourt, 1986)
	<i>Perna canaliculus</i> GLME	Double blind randomised placebo controlled trial involved 47 patients with rheumatoid arthritis consumed 1,050 mg/day mussel extract for 12 weeks compared to a placebo control.	There was improvement in the patient's status. However, NSD noticed between the treatment with mussel extract and the placebo.	(Caughey et al., 1983)

		120 patients in the 60-70 age group with osteoarthritis of the knee. Consumed 2-3 capsules of 500 mg GLME/day for one year.	Practitioner's assessment suggested that 63 of patients had made an excellent improvement, 38 good improvement and 19 with no change. All patients in this study consumed the same treatment, hence, no negative control group.	(Kendall, Lawson et al. 2000)
		Double-blind, parallel comparison placebo controlled study included a total of 38 patients with osteoarthritis and 28 with rheumatoid arthritis received a daily dose of 300 mg of GMLE or placebo control three times daily for 90 days.	68% of rheumatoid arthritis and 39% of osteoarthritis patients showed improvement; 10% demonstrated a transient worsening of the symptoms. However, NSD compared to the placebo.	(Gibson et al., 1980)
	PCSO-524™ Lipid extract	A total of 71 children aged 6 to 13 years with asthma were enrolled in a 16-week, single centre, double-masked, placebo-controlled. Children consumed 2 capsules of Lyprinol® or placebo daily.	Improved the percentage of children reporting little or no trouble with their asthma at three months of treatment (97% compared to 76% $p = 0.057$).	(Lello et al., 2012)
	PCSO-524™ Lipid extract (Lyprinol®)	Pain relief changes in people with Osteoarthritis taking PCSO-524™ (1200mg per day) compared to patients taking fish oil for 12 weeks.	Significantly relieved the pain and discomfort. Significantly improved the quality of life of patients compared to fish oil	(Zawadzki et al., 2013)
	Form of GMLE, Biolane®	Placebo controlled study. Well trained cyclists consumed Biolane® GLME (dosage information unavailable) or placebo 3 weeks prior to the induction of muscle injury.	Increased the Peak power compared to the control. Accelerated the restoration of peak power faster than control group.	(Lambert et al., 1998)

2.8. Nutraceutical and Cosmeceutical Development

There are few commercialized molluscan products currently available. Extensive preclinical and clinical studies have led to the acceptance of the GLME as an anti-inflammatory nutraceutical under the name of Lyprinol® ([Halpern, 2008](#), [Halpern, 2000](#), [Treschow et al., 2007](#), [Whitehouse et al., 1997](#), [Lyprinol, 2017](#)). Lyprinol® is also marketed as an over-the-counter oral treatment for rheumatism and arthritis and fractions derived from Lyprinol can also be used to relieve moderate-asthma in children ([Lyprinol, 2017](#)). Biolane™ is arguably more effective than Lyprinol as a nutraceutical with anti-inflammatory activity and may be due to the preparation not containing any further additives other than powdered GLM ([Cheras et al., 2005](#)). The Indian green mussel extract *P. viridis* (Linnaeus, 1758) has also been commercialised as an anti-inflammatory OTC under the name Cadalmin™ ([Chakraborty, 2012](#)).

Overall it would appear that mollusc extracts with a long history of human ethnomedical consumption are more likely to be relatively safe for use as nutraceuticals. Further research to identify the active constituents in the ethnomedical preparations from molluscs that are used specifically for anti-inflammatory treatments could facilitate strategic development of scientifically validated products that can be value-added as nutraceuticals or pharmaceuticals. However, safety should never be assumed for concentrated natural products, requiring industry investment for comprehensive preclinical and clinical trials.

Products that are not safe for human consumption may still have potential for topical application in skin repair and wound healing. Cosmeceuticals do not have to undergo the same rigorous level of clinical testing for safety and efficacy as do pharmaceuticals and/or nutraceuticals. However, efficacy under-pinned by evidence-based science will be an essential requirement if marketing the product with specific efficacy statements is being considered. In recent years there has been a proliferation in the range of snail creams available for skin care, suggesting a need for improved quality control measures. Allantoin and glycolic acid are two bioactive ingredient in mollusc's mucus that contribute

to their use as constituents in cosmetic skin creams. [El Mubarak et al. \(2013\)](#) validated a method for simultaneous detection of these compounds and demonstrated that there was significant variability in the levels of these compounds between different cosmetic creams, which is likely to reflect the efficacy of snail mucus content and potentially the quality of the product. This further demonstrates the importance of improved quality control for over the counter cosmeceutical and nutraceuticals. Overall more research is required on molluscan bioactive compounds to identify the bioactive agents, optimise methods for their detection and quantification in extracts and formulations for standardisation, as well as validate their *in vivo* efficacy and confirm their safety. With a greater level of clinical testing, the products can be considered for registration as therapeutic agents (e.g. by US Food and Drugs Administration, European Medicines Agency or Therapeutic Goods Administration in Australia) thus leading to not only wider commercial value but also providing safer and more effective options than NSAIDs.

The safety of using the traditional or natural medicine should never be assumed due to the toxicity of some natural products. In some traditional medicine systems, traditional methods of preparation could reduce or remove the toxic components. In addition, mostly the shells are used for species known to produce toxic venoms e.g. cone snails and neogastropods. Flesh from Muricidae species in particular are only used after boiling in TCM which may effectively break down the muscle relaxing choline esters produced in their hypobranchial glands ([Roseghini et al., 1996](#), [Benkendorff et al., 2015](#)). Buccinidae is another family of neogastropod molluscs that have a relatively large salivary glands containing significant quantities of tetramine, which blocks nicotinic acetylcholine receptors e.g. *Neptunea* (Röding, 1798) spp. ([Watson-Wright et al., 1992](#), [Fujii et al., 1992](#), [Modica and Holford, 2010](#)). Reports have demonstrated that people can become intoxicated as a result of consuming these species ([Modica and Holford, 2010](#)) but only one traditional medicine from *Neptunea cumingii* (Crosse, 1862) was identified here for anti-inflammatory applications (Table 1) and this involved a decoction of the shell, so is unlikely to contain tetramine from the salivary gland. However, the TCM using the sea hare (*Aplysia* Linnaeus, 1767) eggs to treat tuberculosis of lymph nodes (Table 2) is of

concern because consumption of *Aplysia* eggs is known to cause liver damage and they contain toxic glycoprotein compounds ([Hino et al., 1994](#), [Kicklighter et al., 2005](#), [Johnson and Willows, 1999](#)). Nevertheless, the lack of other soft-bodied gastropods i.e. Nudibranchia species in the traditional medicines is most likely because many of them contain toxic compounds that are derived from their diets on sessile invertebrates including sponges, ascidians and cnidarians ([Benkendorff, 2010](#), [Benkendorff, 2014](#)). Caution is also required for filter feeding bivalves and their predators, which can accumulate environmental toxins including heavy metals ([Lau et al., 1998](#), [Usero et al., 2005](#), [Bryan et al., 1977](#)) and paralytic shell fish toxins from toxic algal blooms ([Hallegraeff et al., 2003](#), [Lagos et al., 1999](#), [Hallegraeff and Bolch, 2016](#), [Callejas et al., 2015](#)). As well as the toxic substances molluscs can also contain human pathogens and there have been many infectious outbreaks associated with consumption of oysters, clams and mussels ([Potasman et al., 2002](#)). Consequently any molluscs used as functional food should be subject to shellfish safety and quality assurance programs. The potential for toxic metabolites also suggest the need for identification of the bioactive compounds, safety testing and quality assurance on any nutraceutical or traditional medicine to ensure safety.

2.9. Conclusion

Mollusc products have been used as anti-inflammatory remedies and ethnomedicines for centuries by a vast range of ethnocultural groups. In this review we have found some 104 different anti-inflammatory remedies in the literature. Only a few of these preparations have been clinically tested or had their anti-inflammatory activity examined by evidence based scientific methods. However, to date the few preparations tested for anti-inflammatory activity in either *in vitro* or *in vivo* assays have shown promising results. Lyprinol® and Biolane™ from the New Zealand Green Lipped mussel *Perna canaliculus* and Cadalmin™ GMe from the green mussel *Perna viridis* are prime examples of the strategic development of anti-inflammatory remedies from mollusc species that have a sound ethnomedical history.

This review clearly indicates the significance of molluscan natural products as potential leads for anti-inflammatory nutraceutical and cosmeceutical agents. In addition, this review also indicates that there is a paucity of studies that have investigated the anti-inflammatory activity of molluscs, considering the high diversity of species within this phylum. Furthermore, despite the vast use of molluscan preparations in the ethnomedicine and the *in vitro* and *in vivo* anti-inflammatory activity results, only few molluscan natural products have yet been chemically characterised. Therefore, more attention should be paid toward identifying molluscan natural products as a potential source of anti-inflammatory drug leads.

Chapter 3: Anti-Inflammatory Activity and Structure- Activity Relationships of Brominated Indoles from a Marine Mollusc

Chapter 3 of this thesis incorporates the following publication:

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3. Chapter 3: Anti-Inflammatory Activity and Structure- Activity Relationships of Brominated Indoles from a Marine Mollusc

3.1. Abstract

Marine molluscs are rich in biologically active natural products which provide new potential sources of anti-inflammatory agents. Here we used bioassay guided fractionation of extracts from the muricid *Dicathais orbita* to identify brominated indoles with anti-inflammatory activity, based on inhibition of nitric oxide (NO) and tumour necrosis factor α (TNF α) in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages and prostaglandin E2 (PGE2) in calcium ionophore-stimulated 3T3 ccl-92 fibroblasts. Muricid brominated indoles were then evaluated against a range of synthetic indoles to determine structure-activity relationships. Hypobranchial gland extract and the egg extract inhibited the production of NO significantly with IC₅₀ of 30.8 and 40 μ g/mL respectively. The hypobranchial gland extract also inhibited the production of TNF α and PGE2 with IC₅₀ 43.03 μ g/mL and 34.24 μ g/mL respectively. The purified mono-brominated indole and isatin compounds showed significant inhibitory activity against NO, TNF α and PGE2 and were more active than dimer indoles and non-brominated isatin. The position of the bromine atom on the isatin benzene ring significantly affected the activity, with 5Br > 6Br > 7Br. The mode of action for the active hypobranchial gland extract, 6-bromoindole and 6-bromoistain was further tested by the assessment of the translocation of the NF κ B in LPS-stimulated RAW264.7 mouse macrophage. The extract (40 μ g/ml) significantly inhibited the translocation of NF κ B in the LPS-stimulated RAW264.7 macrophages by 48.2 %, whereas (40 μ g/ml) of 6-bromoindole and 6-bromoistain caused a 60.7 and 63.7 % reduction in NF κ B, respectively. These results identify simple brominated indoles as useful anti-inflammatory drug leads and support the

development of extracts from the Australian muricid *D. orbita*, as a new potential natural remedy for the treatment of inflammation.

3.2. Introduction

Inflammation is a complex mechanism involving the activation and deactivation of immune cells in response to stimuli and tightly regulated signalling pathways. If left unchecked, inflammation will result in cellular and tissue damage and can lead to chronic disease ([Alessandri et al., 2013](#)). Macrophages play a key role in initiating and maintaining the inflammatory response, and are activated by pathogen associated molecules and cytokines, which stimulate proinflammatory signalling pathways ([Fujiwara and Kobayashi, 2005](#)). NF κ B has long been considered a prototypical pro-inflammatory signalling molecule, activated by pro-inflammatory cytokines such as tumour necrosis factor α (TNF α), in the presence of viruses, genotoxic agents or when stimulated by microbial constituents including lipopolysaccharide (LPS) from bacterial cell walls ([Karin and Greten, 2005](#)). Activation of the inhibitory subunit of NF κ B (I κ B) kinase results in the phosphorylation of I κ B proteins bound to NF κ B. As a consequence, NF κ B translocates to the nucleus, where it regulates the expression of a variety of transcription factors and co-factors, leading to the expression of pro-inflammatory enzymes like cyclooxygenase 2 (COX 2) and inducible nitric oxide synthase (iNOS), which are responsible for stimulating production of inflammatory signalling molecules ([Lo et al., 1999](#), [Karin and Greten, 2005](#)). TNF α in particular, is considered one of the key inflammatory mediators in endotoxin-induced tissue injury and high levels of TNF α have been correlated with the severity of the inflammatory response ([Moraes et al., 1996](#)). Moreover, NO produced by iNOS is a prominent marker of inflamed tissue and can cause localised toxicity by producing reactive nitrogen oxide species (RNOS) ([Wink et al., 1996](#)) or by reacting directly with proteins or DNA ([Tamir et al., 1996](#)). Furthermore, prostaglandin E₂ (PGE₂) is produced as a result of the combined enzymatic activity of phospholipase A₂ (PLA₂) and COX₂, which is also regulated by the activation of NF κ B. The suppression of excessive production of NO, TNF α and/or PGE₂ in

stimulated cells using *in vitro* assays provides an effective means to evaluate the anti-inflammatory activity of natural products and extracts ([Yuan et al., 2006](#)).

For many centuries, natural products have provided a significant source of leads for drug development. Thirty-one marine compounds have now entered the clinical trials pipeline for drug development, of which 7 have been approved by the Food and Drug Administration (FDA-approved) ([Mayer, 2017](#)). In particular, a significant number of anti-cancer drug leads have been isolated from marine molluscs ([Ciavatta et al., 2016](#)). However, only a small proportion of natural products isolated from marine molluscs have been investigated for anti-inflammatory potential ([Benkendorff, 2010](#)). However, a lipid extract from the New Zealand Green lipped mussel *Perna canaliculus* has been clinically tested for the treatment of chronic inflammation and is commercially available as a nutraceutical ([Brien et al., 2008](#)). Preliminary studies on the crude extracts from whelks in the Muricidae family of predatory gastropods indicate that these may also yield secondary metabolites with interesting anti-inflammatory properties. The acetone extracts of *Drupella (Drupa) margariticola* ([Chellaram and Edward, 2009a](#)) and chloroform extracts from *Purpura persica* significantly reduced carrageenan-induced paw edema in rats at concentrations well below the toxic limits ([Santhi et al., 2011](#)). Furthermore, lipid extracts from *Rapana venosa* were found to reduce inflammation and improve healing of skin burns in Wistar rats ([Badiu et al., 2008](#)). These studies support further investigation of the bioactive compounds in muricid molluscs for potential development of anti-inflammatory treatments to replace the conventional steroidal and non-steroidal anti-inflammatory drugs which have numerous side effects.

The Muricidae are well-known for their production of the dye molecule Tyrian purple (6,6-dibromoindigo, **1**, Table 3.1) and related biologically active brominated indoles ([Benkendorff et al., 2015](#)). These compounds are generated from the precursor indole tyrindoxyl sulfate (**2**, Table 3.1) stored as a salt of the choline ester murexine in the hypobranchial glands and egg masses ([Benkendorff, 2013](#)). The intermediate dye precursors, tyrindoleninone (**3**, Table 3.1) and 6-bromoindigo (**4**, Table 3.1) have been shown to have specific anticancer activity, inducing apoptosis

in a range of cancer cell lines *in vitro* ([Vine et al., 2007](#), [Benkendorff et al., 2011](#), [Edwards et al., 2012](#), [Esmaeliani et al., 2013](#)) and DNA damaged cells to prevent colon cancer *in vivo* ([Westley et al., 2010b](#), [Esmaeliani et al., 2014](#)). These bioactive monobrominated indoles dimerise to form the green coloured pigment tyriverdin (**5**, Table 3.1), the immediate precursor to Tyrian purple, which has no known anticancer activity but it is a potent bacteriostatic agent ([Benkendorff et al., 2000](#)). Minor pigments of Tyrian purple 6, 6'-dibromoindirubin and 6-bromoindirubin (**6**, Table 3.1) have anti-proliferative properties and are potent inhibitors of glycogen synthase kinase-3 (GSK-3) ([Meijer et al., 2003](#)). Synthetic bromoindirubin derivatives with improved solubility and biological selectivity have been developed ([Nam et al., 2005](#), [Kim et al., 2007](#), [Leclerc et al., 2001](#)), leading to several patents over the last decade ([Karabelas et al., 2002](#), [Wang et al., 23 August 2005](#), [Carson et al., 2006](#)).

Depending on the specific mode of action, anticancer compounds can often also exhibit anti-inflammatory activity ([Corson and Crews, 2007](#), [Thun et al., 2002](#), [Karin and Greten, 2005](#)). In addition to anticancer properties, indirubin (**7**, Table 3.1), a minor pigment in Tyrian purple dye mixture, inhibits reactive oxygen species (ROS) resulting in attenuation of phagocytosis and induces ATP stimulated cell death ([Man et al., 2012b](#)). Indirubin derivatives also inhibit the release of pro-inflammatory cytokines interleukin (IL)-1 β and IL-6 in mouse macrophage RAW 264.7 cells stimulated with LPS ([Kim and Park, 2012](#)). The anti-cancer monobrominated indoles from Muricidae molluscs are yet to be tested for anti-inflammatory activity. However, the simple indole derivative, isatin (**8**, Table 3.1) inhibits iNOS, COX-2, and TNF α , which results in reduced PGE2 and NO levels in LPS-stimulated mouse macrophages ([Matheus et al., 2007](#)). Some derivatives of isatin and indole have been patented as potential treatments for inflammation ([Kitaura et al., 1991](#), [Stevens et al., 1 March 1994](#), [Pelcman et al., 5 August 2010](#)). Consequently, further investigation of the anti-inflammatory properties of the brominated indoles from Muricidae are warranted. This study aims to investigate the inhibition of NO, TNF α , PGE2 and NF κ B by extracts and brominated indoles isolated from hypobranchial gland and other reproductive organs/tissues of the Muricidae mollusc *D. orbita*. In addition, the structure activity relationships of a number of brominated indoles were examined,

using a suite of synthetic indole derivatives to determine if dimerization and the position of halogenation had any influence on activity.

3.3. Materials and Methods

3.3.1. Chemicals and reagents:

Escherichia coli LPS (O128:B12, Sigma), sulfanilic acid, N-(1-Naphthyl) ethylenediamine (NED), 85 % orthophosphoric acid sodium nitrite (NaNO₂), isatin (**8**, Table 3.1), 5-bromoisatin (**9**, Table 3.1), 6-bromoisatin (**4**, Table 3.1), 7-bromoisatin (**10**, Table 1), 6-bromoindole (**11**, Table 3.1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tyrian purple (6,6 dibromoindigo (**1**, Table 3.1), indirubin (**7**, Table 3.1) and 6-bromoindirubin (**6**, Table 3.1) were obtained from Prof. A. L. Skaltsounis (University of Athens). Penicillin–streptomycin solution, Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine were from Life Technology Australia (Mulgrave, VIC Australia). RAW264.7 mouse macrophages and 3T3 Swiss albino (ATCC[®] CCL92[™]) cell lines were obtained from American Type Culture Collection (ATCC[®]). **Cell culture:**

The Murine RAW264.7 and 3T3 fibroblast cell culture were maintained in 10 % FBS supplemented DMEM, 100 µg/L streptomycin, and 100 IU/mL penicillin at 37°C and 5 % CO₂ atmosphere. Cells were passaged every 48-72 hrs in a split ratio of 1:10-1:20. Passages used in this study were between passage 7 to passage 25.

3.3.3. Mollusc Collection, dissection and extraction:

Medium sized *Dicathais orbita* (40-90mm shell length) were collected from inter-tidal reefs along the north coast of NSW, Australia, under the Southern Cross University NSW Fisheries exemption permit F89/1171-6.0. The collected snails were kept frozen until needed. In addition, the egg capsules were collected from the same sites in the breeding season in August and stored at -80C until needed. The snails were defrosted under running water and the shell was removed by rupturing it carefully using a bench vice. The hypobranchial glands were excised according to [Westley and Benkendorff \(2008\)](#) then weighed on an analytical balance (Mettler-Toledo, precision 0.0001g).

The extraction of the secondary metabolites from the collected hypobranchial glands (16.06 g) or egg capsules (~12 g) was undertaken according to established procedures ([Edwards et al., 2012](#)). Glands or egg masses were repeatedly soaked for 2 h in solvent (chloroform: methanol, 1:1), replenished until a clear extract was obtained. The extract was then filtered through Whatman filter paper 1 (90mm, Sigma-Aldrich) to remove the tissue. The filtered solvent was then decanted into a separating funnel, where the chloroform and the methanol partition were separated with addition of a small amount of MilliQ water. After the two phases formed, both solvent layers were collected separately then evaporated to dryness on a rotary evaporator (Buchi). The chloroform partition was kept covered in aluminium foil to protect from photolytic degradation and dried using a vacuum pressure of 474 mbar at 40 °C, then transferred to an amber vial and dried under high purity nitrogen gas before storage at -80 °C (the extract yield was 3-5 % w/w from the extracted tissue). The methanol-water partition was dried using a rotational vacuum concentrator (Alpha-RVC, Martin Christ, Osterode, Germany) until totally dried. In a preliminary extraction, *D. orbita* stored for 12 months at -20°C were dissected and the hypobranchial glands, which has already commenced oxidative and photolytic colour reactions were used in the extraction. This extract is referred to as the “degraded HBG extract”. To facilitate dissolving this extract in DMSO for anti-inflammatory testing, it was sonicated in a water bath for 30min, then heated at 60 °C for 10min, then further sonicated for 10min and heated for 10min two more times. For all other extracts and compounds examined, the sonication and heating steps were not required to facilitate solubility in DMSO prior to testing.

3.3.4. Purification of brominated natural products:

The crude chloroform extract from the hypobranchial glands was further fractionated using flash silica gel column chromatography in order to purify the main indole compounds. The separation of the compounds was based on their colour and polarity by eluting from the silica column with increasingly polar solvents starting with hexane, then dichloromethane (DCM), then increasing concentrations of methanol under nitrogen pressure, according to [Esmaeelian et al. \(2013\)](#). Six of the fractions were subject to preliminary bioassay and LC-MS screening and based on these results,

further purification focused on the main brominated indoles tyrindoleninone, tyriverdin and 6-bromoisatin.

For the separation of tyriverdin from the HBG chloroform extract, the extract was dissolved in hexane, which results in precipitation out of the solution. The precipitate was then centrifuged and the solvent removed, before washing again in hexane, and repeating the procedure until a clean dried crystalline powder was obtained. To purify tyrindoleninone, the crude hypobranchial chloroform extract was separated using flash column chromatography pressurised with nitrogen gas. The stationary phase consisted of a slurry of silica gel 60 (63-200 μm particle size, 70-230 mesh) mixed with hexane for pouring. Crude extract (426 mg), was loaded onto the column and eluted using a gradient of solvents. Fraction 1 (~120 mL) was eluted with 100 % hexane, fraction 2 (~70 mL) was eluted with 20 % DCM in hexane, fraction 3 (~230 mL) was collected using 25 % DCM in hexane and fraction 4 with 100 % DCM (~250 mL). Fraction 2 containing tyrindoleninone was concentrated in a rotary evaporator at 40°C and finally dried under N₂ gas within amber vials for LC-MS analysis and activity testing.

3.3.5. Chemical analysis of the extracts and purified compounds:

Extracts were analysed using an Agilent 1260 infinity High Performance Liquid Chromatography (HPLC) system coupled with a 6120 Quadrupole mass spectrometer (MS) according the method outlined in [Valles-Regino et al. \(2016\)](#). The HPLC was undertaken on a Phenomenex Luna C18 reversed phase column (100 x 4.6 mm) using a solvent gradient from 10 to 95 % acetonitrile (ACN), both with the addition of 0.005 % trifluoroacetic acid (TFA) over 18 min at a flow rate of 0.75 mL/min. Eluent was also monitored using parallel UV/Vis diode-array detection (DAD). The mass spectrometer used electrospray ionisation (ESI) in both positive and negative ion modes. Agilent ChemStation was used to analyse the LC-MS data. Brominated indoles were identified by comparison of their retention times and characteristic mass spectra (with major doublet (singly brominated) or

triplet (doubly brominated) mass ion clusters separated by 2 mass units for Br⁷⁸ and Br⁸¹) as reported in previous studies ([Valles-Regino et al., 2016](#), [Westley and Benkendorff, 2008](#)).

The chloroform extracts and purified tyrindoleninone were also analysed using Gas chromatography-mass spectrometry (GC-MS) on a Hewlett Packard (HP) 6890 GC coupled to HP 5973 mass selective detector (MSD). The GC utilised a column of HP-5MS (Crosslinked 5 % PH ME Siloxane) 30 m × 0.25 mm × 0.25 µm film thickness. Column temperature was controlled by HP ChemStation programmed from 50 °C (5 min hold) to 250 °C (5 min hold) @ 10 °C/min. The injection volume was 0.2 µL using an Agilent 7683 series injector with the injection port (split 1:10) at 250 °C. Helium was used as the carrier gas with the flow rate of 0.7 ml/min, MS detector source was set at 250 °C and quad at 150 °C, with the transfer line at 280 °C and ion source voltage 69.9 eV. Data processing was done using HP ChemStation software and the identification of the volatile compounds was based on matches to the library mass spectra and fragmentations patterns (NIST02, WILEY 6, and ESSOILS in house).

3.3.6. Preparation of extracts and compounds:

All extract/compounds were dissolved in DMSO and tested at 5 different concentrations 50, 10, 2, 0.4 and 0.08 µg/mL for all anti-inflammatory assays unless otherwise noted. The final concentration of DMSO was 0.35 % v/v for extracts and compounds. All samples were tested in triplicates at each concentration and each assay was independently repeated at least 3 times. The concentrations used the cytotoxicity assays were 50, 25, 12.5, 6.25 and 3.125 µg/mL.

3.3.7. Cytotoxicity assay:

To check for cytotoxicity of the extracts and compounds against RAW264.7 mouse macrophage and 3T3 ccl-92 mouse fibroblast cell lines, the crystal violet cytotoxicity test was used according to the published protocol ([Feoktistova et al., 2016](#)). In brief, RAW264.7 cells were seeded in a density of 2×10^4 cells/well in a 96-well plate then incubated for 18-24 h. The following day the compounds/extracts were added and then incubated for 24 h before the media was aspirated and the

cells washed twice in a gentle stream of water. After removing the water, 50 μ L of 0.5 % crystal violet staining solution were added and the cells incubated for 20 min at room temperature. The plate was then washed 4 times with water and air dried for 2 h. Finally, 200 μ L of methanol was added to each well and incubated for 20 min at room temperature on a rocker. This was followed by measuring the optical density at 570 nm with a plate reader Anthos Zenyth 200rt (Anthos Labtech Instruments). Chlorambucil in gradient concentration (300, 100, 33.3, 11.1, 3.7 and 1.2 μ g/mL) was used as a positive control.

3.3.8. NO inhibition assay:

The NO inhibition assay was conducted according to published procedures ([Gunawardena et al., 2014b](#), [Whitehouse et al., 1997](#), [Yun et al., 2008](#), [Phanse, 2012](#), [Kim et al., 2015](#), [Wei et al., 2013](#)). RAW264.7 macrophages were seeded in a 96 well plate at a concentration of 6×10^5 cells/mL in colour-free 10 % FBS DMEM and incubated at 37 °C in 5 % CO₂ overnight. RAW264.7 macrophages were incubated 1hr with extracts/compounds prior to the addition of LPS. Dexamethasone at a concentration of 2.5 μ M was used as a positive control and 0.35 % DMSO as a negative control. Then, 20 h after the addition of LPS, the supernatant was collected. The concentration of nitrite in the supernatant was measured using the Greiss reaction by adding equal volume of the supernatant and Greiss reagent (0.1 % NED; 1 % sulfanilic acid in 5 % orthophosphoric acid) in a 96 well plate. The plates were incubated for 15-20 min in the dark, then read at 550 nm wavelength on Anthos Zenyth 200rt (Anthos Labtech Instruments). Sodium nitrite was used to prepare a standard curve to quantify the nitrite from the absorbance readings in this assay.

3.3.9. TNF-alpha inhibition assay:

The amount of TNF α was quantified in cell culture media using murine TNF α ELISA kits (BD bioscience). In brief, the RAW264.7 mouse macrophages (6×10^5 cells/mL) were stimulated with LPS (100 ng/mL) 1h after treatment with several concentrations of extracts (0.08–50 μ g/mL). After 18 h, the supernatant was collected and TNF α production was measured by reading the absorbance

at 450 nm wavelength on Anthos Zenyth 200rt microplate reader (Anthos Labtech Instruments). Dexamethasone at a concentration of a 2.5 μ M was used as a positive control, with a negative control of 0.35 % DMSO.

3.3.10. PGE2 inhibition assay:

The levels of PGE2 were measured in cell media using Cayman Prostaglandin E₂ Express ELISA Kit (Cayman) according to manufacturer's instruction. In brief, the 3T3 ccl-92 fibroblasts (1×10^5 cells/mL) were grown overnight in a 96-well plate and then treated with extracts/compounds 3 h before stimulated with Calcium ionophore (50 μ M). After 20 min supernatants then were collected and PGE2 production was quantified by measuring the absorbance at 405 nm wavelength with Anthos Zenyth 200rt (Anthos Labtech Instruments). Indomethacin at a concentration of 100 μ M was used as a positive control and ~0.5% DMSO as a negative controls.

3.3.11. Assessment of NF κ B translocation:

This assay was performed according to the published protocol in [Olivera et al. \(2012\)](#). RAW264.7 mouse macrophages were seeded at 400,000 cells/well in 800 μ L in 4-well chambered slides and incubated overnight. The cells were then incubated with the test samples (40 μ g/mL HBG extract, 6-bromoisatin or 6-bromoindole) for 1 h prior to stimulation with LPS (700 ng/mL) for 30 min. After stimulation, the cells were washed with PBS and fixed with 3.7 % paraformaldehyde for 15 min. The fixed cells were then washed with PBS three times and permeabilised using 0.2 % Triton-X 100 for 10 min, followed by a PBS wash and finally blocked using the 0.1 % bovine serum albumin (BSA) for 1h. The cells were washed with PBS and incubated with rabbit anti-p65 NF κ B antibody (1:500) and goat anti-mouse I κ B (1:500) overnight at 4°C. The following day, the cells were washed again three times with PBS and incubated 1h with goat-anti-rabbit IgG conjugated with Alexa Fluor 594 (1:1000) and donkey anti-goat IgG conjugated with Alexa fluor 488 (1:1000) at room temperature. Finally, the cells were washed with PBS then mounted with Prolong mounting media containing DAPI before visualising using the Olympus Flouview i10 confocal microscope. Using ImageJ

(v1.50i, National Institutes of Health, USA), an outline was drawn around each nucleus by adjusting the colour threshold of DAPI. Mean fluorescence inside the nucleus was measured, along with several adjacent background readings. The total corrected cellular fluorescence (TCCF) was calculated by applying the equation $TCCF = \text{integrated density} - (\text{area of selected cell} \times \text{mean fluorescence of background readings})$ (McCloy et al., 2014). This TCCF was then used to calculate the %inhibition of the NF κ B translocation against the positive control where the cells were treated with LPS in the presence of the Vehicle (0.35 % DMSO v/v) in Microsoft Office Excel 2013 (MS).

3.3.12. Statistical analysis:

All data were processed in Microsoft office Excel 2013 (MS) first to obtain descriptive statistics. One-way Analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com), with $p < 0.05$ considered significant. The inhibitory concentration responsible for a 50 % reduction in cell viability (LD₅₀) or production of inflammatory markers (IC₅₀) was calculated using the regression analysis, Probit in SPSS v21.

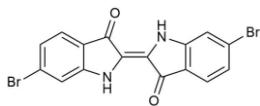
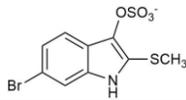
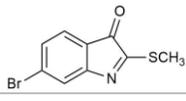
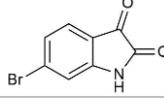
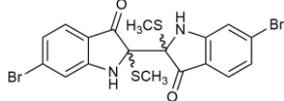
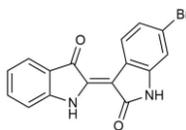
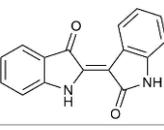
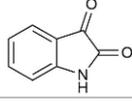
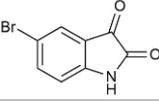
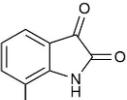
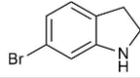
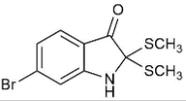
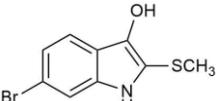
3.4. Results

3.4.1. Chemical analysis of the crude extracts:

LC-MS separations of the chloroform extract confirmed the presence of brominated indoles typically found in *D. orbita* (Figure 3.1, Appendix 3) (Benkendorff, 2013). The fresh hypobranchial gland chloroform extract and the egg mass chloroform extract were dominated by 6-bromoistain (pseudomolecular ion [M+H]⁺ m/z 226, 228; [M+Na]⁺ m/z 248, 250, UV λ_{max} 212, 256, 308, and 408 nm) and tyrindoleninone (pseudomolecular ion [M+H]⁺ m/z 256, 258, UV λ_{max} 236, 248, 274, 352, 402 nm) respectively, and contained smaller amounts of 6-bromoindole (pseudomolecular ion [M+2H]⁺ m/z 198, 200, UV λ_{max}) and tyriverdin (pseudomolecular ion [M+Na]⁺ m/z 535, 537, 539; UV λ_{max} 236, 252, 274, 352, 402, and 596 nm) (Figure 3.1A). Another brominated indole, tentatively identified as tyrindolinone (C₁₀H₁₀BrNOS₂) (Benkendorff, 2013), detected in hydrated form

pseudomolecular ion $[M+H]^+$ 306, 308, UV λ_{\max}), was more abundant in the hypobranchial gland extracts (Figure 3.1A). The degraded hypobranchial gland extract did not appear to contain bromoistain or tyrindoleninone (Figure 3.1C), and instead was dominated by unidentified compounds that did not produce paired isotopic ions indicative of brominated indoles. Due to the lack of activity the degraded extract was not further characterized. The methanol extract of the hypobranchial gland was dominated by the precursor tyriindoxyl sulfate (pseudomolecular ion detected in negative ion mode $[MH]^-$ m/z 336, 338, UV λ_{\max} 225 and 300nm) and contained some murexine ($[M]^+$ m/z 224, UV λ_{\max} 266 (Figure 3.1D).

Table 3.1. Chemical structure properties and source of compounds used in study.

	Chemical structure	Molecular Weight¹	Log value²	<i>P</i>	Synthetic vs. purified
1		420.06	4.47		Synthetic
2		337.18	-0.346		Semi-purified (methanol extract)
3		256.12	2.89		Purified
4		226.03	1.61		Synthetic
5		514.25	4.66		Purified
6		356.18	4.08		Synthetic
7		262.27	2.9		Synthetic
8		147.13	0.83		Synthetic
9		226.03	1.61		Synthetic
10		226.03	1.59		Synthetic
11		196.05	2.94		Synthetic
12		304.22	3.00		Semi-purified (chloroform extract)
13		258.13	3.38		Semi-purified (chloroform extract)

¹ The accurate mass based on the average of Br⁷⁹ and Br⁸¹ isotopes. ² The predicted log *P* values were calculated with molecular modelling using Molinspiration© Cheminformatics (2016)

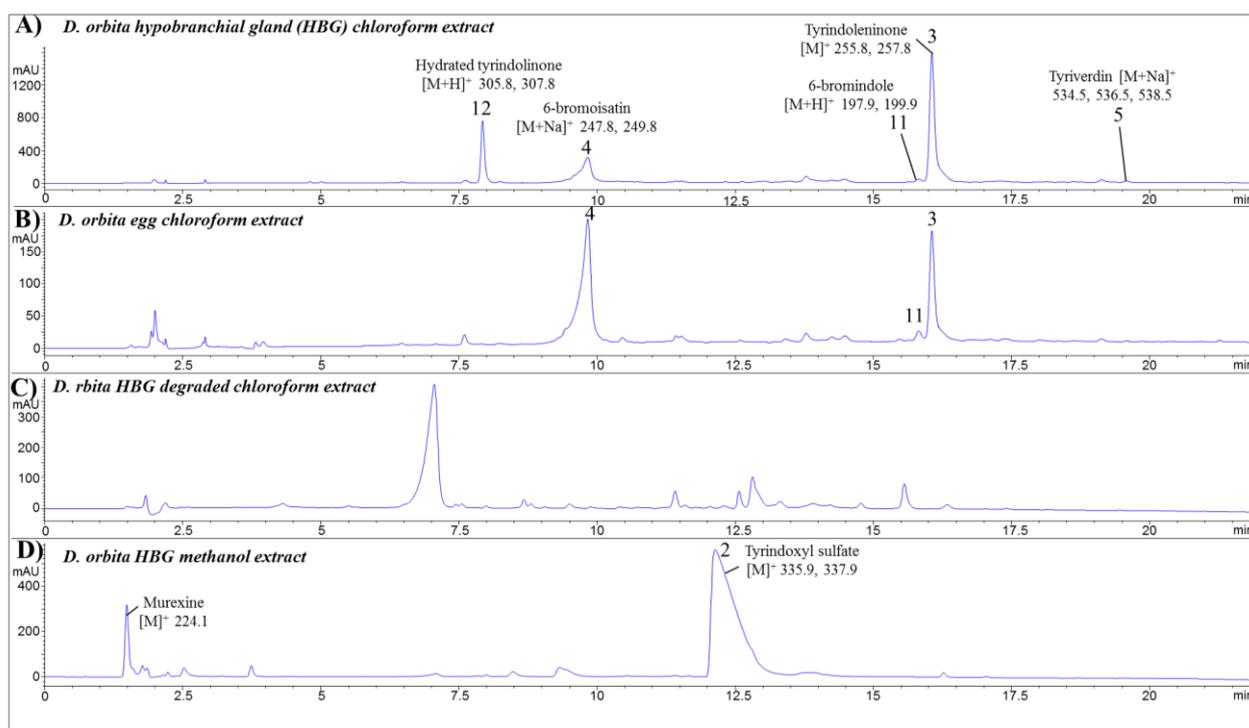


Figure 3.1: LC-MS chromatographs of several extracts from *Dicathais orbita* showing brominated indoles identified by mass spectrometry (molecular ions for Br⁷⁹, Br⁸¹). A) chloroform extract of the hypobranchial gland; B) chloroform extract of the egg mass; C) degraded chloroform extract of the hypobranchial gland and D) methanol extract of the hypobranchial gland. Mass spectra for the main brominated indoles are shown in appendix 2.

GC-MS analysis of the hypobranchial gland chloroform extract further confirmed the dominant volatile compounds in the extract were brominated indoles (Figure 3.2, Appendix 2). Based on comparison to mass spectral databases (≥ 98 % match) these compounds were identified as methyl 6-bromoisatin, 6-bromo-2,3 dihydro indole-2,3-diol, 6-bromindoxyl, tyrindolinone, tyrindoxyl and 6-bromoisatin (Figure 3.2A). Tyrindoxyl is a transient compound that readily oxidizes to tyrindoleninone (Cooksey, 2001, Baker and Duke, 1973). GC-MS confirm that the purity of tyrindoleninone in the isolated orange fraction eluted from the silica column with 20 % DCM in hexane.

Tyriverdin is not volatile and highly insoluble in all solvent. It precipitated out of solution during LC-MS, hence this semi-purified compound could only be tentatively identified using TLC (r.f value = 0.32) and the change from a green to purple colour following exposure to light confirmed it as mostly likely to be tyriverdin (Benkendorff et al., 2000).

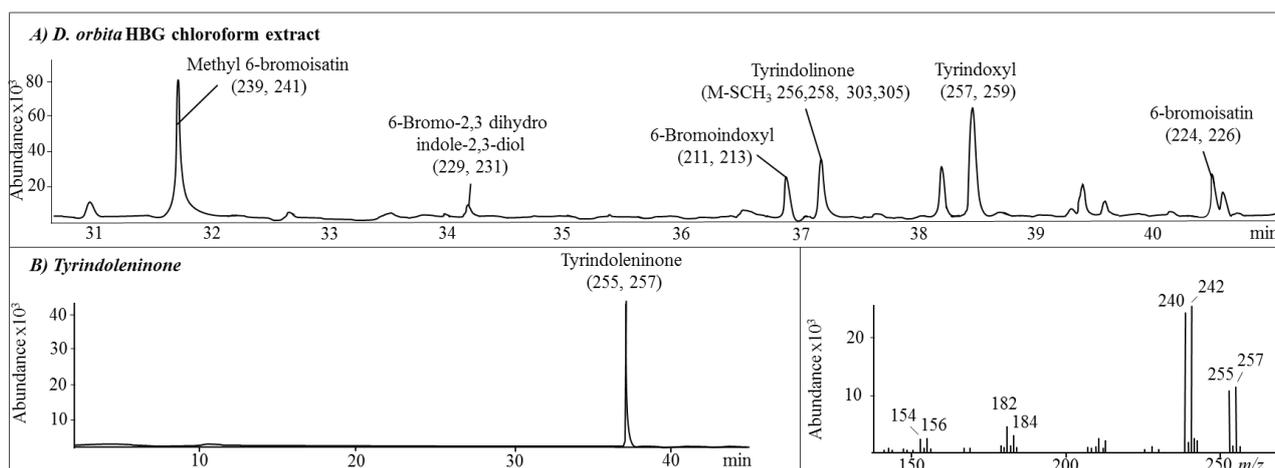


Figure 3.2: Gas chromatography mass spectrometry of the chloroform extracts from *Dicathais orbita* showing brominated indoles identified by mass spectrometry (molecular ions for Br^{79} , Br^{81}). GC-MS chromatograms of the A) hypobranchial gland chloroform extract; B) purified fraction containing tyrindoleninone, with associated mass spectral fragmentation pattern. The brominated compounds were identified based on characteristic mass isotopic patterns for brominated indoles (Br^{79} and Br^{81}), and matched to the NIST02 database. The structures for the main compounds tested are provided in Table 2. The mass spectra for the brominated indoles in the extract are shown in Appendix 3.

3.4.2. Cytotoxicity assays:

There was no cytotoxicity observed for any of the *D. orbita* extracts used in this study, at concentrations up to 50 $\mu\text{g}/\text{mL}$, against both RAW264.7 and 3T3 cell lines (Table 3.2) (cell viability was 100 % in all triplicate wells after 24h of incubation). Therefore, this concentration was chosen as the highest concentration for anti-inflammatory testing. The LD_{50} for all test compounds and extracts was much higher than the maximum test concentration (Table 3.2). However, at 50 $\mu\text{g}/\text{mL}$, 6-bromoindirubin, tyrindoleninone, 5-bromoisatin, 7-bromoisatin and 6-bromoindole showed some toxicity toward RAW264.7 cells after 24h of incubation with an average % viability of 56.2 %, 84.1 %, 55.0 %, 81.9 % and 66.4 % respectively, in comparison to solvent controls. Only 7-bromoisatin showed slight toxicity at 50 $\mu\text{g}/\text{mL}$ against 3T3 fibroblast cell line after 24h of incubation, with 80.9 % viability when compared to the DMSO-treated wells. The LD_{50} of the positive control drug chlorambucil was 14 $\mu\text{g}/\text{mL}$ (46.02 μM) for RAW 264.7 macrophages and 17 $\mu\text{g}/\text{mL}$ (55.88 μM) for 3T3 fibroblasts.

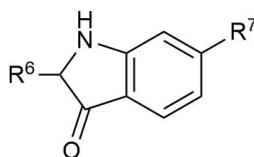
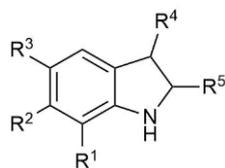
3.4.3. NO inhibition assay:

The chloroform extracts from *D. orbita* (hypobranchial gland and egg extract) used in this study showed significant inhibition of NO production by LPS-stimulated RAW 264.7 cells at concentrations down to 10 µg/mL, in comparison to the DMSO control ($p < 0.001$) (Figure 3.3A). At the maximum test concentration of 50 µg/ml, the hypobranchial and the egg extract reduced NO production on average by 80 % and 57 % respectively, compared to the well-known anti-inflammatory drug dexamethasone, which caused 56 % inhibition at the working concentration at 2.5 µM (Figure 3.3A). The methanol extract and the degraded hypobranchial gland chloroform extract, however, showed no inhibition of NO production in the LPS- stimulated RAW264.7 mouse macrophages. The methanol extract was dominated by tyrindoxyl sulfate (Figure 3.1B), so the lack of NO inhibitory activity in this extracts indicates that this brominated indoxyl-sulfate salt also does not have any associated activity (Table 3.2).

Table 3.2: Anti-inflammatory Inhibitory Concentration 50 (IC₅₀) for the brominated indole natural products from *Dicathais orbita* and synthetic analogues. NA = not active at maximum test concentration 50 µg/mL; NT = Not tested.

X =

Y =



Compound	R ₁	R ²	R ³	R ⁴	R ⁵	R ⁶	R ⁷	Cytotoxicity* RAW264.7	Cytotoxicity* 3T3 ccl-92	NO IC ₅₀ µM	TNFα IC ₅₀ µM	PGE2 IC ₅₀ µM
6,6 dibromoindigo (1)	H	Br	H	O	Y	X	Br	NA	NA	NA	NT	NT
Tyrindoxyl sulfate (2)	H	Br	H	OSO ₃ ⁻	SCH ₃	-	-	NA	NA	NA	NT	NT
Tyrindoleninone (3)	H	Br	H	O	SCH ₃	-	-	>195.22	NA	103	157	NT
6-bromoisatin (4)	H	Br	H	O	O	-	-	NA	NA	120	123	>221.21
Tyriverdin (5)	H	Br	H	O	SCH ₃ , Y	SCH ₃ , X	Br	NA	NA	>97.23	NT	NT
6-bromoindirubin (6)	H	Br	H	Y	O	X	H	~140.38	NT	NA	NT	NT
Indirubin (7)	H	H	H	Y	O	X	H	NA	NA	NA	NT	NT
Isatin (8)	H	H	H	O	O	-	-	NA	NA	339.83	>339.83	NT
5-bromoisatin (9)	H	H	Br	O	O	-	-	~221.21	NA	152	38	NT
7-bromoisatin (10)	B r	H	H	O	O	-	-	>221.21	>221.21	NA	NT	NT
6-bromoindole (11)	H	Br	H	H	H			>255.04	NA	187	150	223

* The test concentration (µM) which cause toxicity (reduction in cell viability) toward the cell line after 24 h of incubation.

The purified brominated indoles tyrindoleninone and tyriverdin significantly inhibited the production of NO, down to 0.08 µg/mL and 10 µg/mL respectively ($p < 0.05$, Figure 3.3B). The IC₅₀ for tyrindoleninone was 26.4 µg/mL (103 µM), whereas tyriverdin was greater than the maximum test concentration (Table 3.2). The synthetic version of the natural product 6-bromoisatin significantly inhibited NO production down to 0.4 µg/ml (Figure 3.3B), with an IC₅₀ 27.1 µg/mL (120 µM) (Table 3.2). The non-brominated compound isatin showed weak NO inhibitory activity causing on average 34 % reduction at 50 µg/mL (Figure 3.3C), with a predicted IC₅₀ of 63.3 µg/mL (430 µM). The synthetic 5-bromoisatin had higher activity (Figure 3.3C) with predicted IC₅₀ of 34.3 µg/mL (151.6

μM , Table 3.1), whilst 7-bromoisatin showed lower activity, with less than 5 % inhibition at 50 $\mu\text{g}/\text{mL}$ (Figure 3.3C). The 6-bromoindole showed similar activity to 5 and 6-bromoisatin (Figure 3.3C, Table 3.2). However, all the synthetic dimers including indirubin, 6,6 dibromoindigo and 6-bromoindirubin showed minimal inhibitory activity (Figure 3.3D).

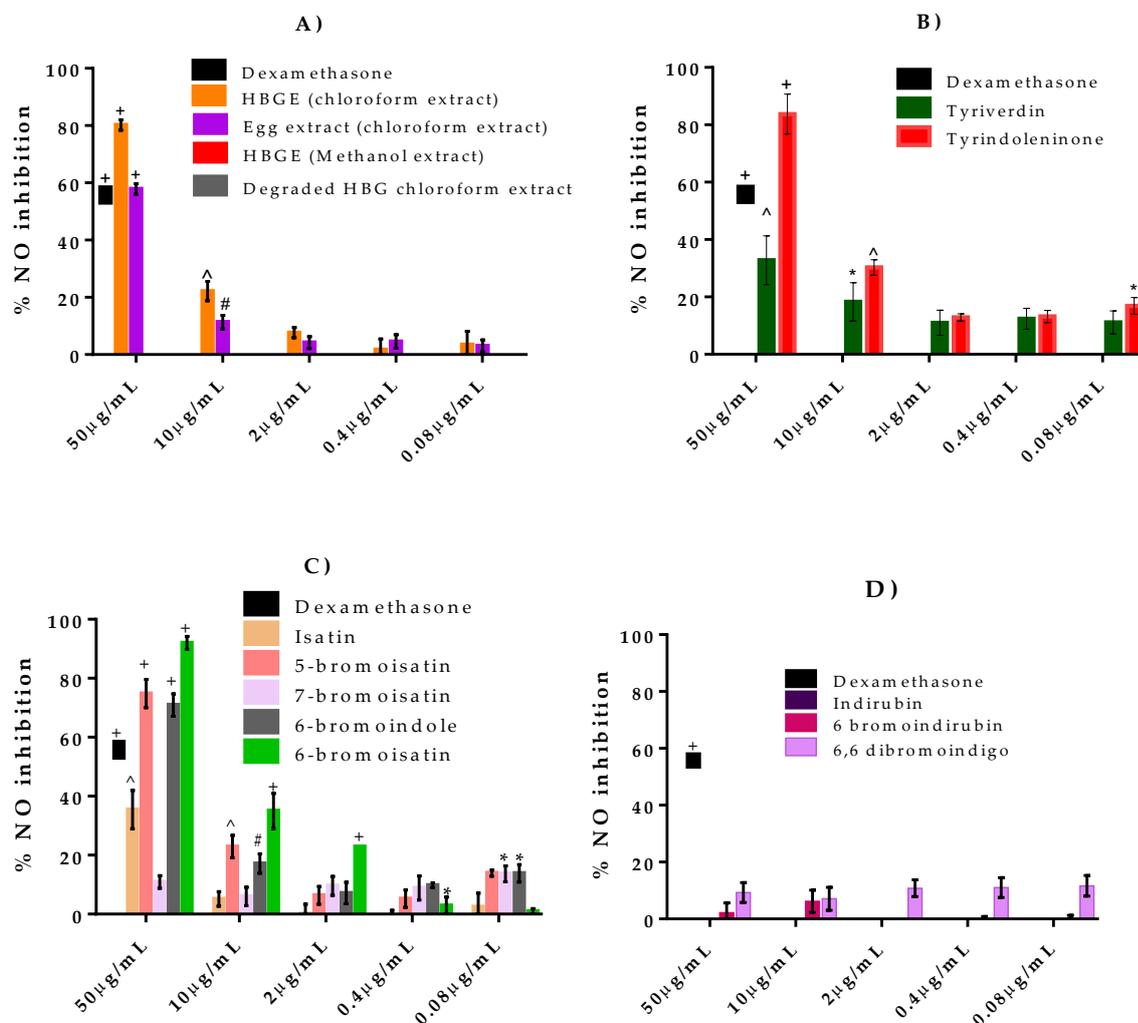


Figure 3.3: NO inhibition in RAW264.7 mouse macrophages stimulated with LPS and treated with extracts from *D. orbita*, purified brominated indoles and synthetic analogues. Data are expressed as % inhibition of NO production relative to the negative control DMSO: A) extracts from the hypobranchial glands (HBG) and egg capsules; B) the purified natural products tyrindoleninone and tyriverdin; C) the monomer synthetic indoles isatin, 5-bromoisatin, 7 bromoisatin and 6-bromoindole; D) the dimer synthetic indoles indirubin, 6-bromoindirubin and 6,6 dibromoindigo. Data shown are means \pm SEM from three separate experiments performed in triplicates. The symbols above the bars indicate statistically significant differences in the amount of nitrite in the treatments compared to DMSO control. * $p < 0.05$, # $p < 0.01$, ^ $p < 0.001$, + $p < 0.0001$ versus untreated, stimulated cells (LPS + DMSO). Dexamethasone at a concentration of 2.5 μM was used as a reference drug.

3.4.4. TNF α inhibition:

The levels of TNF α in the supernatant were measured in LPS-stimulated RAW264.7 pre-treated for one hour with the chloroform extract from the hypobranchial gland (HBGE), isatin, 5-bromoisatin, 6-bromoisatin, 6-bromoindole and tyrindoleninone (Figure 3.4). The HBGE showed significant inhibition of TNF α , down to 0.4 μ g/mL ($p < 0.01$). At 50 μ g/ml this extract caused > 60 % inhibition of TNF α , which was greater than the positive control dexamethasone (Figure 3.4A). All the brominated indoles tested also showed significant inhibition and for 5-bromoisatin at 50 μ g/ml, inhibition reached 100 %, with IC₅₀ 38.05 μ M (Table 3.2). The IC₅₀ of the non-brominated indole, isatin, was predicted to be 717.27 μ M (above the maximum test concentration), and much less active than the brominated indoles 6-bromoisatin (122.65 μ M), 6-bromoindole (150.01 μ M) and tyrindoleninone (157.12 μ M) (Table 3.2).

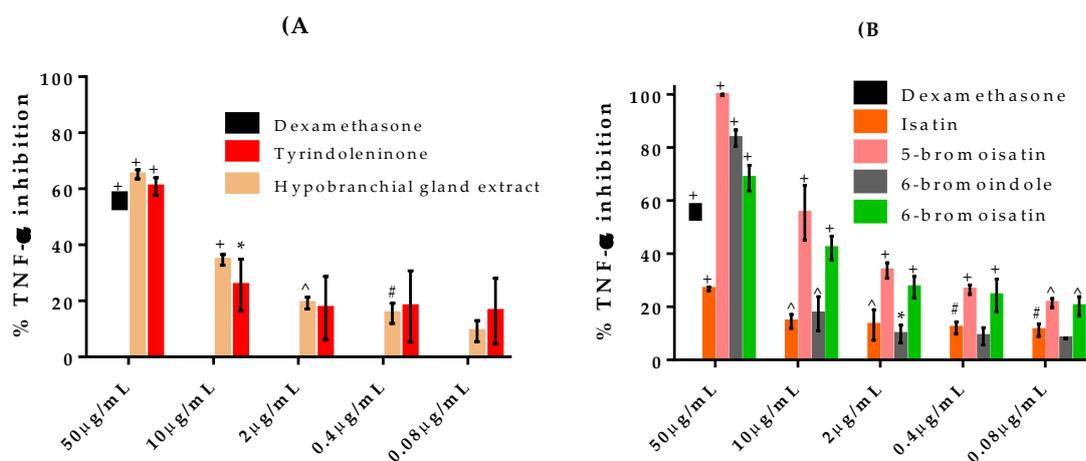


Figure 3.4: Percent inhibition of TNF α in LPS stimulated RAW264.7 macrophages after treatment with *Dicathais orbita* hypobranchial gland (HBG) extract and associated brominated indoles. A) chloroform extract and tyrindoleninone, purified from the HBG extract; B) synthetic isatin and indole compounds. Data shown are means \pm SEM from three separate experiments performed in triplicate. The symbols above the bars indicate statistical significance of the differences in the amount of TNF α in the samples compared to DMSO control. * $p < 0.05$, # $p < 0.01$, ^ $p < 0.001$, + $p < 0.0001$ versus LPS + DMSO. Dexamethasone at a concentration of 2.5 μ M was used as a reference drug.

3.4.5. PGE2 inhibition assay:

The effect of different concentrations of the HBG chloroform extract and the naturally occurring compounds, 6-bromoisatin and 6-bromoindole, on the generation of PGE2 in 3T3 ccl-92 fibroblast was examined. The highest inhibition rate was found using the HBG extract, which inhibited the

production of PGE2 by more than 65 % at 50 $\mu\text{g}/\text{mL}$ ($p < 0.0001$), which is greater than the inhibition caused by Indomethacin at the 100 μM concentration (Figure 3.5). In addition, 6-bromoindole caused a significant dose response for PGE2 inhibition, with significant inhibition compared to the solvent control even at the lowest concentration (0.08 $\mu\text{g}/\text{mL}$) ($p < 0.05$) and IC_{50} of 223.28 μM (Table 3.2). Similarly, 6-bromoisatin caused a significant dose response with 40 % inhibition at 50 $\mu\text{g}/\text{mL}$ and just 10 % inhibition at 0.08 $\mu\text{g}/\text{mL}$ ($p < 0.0001$), with an IC_{50} of 293.02 μM (Table 3.2).

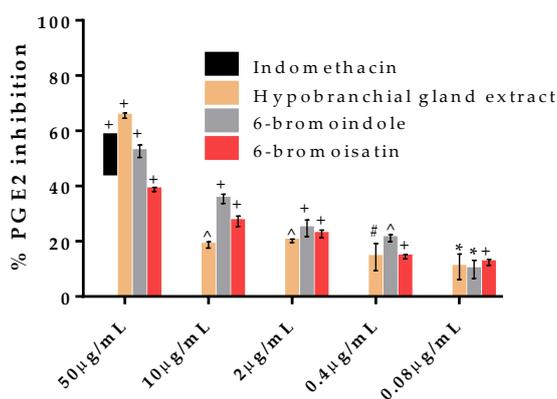


Figure 3.5: PGE2 inhibition in calcium ionophore stimulated 3T3 ccl-92 fibroblasts after exposure to a chloroform extract from the hypobranchial glands of *Dicathais orbita* and the associated brominated compounds 6-bromoisatin and 6-bromoindole. Data shown are means \pm SEM from three separate experiments performed in triplicates. Symbols above the bars indicate statistically significant differences in the amount of PGE2 produced in the samples compared to DMSO treatment. * $p < 0.05$, # $p < 0.01$, ^ $p < 0.001$, + $p < 0.0001$ versus LPS + DMSO. Indomethacin at a concentration of 100 μM was used as a reference drug.

3.5. Assessment of NF κ B translocation:

To investigate the mode of action of the active extract and brominated indoles, the ability to inhibit the translocation of the NF κ B in LPS-stimulated RAW264.7 was assessed. LPS caused marked increase in the translocation of NF κ B into the nucleus, as indicated by significant increases in the red fluorescence intensity inside the nucleus (Figure 3.6A&B). The HBG chloroform extract, 6-bromoisatin, isatin and 6-bromoindole at 40 $\mu\text{g}/\text{mL}$ concentration were all found to cause a noticeable inhibition of the translocation of NF κ B in the LPS-induced RAW264.7 cells, compared to DMSO (Figure 3.6A). 6-Bromoindole inhibited the translocation of NF κ B by 63.2 % on average, whereas 6-bromoisatin, HBG extract and isatin caused 60.7, 48.2 and 45.7 % inhibition respectively (Figure 3.6C).

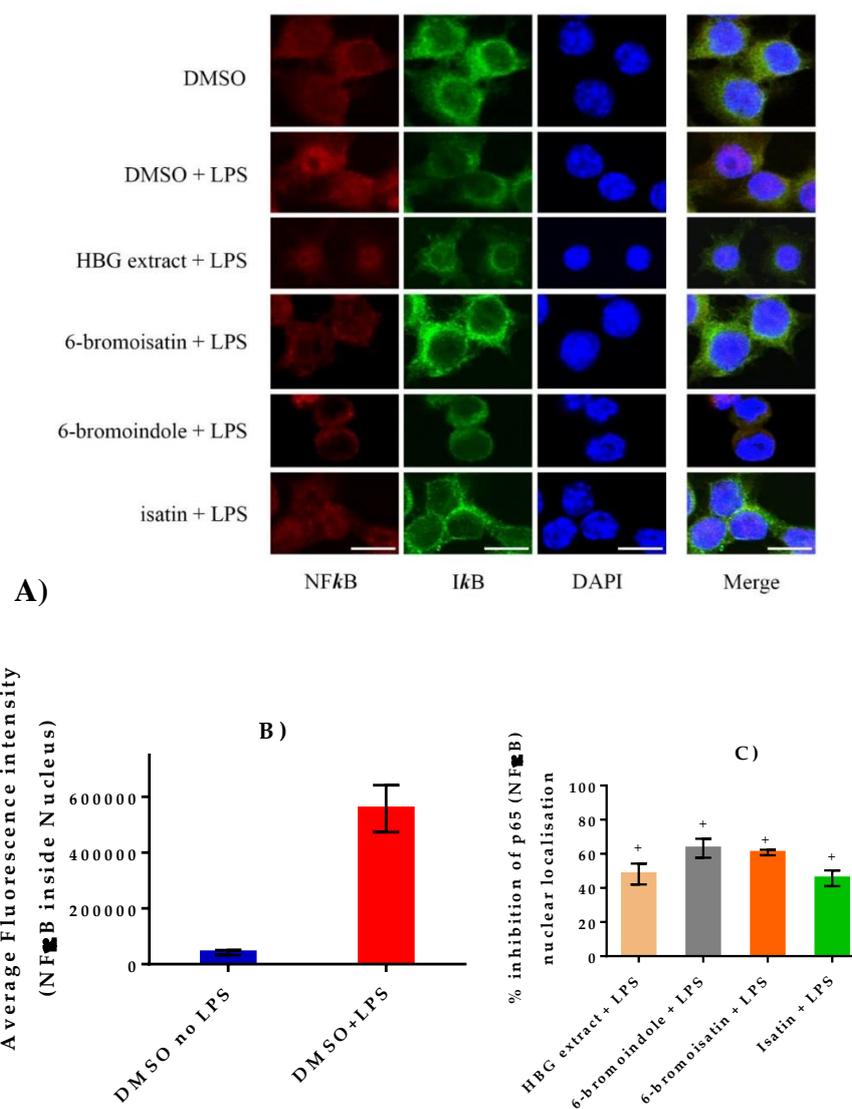


Figure 3.6: The inhibition of NFκB translocation by HBG extract, isatin, 6-bromoisatin, and 6-bromoindole. A) representative images of the RAW264.7 cells obtained by Olympus FV i10 confocal microscope showing the effect of each treatment on the translocation of the p65 subunit of the NFκB stained with Alexa fluor 594 (red fluorescence) into the nucleus. The inhibitor subunit was stained with Alexa fluor 488 (green fluorescence) to highlight the inactivated NFκB in the cytoplasm. DAPI (blue) was used to stain the nucleus. Scale bar set to 10 μm. B) LPS-induced activation of NFκB in RAW264.7 showing the average intensity of the NFκB fluorescence (red) inside the nucleus: C) mean % NFκB inhibitory activity of the HBG extract and the synthetic compounds 6-bromoistatin, isatin and 6-bromoindole based on the reduction of fluorescence intensity relative to DMSO + LPS stimulated control. All test compounds/extracts were tested at a final concentration of 40 μg/mL. Data shown are mean ± SEM from three separate experiments. “+” = $p < 0.0001$ versus LPS + DMSO. All data were obtained using the image processing and analysis software ImageJ (<https://imagej.nih.gov/ij/>).

3.6. Discussion

The development of new natural products or drugs that prevent the over-production of NO and pro-inflammatory cytokines like TNFα and PGE2, has become an important focus of research for the treatment chronic inflammatory diseases. In this study, crude organic extracts from the Australian muricid *D. orbita* were tested and found to have NO, TNFα and PGE2 inhibitory activity, but no

cytotoxicity at the active concentrations. Consistent with previous studies, the active lipophilic (chloroform) extracts from the hypobranchial glands and egg masses were found to contain the brominated indole intermediate precursors to Tyrian purple dye ([Benkendorff, 2013](#)). Purification of the chloroform extract led to the isolation of tyrindoleninone and tyriverdin, both of which were found to significantly inhibit NO production, along with 6-bromoisatin and several other synthetic monobrominated indoles. By comparison, the synthetic indole dimer pigments and the polar methanol extract containing the salt precursors tyrindoxyl sulfate and murexine were not found to inhibit the production of NO in LPS stimulated RAW cells. This indicates that extracts from *D. orbita* containing small brominated indole have potential use as anti-inflammatory agents, in addition to their previously reported anticancer properties ([Esmaelian et al., 2014](#), [Esmaelian et al., 2017](#), [Westley et al., 2010b](#)).

A range of synthetic brominated indole derivatives were tested to establish the structure-activity relationships for NO inhibition. Isatin showed mild anti-inflammatory activity significantly inhibiting the production of NO at 50 at $\sim 339.8 \mu\text{M}$ and TNF α at concentrations down to $0.5 \mu\text{M}$ (but with IC₅₀ over the maximum test concentration $50 \mu\text{g/ml}$). This activity is generally consistent with that reported by [Matheus et al. \(2007\)](#), who found that isatin significantly inhibited LPS and interferon- γ induced NO production in RAW264.7 cells, at even lower concentrations of 10-100 μM , whereas TNF α was inhibited at 100 μM , although they did not establish the minimum effective dose. These researchers also tested a range of simple halogenated isatin derivatives and found that whilst most were active in the test range, the specific activity varied according to the halogen substituent and position. Likewise, our results indicate the presence and position of bromine on the aromatic ring can affect the activity, with 6-bromoistain > 5-bromoistain > isatin > 7-bromoisatin for NO inhibition, whereas for TNF α inhibition 5-bromoisatin > 6-bromoisatin > isatin. ([Matheus et al., 2007](#)) reported that 4-bromoisatin and 5-iodoisatin did not inhibit NO production or PGE₂, whereas three chlorinated isatin derivatives and 5-flouroisatin inhibited NO and PGE₂ at similar concentrations to isatin. In the TNF α production assay, however, 5-iodoisatin = 6-chloroisatin > isatin > 5-chloroisatin = 7-chloroisatin > 4-

bromoisatin = 4-flourisatin ([Matheus et al., 2007](#)). These results indicate that the anti-inflammatory activity of isatins depends on both the position and type of halogen substituent, with different patterns for TNF α compared to NO and PGE2 inhibition suggesting there may be more than potential target site or mode of action ([Matheus et al., 2007](#)). Nevertheless, the results from both studies overall suggest that substitution of bromine into the C₅ and C₆ position on isatin based compounds effectively increases the anti-inflammatory activity. Previous studies on the anticancer activity of isatin derivatives also suggests that bromine substitution on C₅ or C₆ can result in increased biological activity ([Lee et al., 2001](#), [Hoessel et al., 1999](#), [Vine et al., 2007](#)).

In addition to the brominated isatin derivatives, we found similar anti-inflammatory activity with synthetic 6-bromoindole and naturally derived 6-brominated indole derivatives. This suggests that the specific functional group in position 2 on the indole ring may not be important for the inhibition of NO, PGE2 or TNF α . Nevertheless, dimerisation of the indoles was found to substantially reduce the activity. The lack of inhibitory activity associated with the brominated indirubin and indigo dimers could be due to their insolubility, as indicated by their calculated log *p* values of >4 (Table 3.2). [Kim and Park \(2012\)](#) synthesised a soluble 3 monoxime derivative of indirubin and demonstrated significant anti-inflammatory activity, including inhibition of NO and PGE2. However, in our study, the relatively soluble nonbrominated indirubin was also not active and showed a tendency towards stimulating the production of NO in LPS stimulated RAW cells. This result was unexpected given that indirubin is known to inhibit glycogen synthase kinase-3 (GSK3) ([Hoessel et al., 1999](#)), which promotes anti-inflammatory responses including LPS induced NO production ([Yuskaitis and Jope, 2009](#)). Furthermore, indirubin has been identified as an active ingredient in the traditional medicinal plants *Isatis tinctoria* and *Polyglonum tinctorium* used to treat inflammation and has demonstrated *in vivo* anti-inflammatory properties ([Kunikata et al., 2000](#), [Hamburger et al., 2015](#), [Lai et al., 2017](#)). Nevertheless, consistent with our results, indigo and alkaloid fractions obtained from the plant *Indigofera suffruticosa* have been shown to stimulate NO production ([Lopes et al., 2011](#)). This

suggests indigo and indirubin may have immunomodulatory activity that depends on the specific cell type and stimulation.

Recent studies showed that many anti-inflammatory drugs, including dexamethasone, can suppress the production of the pro-inflammatory factors by inhibiting the translocation of NF κ B ([Murakami and Ohigashi, 2007](#), [Krakauer, 2004](#)). Here we provide preliminary evidence that the hypobranchial glands extract of *D. orbita*, along with isatin, 6-bromoisatin and 6-bromoindole exerted anti-inflammatory effects by inhibiting the translocation of NF κ B, thus resulting in the inhibition of NO, TNF α and PGE2 production. Consistent with this, the indirubin analogue indirubin-3-monoxime was found to downregulate NF κ B activation, correlating with a reduction of inducible NO synthase (iNOS) and cyclooxygenase-2 ([Kim and Park, 2012](#)). Inhibiting the translocation of NF κ B prevents the undesirable over production of pro-inflammatory cytokines and NO from the iNOS pathway. [Matheus et al. \(2007\)](#) found that isatin and its halogenated derivatives significantly inhibit iNOS and COX-2 protein expression, thus resulting in a significant reduction of NO and PGE2 production. The inhibition of NF κ B translocation signalling pathway is consistent with the dual anti-inflammatory activities and anticancer activities of halogenated indole, isatin and indirubin derivatives from Muricidae molluscs ([Meijer et al., 2003](#), [Meijer et al., 2006](#), [Edwards et al., 2012](#), [Esmaeelian et al., 2014](#), [Vine et al., 2007](#)).

In conclusion, this study supports the nutraceutical potential of extracts from the hypobranchial glands of the Australian muricid *D. orbita* for anti-inflammatory applications. It also confirms that simple brominated isatins have anti-inflammatory activity including the inhibition of NO, PGE2 and TNF α , which are likely to be mediated by inhibition of NF κ B translocation, thus contributing to potential application and development as anti-inflammatory and anticancer agents

Chapter 4:

Brominated indoles from a marine mollusc inhibit inflammation in a murine model of acute lung injury

Chapter 3 of this thesis incorporates the following publication:

AHMAD, T. B., RUDD, D., BENKENDORFF, K., MAHDI, L. K., PRATT, K. A., DOOLEY, L., WEI, C. & KOTIW, M. 2017. Brominated indoles from a marine mollusc inhibit inflammation in a murine model of acute lung injury. *PLoS One*, 12, e0186904.

4. Chapter 4: Brominated indoles from a marine mollusc inhibit inflammation in a murine model of acute lung injury

4.1. Abstract

New drug leads for the treatment of inflammation are urgently needed. Marine molluscs are widely used as traditional medicines for the treatment of inflammation. Here we report the positive effects of a hypobranchial gland (HBG) extract and the dominant bioactive compound 6-bromoisatin from the Muricidae mollusc *Dicathais orbita*, for reducing lipopolysaccharide (LPS) induced acute lung inflammation in a mouse model. Both 6-bromoisatin and the HBG extract, suppressed the inflammatory response in mice that were pre-treated by oral gavage at 48, 24 and 1 h prior to LPS infusion. The inflammatory antagonists were tested at concentrations of 0.5 mg/g and 0.1 mg/g HBG extract and 0.1 mg/g and 0.05 mg/g 6-bromoisatin in carrier oil and all treatments reduced inflammation as indicated by a significant suppression of inflammatory markers present in bronchoalveolar lavage fluid (BALF), in comparison to LPS induced positive control mice administered the carrier oil alone ($p < 0.0001$). Tumour necrosis factor-alpha ($\text{TNF}\alpha$) and interleukin-1 beta ($\text{IL-1}\beta$) levels, in addition to total protein concentration were all significantly reduced in BALF from mice treated with the extract or 6-bromoisatin. Furthermore, all treatment groups showed significant reductions in neutrophil sequestration and preservation of the lung tissue architecture compared to the positive control ($p < 0.0001$). The combined results from this study and our previous *in vitro* studies indicate that 6-bromoisatin in the HGB extracts inhibit the activation of inflammatory signalling pathway. The results from this study further confirm that the HBG extract from Muricidae molluscs and 6-bromoisatin are bioavailable and effective *in vivo*, thus have potential for development as natural therapeutic agents for inflammation.

4.2. Introduction

Inflammation is a natural immune response to infection, but if left unchecked can lead to tissue damage and chronic disease. Acute lung inflammation (ALI) is a life-threatening syndrome that can lead to multisystem organ failure and is a significant cause of morbidity and mortality worldwide ([Kumari et al., 2015](#), [von Dossow-Hanfstingl, 2012](#)). A high incidence of ALI is associated with infection by Gram negative pathogenic bacteria, as these bacteria contain lipopolysaccharides (LPS) as a major component of the outer cell membrane. The Toll-Like Receptors-4 (TLR-4) found on alveolar macrophages and epithelial cells recognise LPS during lung infection and play a critical role in initiating the host's immune response ([Kumari et al., 2015](#)). This response often leads to the activation of the inflammatory nuclear transcription factor Kappa B (NFκB) pathway in the alveolar macrophages and epithelial cells, resulting in a marked increase in the production of key pro-inflammatory cytokines such as tumour necrosis factor-α (TNFα) and interleukin-1β (IL-1β) ([Miyake, 2004](#), [Beutler and Poltorak, 2001](#), [Wheeler and Bernard, 1999](#)). The production of pro-inflammatory chemokines and cytokines leads to the recruitment of neutrophils into the lung interstitium and alveolar spaces ([Kumari et al., 2015](#)). In this environment, activated neutrophils produce large amounts of free radicals and reactive oxygen species (ROS), such as nitric oxide (NO), O₂⁻ and OH⁻, which cause pulmonary endothelial damage and damaging oxidation of lipid and protein components of cells ([Chow et al., 2003](#)). All these events can eventually lead to impairment of gas exchange and non-cardiogenic protein-rich oedema inside the alveolar space due to the damage to the endothelial and epithelial cells ([Guidot et al., 2006](#)). Consequently, these physiological changes can eventually lead to respiratory failure and death.

There is an urgent need for anti-inflammatory agents that downregulate the release of inflammatory mediators and thus reduce the symptoms of ALI without serious side effects. Mouse models of ALI are well established for investigating acute lung injury such as respiratory distress syndrome (ARDS) in humans and for exploring potential therapeutic treatments. Airway administration of LPS leads to the development of an acute inflammatory process characterised primarily by the infiltration of

neutrophils into the airspace leading to areas of haemorrhage in lung tissues, generation of pro-inflammatory cytokines and mediators, and damage to the alveolar architecture ([Asti et al., 2000](#), [Lefort et al., 2001](#)). The inflammatory response in the lungs peaks within the first six hours and reaches the plateau after around 12 h before the mice fully recover after approximately 72 h ([Lefort et al., 2001](#)). The mouse ALI model is well characterised and the progression of the inflammatory cascade is also well known, so novel anti-inflammatory agents can be effectively compared to established anti-inflammatory agents with known modes of action.

Many natural products are sourced from marine organisms due to their inherent broad biological and chemical diversity. Marine natural products have been shown to have an extensive array of therapeutic properties, including anticoagulant, antimicrobial, wound healing and immune modulation, antioxidant, anticancer, anti-inflammatory, antihypertensive, and other reported bioactivity ([Senthilkumar and Kim, 2013](#), [Perdicaris, 2013](#)). There is significant data demonstrating that molluscs have been used to treat inflammatory conditions in many traditional medicine regimes ([Benkendorff et al., 2015](#), [Benkendorff, 2010](#)). The potential anti-inflammatory activity of molluscs has been supported by many *in vitro* and *in vivo* studies, as well as in human clinical trials. Preliminary studies on extracts from whelks in the Muricidae family of predatory gastropods indicate that these yield secondary metabolites with interesting anti-inflammatory properties ([Benkendorff et al., 2015](#), [Ahmad et al., 2018](#)).

The Muricidae family of marine gastropod molluscs is well known for the production of brominated indoles ([Benkendorff, 2013](#), [Benkendorff et al., 2015](#)), including 6-bromoisatin (Figure 4.1), which has well established anti-cancer and chemopreventative properties via the induction of apoptosis ([Edwards et al., 2012](#), [Esmaelian et al., 2013](#), [Esmaelian et al., 2014](#), [Esmaelian et al., 2017](#)). Indoles are aromatic nitrogen-containing compounds of particular interest because of their bioactivity and pharmaceutical potential ([Pauletti et al., 2010](#)). In muricid molluscs, brominated indoles are produced in a specialized biosynthetic organ called the hypobranchial gland (HGB) ([Westley et al., 2010a](#)). Both the HGB extract and 6-bromoisatin from *D. orbita* have anti-inflammatory activity in

in vitro assays with evidence that they inhibit the production of inflammatory mediators and pro-inflammatory cytokines including NO, TNF α in LPS-stimulated RAW 264.7 cells, PGE2 in calcium ionophore-triggered 3T3 ccl-92 fibroblasts and significantly inhibit the translocation of NF κ B into the nucleus in LPS-stimulated RAW 264.7 cells ([Ahmad et al., 2017b](#)). However, the anti-inflammatory activity of HBG extract and the dominant brominated indole, 6-bromoisatin, is yet to be confirmed *in vivo*. The efficacy and safety of both HBG extract and 6-bromoisatin has been validated in previous *in vivo* cancer models ([Esmaeelian et al., 2014](#), [Westley et al., 2010b](#), [Esmaeelian et al., 2017](#)) and their low *in vivo* toxicity ([Esmaeelian et al., 2014](#), [Esmaeelian et al., 2017](#), [Westley et al., 2013](#), [Yazbeck et al., 2015](#)) confirms the potential for development as natural drugs. Consequently, this study aims to test whether the HBG extract from the Australian Muricidae *Dicathais orbita*, along with the main bioactive constituent 6-bromoistain, can systemically ameliorate the early inflammatory response and protect lung architecture in a LPS-mediated ALI mouse model.

4.3. Materials and Methods

4.3.1. Chemicals and reagents

Synthetic 6-bromoisatin was obtained from Tokyo Chemical Industry (Chuo-ku, Tokyo, Japan) with a high level of purity confirmed by ¹H-NMR (Figure 4.1B). *Escherichia coli* O128:B12 LPS was obtained from Sigma (St. Louis, Missouri, United States). Pure filtered grape seed oil for use as a carrier oil was obtained from Australian Botanical Products (United States Pharmacopeia grade) (Hallam, VIC, Australia). Solvents were HPLC grade from Sigma Aldrich (St. Louis, MO, USA). Mouse TNF α ELISA kit was purchased from BD biosciences (Sparks, MD, USA). IL-1 β ELISA kit was purchased from R&D Systems (Minneapolis, MN, USA). Pierce™ BCA Protein Assay was obtained from Thermo Fisher Scientific (Waltham, Massachusetts, United States). Isoflurane and Lethabarb® were purchased from Virbac Pty Ltd., (Wetherill Park, NSW, Australia).

4.3.2. Preparation of mollusc extract and compound

D. orbita snails were collected from Northern New South Wales inter-tidal reefs, Australia, under Fisheries exemption permit F89/1171-6.0. Snails were kept frozen at -80 °C until required. Snails were subsequently thawed and the shell carefully ruptured using a bench vice. Hypobranchial glands were excised according to the procedure described by [Westley and Benkendorff \(2008\)](#). A specimen of *Dicathais orbita* mollusc was lodged into the National Marine Science Centre (NMSC) Molluscan Reference Collection.

Extraction of secondary metabolites from the collected HBG (40 g) was processed according to established procedures described by [Edwards et al. \(2012\)](#). Glands were repeatedly soaked for 2 h in solvent (chloroform: methanol, 1:1), which was replenished until a clear extract was acquired. The extract was then filtered through Whatman filter paper 1 (90 mm, Sigma-Aldrich) to remove the tissue. A chloroform/methanol partition was induced using a small amount of MilliQ water in a separation funnel. After the two phases formed, the chloroform layer was collected and kept covered in aluminium foil to protect from photolytic degradation and subsequently evaporated to dryness on a rotary evaporator (Buchi), using a vacuum pressure of 474 mbar at 40°C, then transferred to an amber vial and dried under high purity nitrogen gas. The extracts were then stored at -80°C until use.

4.3.3. Liquid Chromatography-Mass Spectrometry (LC-MS) analysis

The chloroform extract of the HBG was analysed using an Agilent 1260 infinity High Performance Liquid Chromatography (HPLC) system coupled with a 6120 Quadrupole mass spectrometer (MS) according to validated procedures ([Valles-Regino et al., 2016](#)).

4.3.4. Animals

All animal experiments were approved by the Animal Ethics Committee at the University of Southern Queensland (application number 15REA014). A total of 35 male and female C57 Black/6 mice were obtained from the Animal Resources Centre (ARC), Perth, Western Australia. Mice were housed in the University of Southern Queensland Animal House, maintained on an automated time cycle of 12

h light and 12 h dark and had access to rodent chow and water *ad libitum*. Mice were separated according to their gender where males were housed in a different room away from females to reduce stress. The mice were divided randomly into 6 groups (six mice per group except for the negative control group where five mice were used)

4.4. Acute lung inflammation model

The procedure for the LPS mediated mouse model of acute lung inflammation followed the method described by [Moffatt et al. \(2006\)](#). In brief, C57 Black/6 mice were randomly divided into 6 groups (n = 6 except for PBS negative control n = 5). Mice received three oral doses (administered at 48 h, 24 h and 1 h prior to the administration of LPS) of HBG extract at 0.5 mg/g or 0.1 mg/g, or 6-bromoisatin at 0.05 mg/g or 0.1 mg/g, dissolved in 100 µl analytical grade grape seed oil. Both LPS positive and negative controls received three doses of 100 µl of grape seed oil following the same timeframe. Acute lung inflammation was induced by intranasal (i.n.) administration of 1.25 mg/kg of LPS in 50 µL of sterile Phosphate buffered saline (PBS) ([Moffatt et al., 2002](#), [Moffatt et al., 2006](#), [Corteling et al., 2002](#)), whilst i.n administration of 50 µL of sterile PBS was used as the negative control. Three hours post i.n. administration of LPS, mice were euthanised following isoflurane anaesthesia by intra-peritoneal (i.p.) injection of 0.2 mL Lethabarb® (pentobarbitone sodium) ([Corteling et al., 2002](#)). All handling procedures during experiments (oral gavage, intranasal administration) were performed under light anaesthesia according to USQ HP016 Rodent (rat or mouse) gaseous anaesthesia (isoflurane) to minimise stress during handling. Mice were placed inside an induction chamber supplied with 100 % O₂ (on a flow rate 1.5 litres/min) and a small volume of isoflurane (4 % induction and 1.5 % maintenance) was supplied using an isoflurane vaporising machine (Northern Vaporisers, Skipton, UK). Respiration and response to stimulation were monitored during the procedure and the supply of isoflurane was adjusted accordingly. When returned to their allocated box, mice were monitored until complete recovery was confirmed, via active behavioural signs. Deep anaesthesia was only used prior to i.p. injection of 0.2 mL of Lethabarb® for the euthanasia process. Mice were monitored half-hourly post oral gavage for the first hour and hourly

for the following 3 hrs. The monitoring was more frequent after the intranasal administration of LPS as mice were closely monitored throughout the 3 hrs post administration till euthanasia.

4.4.1. Bronchoalveolar lavage fluid (BALF) collection and analysis

Following euthanasia, mice lungs were lavaged three times with 0.5 mL of ice-cold PBS as described in [Fang et al. \(2015\)](#). The total cell count of the BALF was measured using an automated cell counter (BIO-RAD). The BALF was then centrifuged 1500 rpm for 10 min at 4°C. The recovered supernatant was collected and stored in -80 °C freezer until use. The remaining cell pellet was resuspended in 200 µL of PBS and centrifuged in a Cytospin™ 4 (Thermo Scientific) at 700 rpm for 8 min. Cell deposits were then stained using the Diff Quick staining system (CHEMTREC®). A microscopic differential cell count was then conducted using an Olympus BX61WI microscope at 400x magnification in which 300 cells were counted on the slide (100 in three separate frames of view) and averaged to 100 cells to deduce the percentage of neutrophils (n = 6). Representative images of the cell count were captured using TUCSON camera (NIKON Japan) installed on Leica® light microscope (Leica Microsystems).

4.4.2. Measurement of TNF-alpha levels in BALF

The amount of TNF α was quantified in BALF using a murine TNF α ELISA kit according to the manufacturer's instructions. Plates were read at 405 nm wavelength using an Anthos Zenyth 200rt microplate reader (Anthos Labtech Instruments).

4.4.3. Measurement of IL-1 β levels in BALF

The amount of IL-1 β was quantified in BALF, using mouse IL-1 β ELISA kit according to the manufacturer's instructions. Plates were read at 450 nm wavelength (Anthos Labtech Instruments) with the wavelength correction set on 540 nm.

4.4.4. Total protein in BALF

Total proteins in the BALF were determined using Pierce™ BCA Protein Assay kit according to the manufacturer's instructions.

4.4.5. Lung histopathological studies

Whole lungs were harvested and fixed in 10% neutral-buffered formalin before being histologically processed in an Excelsior™ AS Tissue Processor (Thermo Scientific™), embedded in paraffin, sectioned on a microtome to 4 μm, and stained with hematoxylin and eosin (H&E). The slides were coded and assessed blind using standard histological procedures, under a light microscope for evidence of pathology, the degree of pathology subsequently compared to the negative control using a semiquantitative analysis as described by [Eveillard et al. \(2010\)](#) and [Klopfleisch \(2013\)](#). The scores ranged from 0 (no lesion) to 4 (severe and comprehensive lesion) and were assigned according to the degree of alveolar necrosis, vascular congestion, infiltration by neutrophils, and infiltration by macrophages.

4.4.6. Statistical analysis

One-way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA), with $p < 0.05$ considered significant.

4.5. Results

4.5.1. Chemical analysis of HBG extract

LC-MS analysis of the HGB extract confirmed the presence of the brominated indoles typically found in *D. orbita* (Figure 4.1A) ([Benkendorff, 2013](#)). The extract was dominated by the presence of 6-bromoisatin (35.34%) beside tyrindoleninone (39.98%) and traces of 6-bromoindole and tyriverdin (Figure 4.1A). ^1H NMR confirmed the purity of the synthetic 6-bromoisatin (Figure 4.1B).

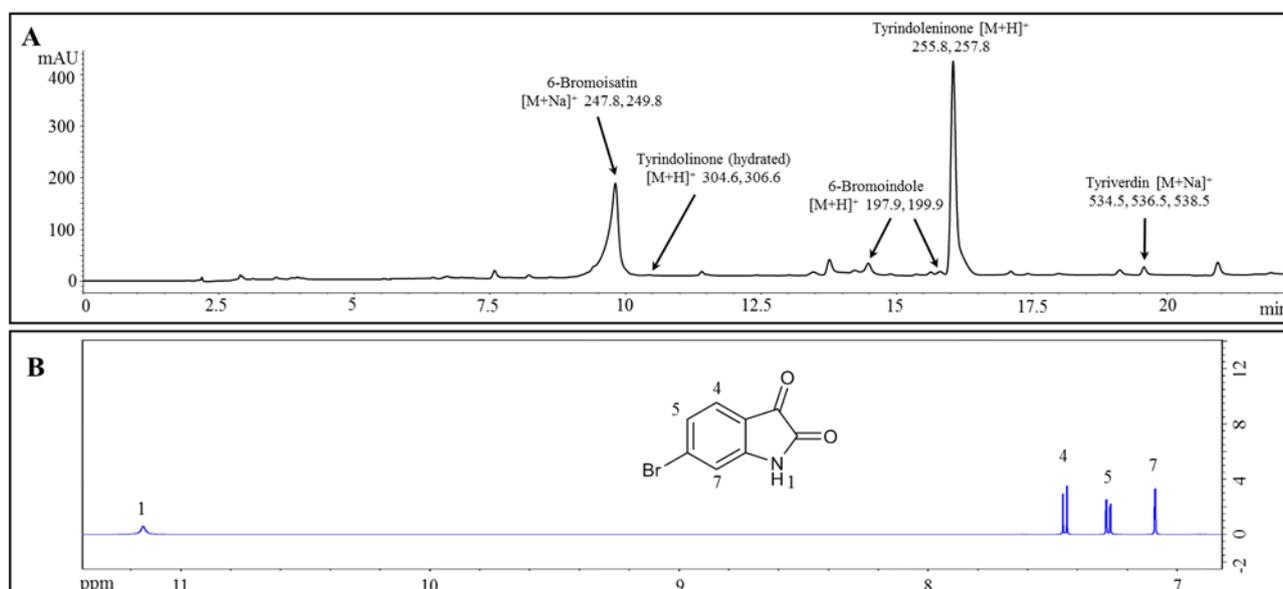


Figure 4.1: Chemical analysis of the hypobranchial gland (HBG) extract and 6-bromoisatin. A) High performance liquid chromatography (HPLC) chromatogram of the (HBG) extract from *Dicathais orbita* showing brominated indole profile. Retention times (tR) and major ions in ESI-MS indicate the presence of 6-bromoisatin (tR = 9.825 min), hydrated tyrindolinone (tR = 10.454 min), tyrindoleninone (tR = 16.056 min) and tyriverdin (tR = 19.571). B) ¹H-NMR spectra of 6-bromoisatin showing purity of the synthetic compound used in this study.

4.5.2. Total and differential cell counts

The total number of cells in BALF of LPS-stimulated positive controls was 6.4×10^5 cells/mL (Figure 4.2A). Relative to the positive control, the number of cells significantly decreased ($p < 0.0001$) in all the treatment groups: means of 2.6×10^5 cells/mL in HBG extract 0.5 mg/g dose and 4.1×10^5 cells/mL in the 0.1 mg/g dose, and for 6-bromoisatin, means of 2.2×10^5 cells/mL in the 0.05 mg/g dose and 3.3×10^5 cells/mL in the 0.1 mg/g dose. All treatments except 0.1 mg/g HGB extract were not significantly different from the PBS negative control or each other (Figure 4.2A, $p > 0.05$).

Figure 4.2B shows that compared to the LPS positive control with neutrophil counts at a mean of 3.6×10^5 cells/mL, mice in all treatment groups had a significantly reduced neutrophil count ($p < 0.0001$), with a total absence of neutrophils in the 6-bromoisatin 0.05 mg/g dose regime and PBS negative control. The other treatment groups also displayed significant inhibition of neutrophil sequestration in the lungs, with only 1.4×10^3 , 5.7×10^3 and 3.1×10^3 neutrophils/mL found in the BALF collected from the HBG extract at 0.5 and 0.1 mg/g, and 6-bromoisatin (0.1 mg/g) treatment groups respectively ($n = 6$; $p < 0.0001$). There was no significant difference between the treatment groups.

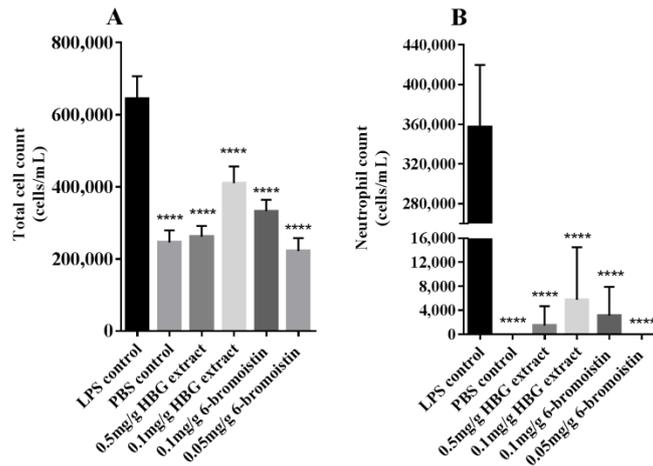


Figure 4.2: Cellularity in the bronchoalveolar lavage fluids (BALF). Cell count in the BALF from mice stimulated with LPS and treated with 6-bromoisatin or a hypobranchial gland (HBG) extract from *Dicathais orbita*. A) Total cell counts; B) Neutrophil counts from the differential staining. *** = $p < 0.001$; **** = $p < 0.0001$.

4.5.3. Measurement of TNF α levels in BALF

The HBG extract treatment significantly decreased the level of TNF α relative to the positive control in a dose dependent manner (Figure 4.3A), with 5 pg/mL TNF α detected at 0.5 mg/g and 133 pg/mL TNF α in the 0.1 mg/g HBG dose, compared to the positive control yield of 1127 pg/mL ($n = 6$; $p < 0.0001$). No TNF α was detected following treatment with 0.1mg/g of 6-bromoisatin and only 3 pg/mL TNF α detected after pre-treatment with 0.05 mg/g 6-bromoisatin. (Figure 4.3A). There was no significant difference between the treatment groups.

4.5.4. Measurement of IL-1 β levels in BALF

The IL-1 β concentration in the BALF from mice that received 0.5 mg/g HBG extract was equal to the level detected in the PBS negative control at 28 pg/mL and significantly lower than the 493 pg/mL in the LPS positive control ($p < 0.0001$, Figure 4.3B). The lower dose (0.1 mg/g) of HBG extract also reduced the levels of IL-1 β significantly (114 pg/mL) compared to the LPS positive control. Treatment with 0.05 mg/g 6-bromoisatin reduced the concentration of IL-1 β to 16 pg/mL (Figure 4.3B). All treatments were significantly lower than the LPS positive control but not from each other.

4.5.5. LPS-induced protein concentration in BALF

After i.n. LPS-administration, protein concentration in BALF from mice significantly increased as a marker of leakage, and was 307 $\mu\text{g}/\text{mL}$ after 3 h compared with the PBS control, which was only at 133 $\mu\text{g}/\text{mL}$ (Figure 4.3C). The BALF protein concentration was reduced in LPS-stimulated mice treated with the *D. orbita* HBG extract and 6-bromoisatin, reaching only 139 $\mu\text{g}/\text{mL}$ in mice receiving 0.1 mg/g 6-bromoisatin and 136 $\mu\text{g}/\text{mL}$ in mice receiving 0.1 mg/g HBG extract. The difference between all treatment groups versus the LPS positive control was significant ($p < 0.0001$), but not different from the baseline protein concentrations in the BALF of negative controls (Figure 4.3C). There was a significant dose effect for 6-bromoisatin with slightly higher protein levels at 0.05 compared to 0.1mg/ml ($p = 0.018$).

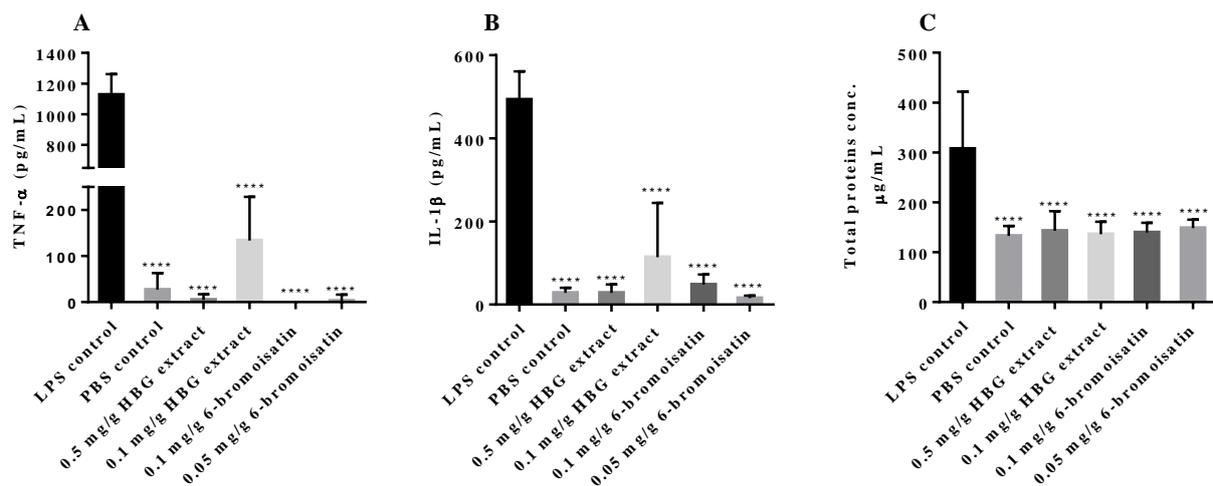


Figure 4.3: Cytokine and total protein concentration in BALF. Inhibitory effect of hypobranchial gland (HBG) extract from *Dicathais orbita* and 6-bromoisatin on the LPS-stimulated acute lung inflammation (ALI) in mice indicated by the levels of pro-inflammatory cytokines and total proteins in bronchoalveolar lavage fluids (BALF). A) Concentration of TNF α ; B) IL-1 β levels obtained using ELISA and; C) total protein concentration in BALF obtained by BCA assay. All BALF were collected from mice 3 h post intranasal (i.n.) administration of LPS or PBS ($n = 6$; **** = $p < 0.0001$). Mice received 3 doses of HBG extract (0.5 mg/g or 0.1 mg/g), 6-bromoisatin (0.1 mg/g or 0.05 mg/g) and both control groups received 100 μL of grape seed oil 48 h, 24 h and 1 h prior to i.n. administration of LPS or PBS.

4.5.6. Histopathology of the lungs

Examination of histological sections of the lungs from the LPS positive control group revealed areas of severe haemorrhages (indicated by orange arrows), damage to the lung architecture, increased

cellularity in the air spaces, and clear evidence of both macrophage (blue arrow heads) and neutrophil (purple arrow heads) infiltration, with a large number of macrophages and neutrophils found in the alveolar spaces (Table 4.1 and Figure 4.4A). Lung histopathology examinations also revealed damage to and thickening of alveolar walls (red arrow heads) in tissue sections from the LPS positive control mice (Table 4.1 and Figure 4.4A). In contrast, damage to lung tissues was greatly reduced in all HBG extract and 6-bromoisatin treated mice. The treated mice showed lung architecture similar to the negative control group, displaying normal lung architecture with no evidence of haemorrhage or neutrophil sequestration into the airspaces, apart from occasional alveolar macrophages (yellow arrow heads) and relatively thin alveolar walls (green arrow heads) (Figure 4.4B-F and Table 4.1). Semi-quantitative scoring of the microscopic histopathological examination confirmed that treatment with all doses of HBG extract and 6-bromoisatin significantly minimised all the indicators of acute inflammatory damage to the lungs, including macrophage and neutrophil infiltration into the airspace, as well as vascular congestion and alveolar destruction, compared to the LPS positive control group ($p < 0.0001$) (Table 4.1). There was an inverse dose response for 6-bromoisatin with significantly higher macrophages ($p = 0.006$) at 0.1mg/g compared to the lower dose of 0.05mg/g.

Table 4.1: Histopathological scores for lungs tissues. Histopathological scores for lungs of mice after LPS stimulation (PBS control received 50 μ L of PBS intranasally (i.n.) instead of LPS) and oral gavage with extracts from the hypobranchial glands of *Dicathais orbita* or the dominant secondary metabolite 6-bromoisatin. Data are means \pm standard error of the means, n= 6 for all groups except for PBS control n= 5. *= $p < 0.05$; **= $p < 0.01$; ****= $p < 0.0001$.

Treatment Group	Macrophage Infiltration	Neutrophil Infiltration	Vascular Congestion	Alveolar Destruction	Total Score
LPS control	3.8 \pm 0.2	3.7 \pm 0.2	3.2 \pm 0.4	2.9 \pm 0.4	13.5 \pm 1.2
PBS control	1.4 \pm 0.2****	0 \pm 0****	0.4 \pm 0.2****	0.6 \pm 0.2****	2.4 \pm 0.7****
0.5 mg/g HBG	2 \pm 0.4****	0.2 \pm 0.2****	1 \pm 0.3****	1.5 \pm 0.2**	4.7 \pm 0.6****
0.1 mg/g HBG	2.8 \pm 0.2****	0.5 \pm 0.3*	0.8 \pm 0.3****	0.7 \pm 0.2****	4.8 \pm 0.8****
0.1 mg/g 6-bromoisatin	2.6 \pm 0.2****	0.3 \pm 0.2**	0.7 \pm 0.2****	0.7 \pm 0.2****	4.3 \pm 0.7****
0.05 mg/g 6-bromoisatin	1.5 \pm 0.2****	0 \pm 0****	0.3 \pm 0.2****	0.5 \pm 0.2****	2.3 \pm 0.3****

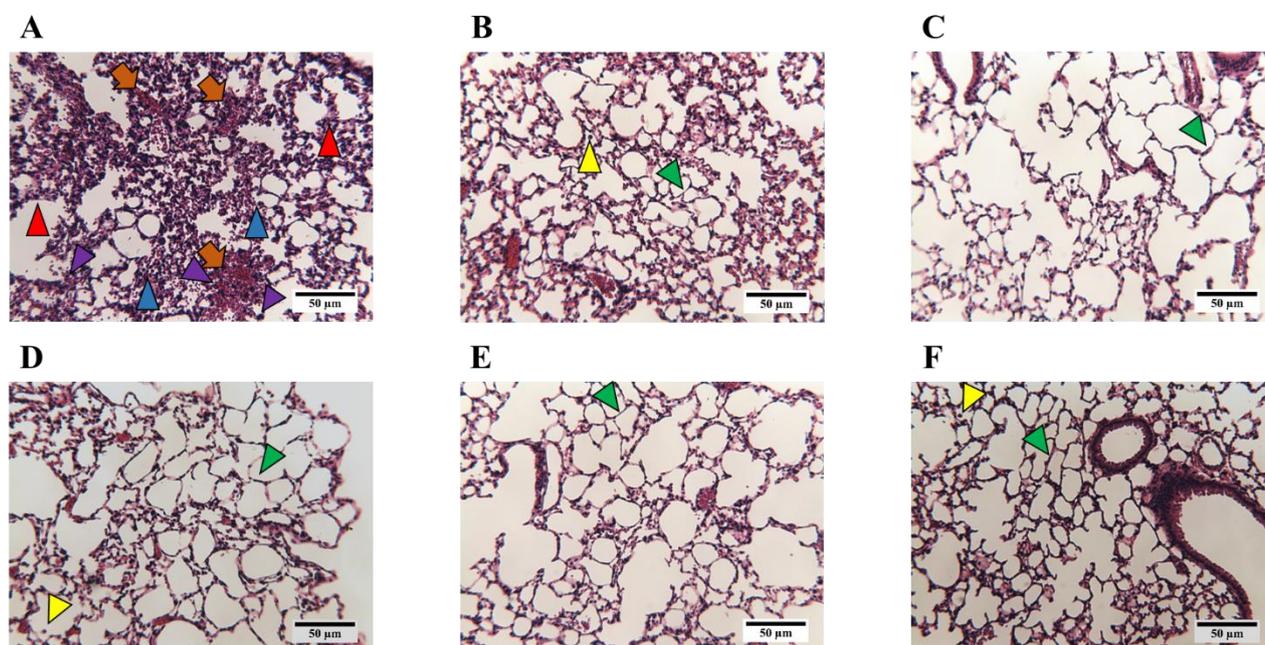


Figure 4.4: HBG extract and 6-bromoisatin treatment protect lung tissues from the adverse effect of LPS. H&E stained lung sections demonstrating the anti-inflammatory effects of hypobranchial gland (HBG) extract from *Dicathais orbita* and 6-bromoisatin on LPS-induced acute lung inflammation (ALI). Lung tissue from A) LPS stimulated positive control showing areas of severe haemorrhage (orange arrows), infiltration of macrophages (blue arrow heads) and neutrophils (purple arrow heads), thickened alveolar walls (red arrow heads). The unstimulated negative control mice that received 50 μ L PBS intranasally (i.n.) (B), 0.5 mg/g HBG extract treated mice (C), 0.1 mg/g HBG extract treated mice with undamaged alveolar space and parenchymal architecture (D), mice treated with 0.1 mg/g of 6-bromoisatin (E), and mice treated with 0.05 mg/g 6-bromoisatin (F) all showing preserved alveolar space and parenchymal architecture and all showed thin alveolar wall (green arrow heads) and lack of signs of macrophage and neutrophil infiltrations, except for occasional alveolar macrophages (yellow arrow heads). Images are representative of 3 different sections on each slide out of 6 slides per group magnified X 200 and scale bars set to 50 μ m.

4.5.7. Correlation between histopathology and inflammatory markers

There were significant positive correlations between histopathological scores and inflammatory markers in the bronchoalveolar lavage fluids ($p < 0.001$, Figure 4.5, Appendices 4-11). Each of the histopathological markers was significantly correlated to the total semi-quantitative score (Appendix 6). The total score is significantly correlated to the cellularity and cytokines in the BALF (Figure 4.5) and demonstrates that the correlations are primarily driven by the higher scores and BALF values in the LPS positive control, compared to the negative control and all treatment groups (Appendices 6-11). The correlations tend to be stronger for neutrophils than total cell count for all histopathological scores (Figure 4.5, Appendices 7-11). The correlations are also stronger for the cytokines TNF α and IL-1 β than total protein (appendices 5-8) and greater than 70% of the variation in the total histological score is explained by these individual cytokines (Figure 4.5). Within the BALF, cell numbers were significantly correlated to the cytokines and total protein, with the strongest relationships found between neutrophils and TNF α (Appendix 11).

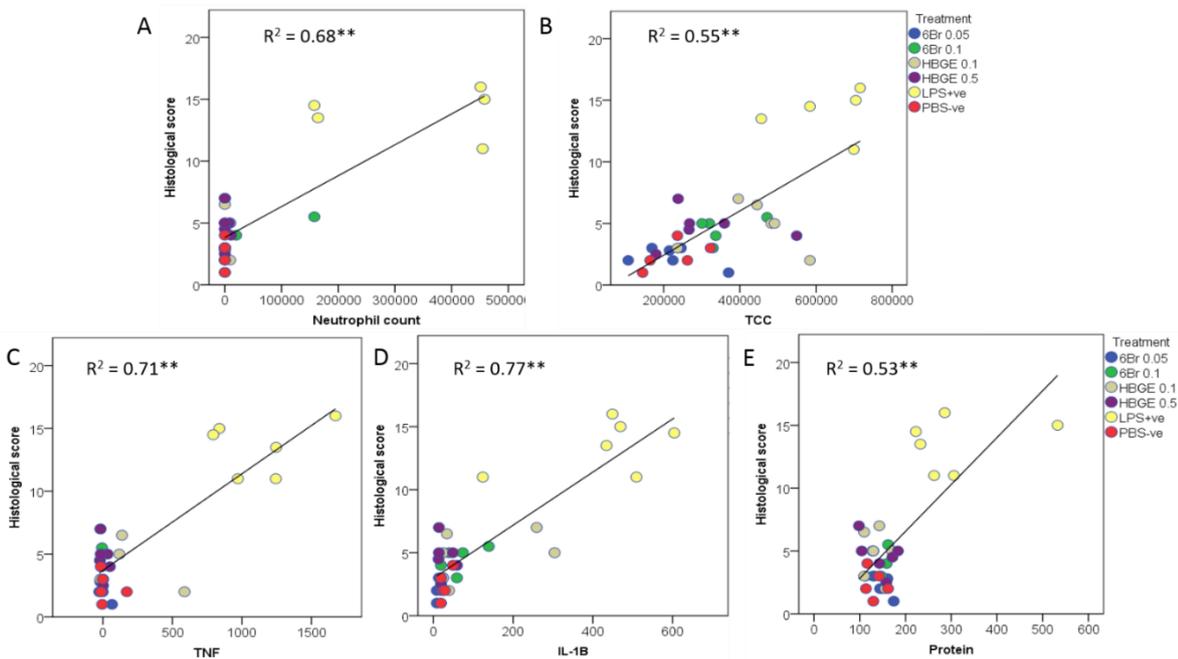


Figure 4.5: Correlations between the histological score and inflammatory markers in the lungs of mice. The relationship between the overall histopathological score and parameters in the bronchoalveolar lavage fluids: A) Neutrophil count; B), total cell count (TCC); C) Tumour necrosis factor (TNF) alpha; D) interleukin I-1B; and E) protein. The linear relationship and R^2 values are determined from all samples pooled across all mice in the LPS-stimulated acute lung inflammation model: PBS -ve negative control; LPS +ve lipopolysaccharide stimulated positive control; HBGE Hypobranchial gland extract at 0.1 and 0.5 mg/g and ; 6Br 6 bromoisatin at 0.1 and 0.05 mg/g. ** significant relationship at $p < 0.01$.

4.6. Discussion

The present study has demonstrated the anti-inflammatory properties of orally administered HBG extract from the marine mollusc *D. orbita* and the major constituent 6-bromoisatin in a LPS mediated mouse model of acute lung inflammation. These findings support previous evidence of *in vitro* anti-inflammatory activity ([Ahmad et al., 2017b](#)) and suggest that HBG extract from muricid molluscs and associated brominated indoles should be explored further as orally active therapeutic alternatives for the treatment of lung inflammation. ALI is a life threatening condition associated with high levels of morbidity and mortality. Despite the significance of these diseases, there has been little progress in the development of alternative treatment regimens to the conventional use of steroids, current NSAID supplemented with antibiotics and assisted ventilation ([Wheeler and Bernard, 1999](#), [Kim et al., 2014](#)).

Isatins are naturally occurring molecules and are well known for their synthetic versatility and diverse pharmacological properties ([Pandeya et al., 2005](#), [Medvedev et al., 2007](#)). 6-Bromoisatin is a brominated form of isatin, which has been shown to have *in vitro* anti-inflammatory activity in our previous studies ([Ahmad et al., 2017b](#)). Modes of action for simple isatin derivatives appear to be associated with inhibiting the transcription of iNOS and COX-2 enzymes ([Matheus et al., 2007](#)) and the translocation of NF κ B, thus suppressing NO and TNF α ([Ahmad et al., 2017b](#)) in mouse macrophage cell lines. Halogenated isatins have been shown to have greater biological activity than non-halogenated forms, as demonstrated by structure-activity relationship studies ([Ahmad et al., 2017b](#), [Vine et al., 2007](#)). As far as we are aware, this is the first study to establish that bromoisatin is active in an animal model for acute inflammation.

Interestingly a tendency towards a reverse dose effect was observed for 6-bromoisatin in the histopathological scores, with a significant difference between doses for macrophage infiltration. Similar reverse dose effects have been observed with synthetic 6-bromoisatin in a 14 day mouse model for colon cancer, specifically for apoptosis ([Esmaelian et al., 2014](#)) and for haematological

white blood cell and neutrophil counts using 6-bromoindole-3-pyruvate semi-purified from *Dicathais orbita* extracts ([Esmaeelian et al., 2017](#)). It is uncertain why this occurs, but it is most likely the result of *in vivo* metabolism and degradative reactions that occur at high versus low doses. 6-Bromoindole-3-pyruvate can form dimers *in vivo* ([Gustafsson et al., 2017](#)) and higher concentrations would increase the opportunity for dimers to form. As reported in our previous *in vitro* studies, brominated indole dimers have lower anti-inflammatory activity than the monomers ([Ahmad et al., 2017b](#)).

All of the semi-quantitative histological parameters and inflammatory markers from the bronchoalveolar lavage fluids were significantly correlated in this study and provide multiple lines of evidence supporting the inhibition of LPS-stimulated inflammation and associated damage to the lungs by 6-bromoindole-3-pyruvate and the natural HBG extract. According to available results from this study and our previous *in vitro* study ([Ahmad et al., 2017b](#)), 6-bromoindole-3-pyruvate appears to inhibit the overproduction of pro-inflammatory mediators and cytokines by inhibiting the translocation of NF κ B (Figure 4.6). Translocation of NF κ B to the nucleus regulates the expression of a variety of transcription factors and co-factors, that lead to the expression of pro-inflammatory enzymes including COX-2 and iNOS, which are responsible for stimulating further signalling molecules (Figure 4.6), including adhesion molecules, pro-inflammatory cytokines (IL-1 β , IL-6, and TNF α) and chemokines ([Baeuerle and Baltimore, 1999](#), [Lo et al., 1999](#), [Kyriakis and Avruch, 2001](#)). It is well known that unresolved inflammatory responses can cause serious damage to the affected tissues, as well as the neighbouring tissues. Hence, agents that inhibit NF κ B translocation and /or the whole TLR4 signalling pathway could be of a significant value as anti-inflammatory agents and may prevent the tissue damage caused during ALI. Nevertheless, based on the current *in vivo* model, we can't rule out potential upstream effects, such as interference with LPS binding to receptors on the surface of cells (Figure 4.6). Further studies are required to elucidate the specific mode/s of action and investigate the pharmacokinetics of 6-bromoindole-3-pyruvate using different treatment regimes. The potential for therapeutic use of *D. orbita* HBG chloroform extract and 6-bromoindole-3-pyruvate as anti-inflammatory agents also relies on low *in vivo* toxicity. In previous rodent models, there was no morbidity, ill health or gastro-

intestinal damage apparent after 2 -14 weeks of daily oral gavage with the muricid extract or pure 6-bromoisatin ([Westley et al., 2013](#), [Yazbeck et al., 2015](#), [Esmaeelian et al., 2017](#), [Esmaeelian et al., 2014](#), [Gustafsson et al., 2017](#)). As gastro-intestinal damage is the most significant side effect of currently used treatments (NSAIDs) ([Mahmud et al., 1996](#)), 6-bromoisatin provides a lead for the development of safer anti-inflammatory drugs. In addition to the anti-inflammatory activity reported here, *D. orbita* chloroform extracts and brominated indoles have been shown to harbour antibacterial properties against a range of Gram positive and negative bacteria ([Benkendorff et al., 2000](#)). Furthermore, 6-bromoisatin has anti-proliferation and apoptotic properties and effectively prevents early stage colorectal cancer formation in rodents ([Esmaeelian et al., 2014](#), [Esmaeelian et al., 2017](#)). Likewise, HBG extracts also induce apoptotic effects in DNA damaged cells in colorectal cancer rodent models ([Westley et al., 2010b](#), [Esmaeelian et al., 2017](#)). However, some of the other brominated indoles in the HGB extracts appear to cause idiosyncratic liver damage ([Westley et al., 2013](#), [Esmaeelian et al., 2017](#)), suggesting that there are benefits associated with using purified 6-bromoisatin. Furthermore, polar extracts from Muricidae hypobranchial glands contain choline esters which can be associated with significant toxicity ([Benkendorff et al., 2015](#), [Roseghini et al., 1996](#)). However, [Rudd and Benkendorff \(2014\)](#) have published a method for supercritical fluid extraction of *D. orbita* HGBs that concentrates the anti-inflammatory brominated indoles without the toxic choline esters. This safe extraction method is suitable for future nutraceutical development and will facilitate quality control of the natural product.

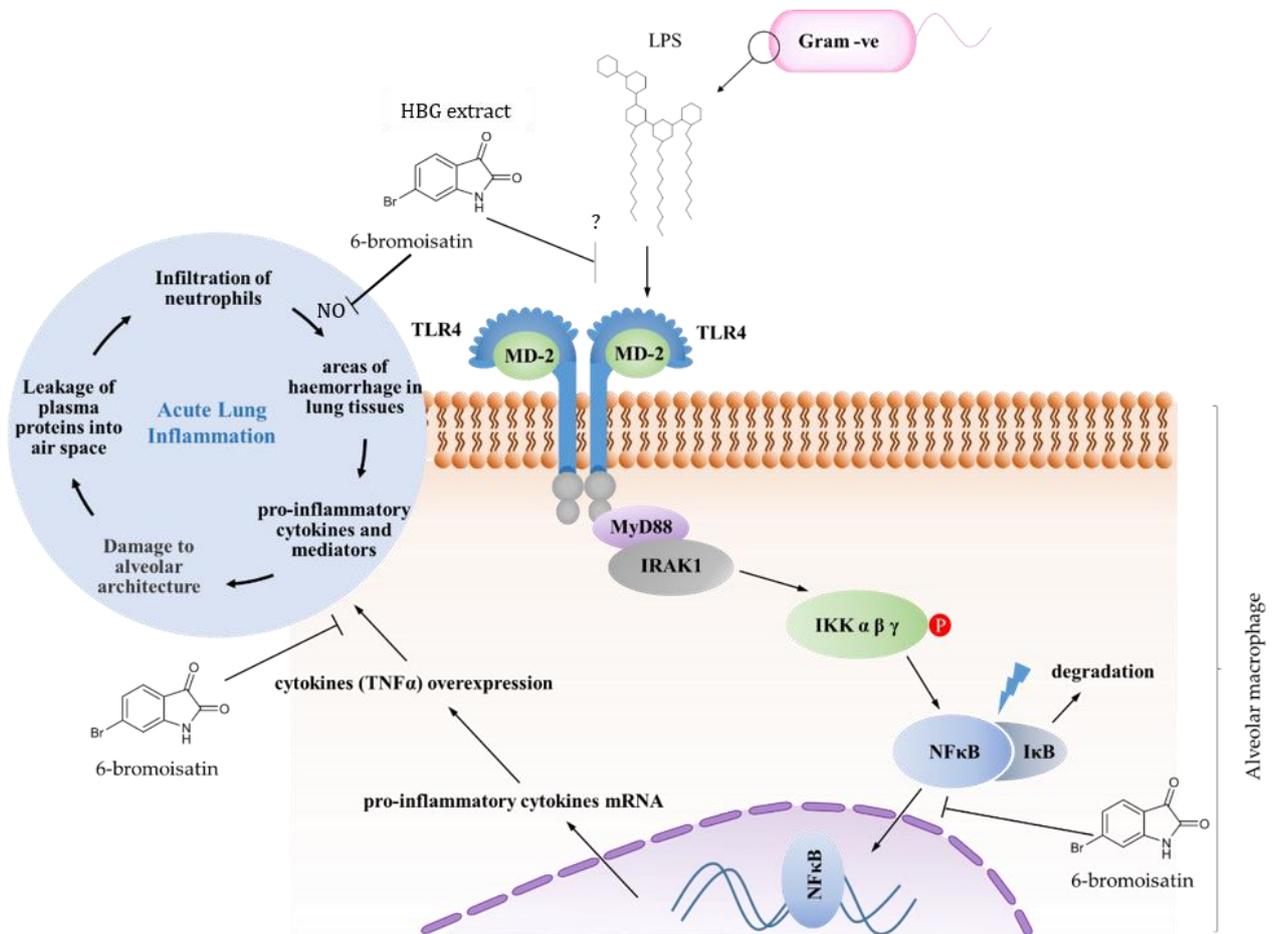
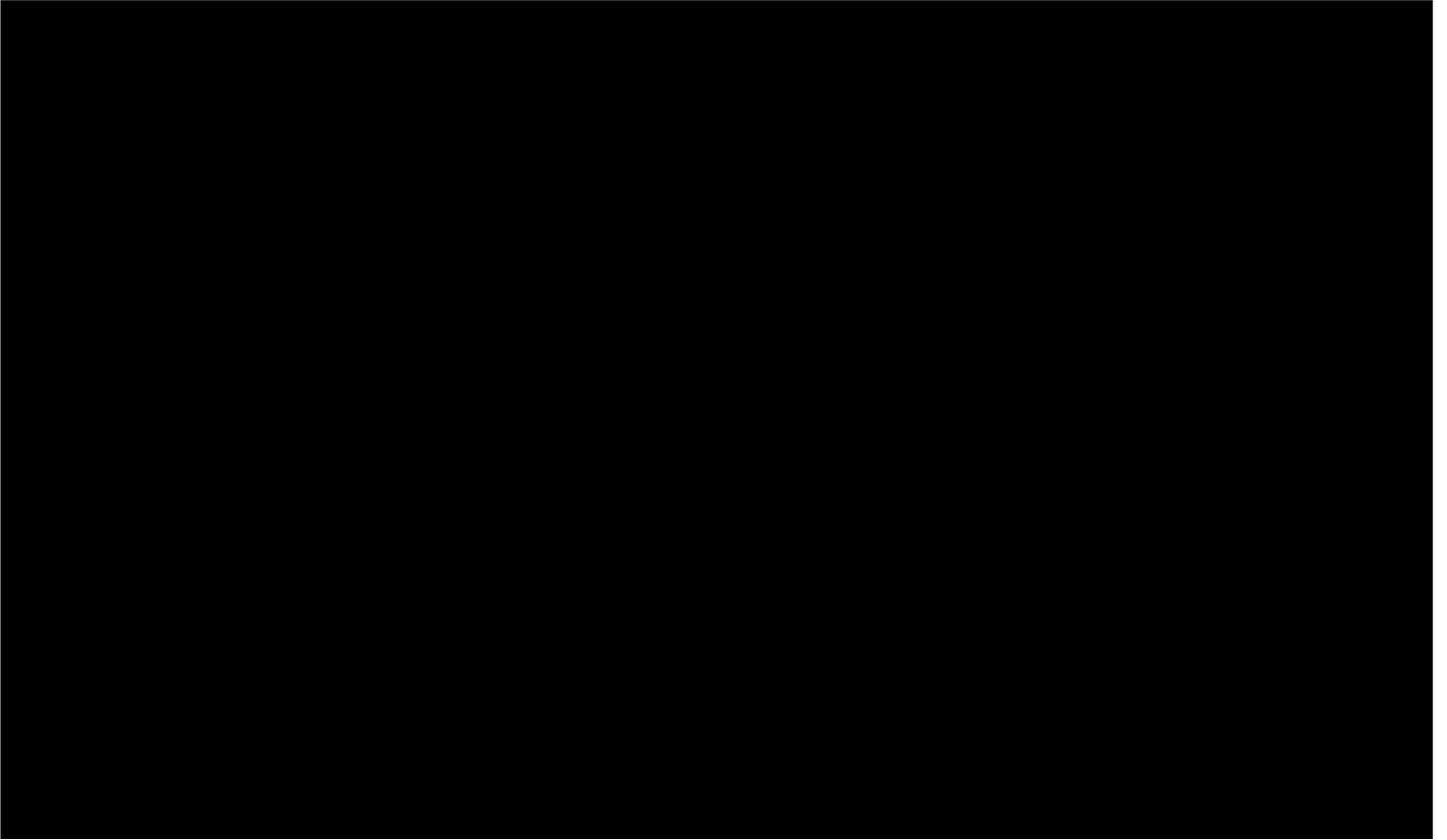


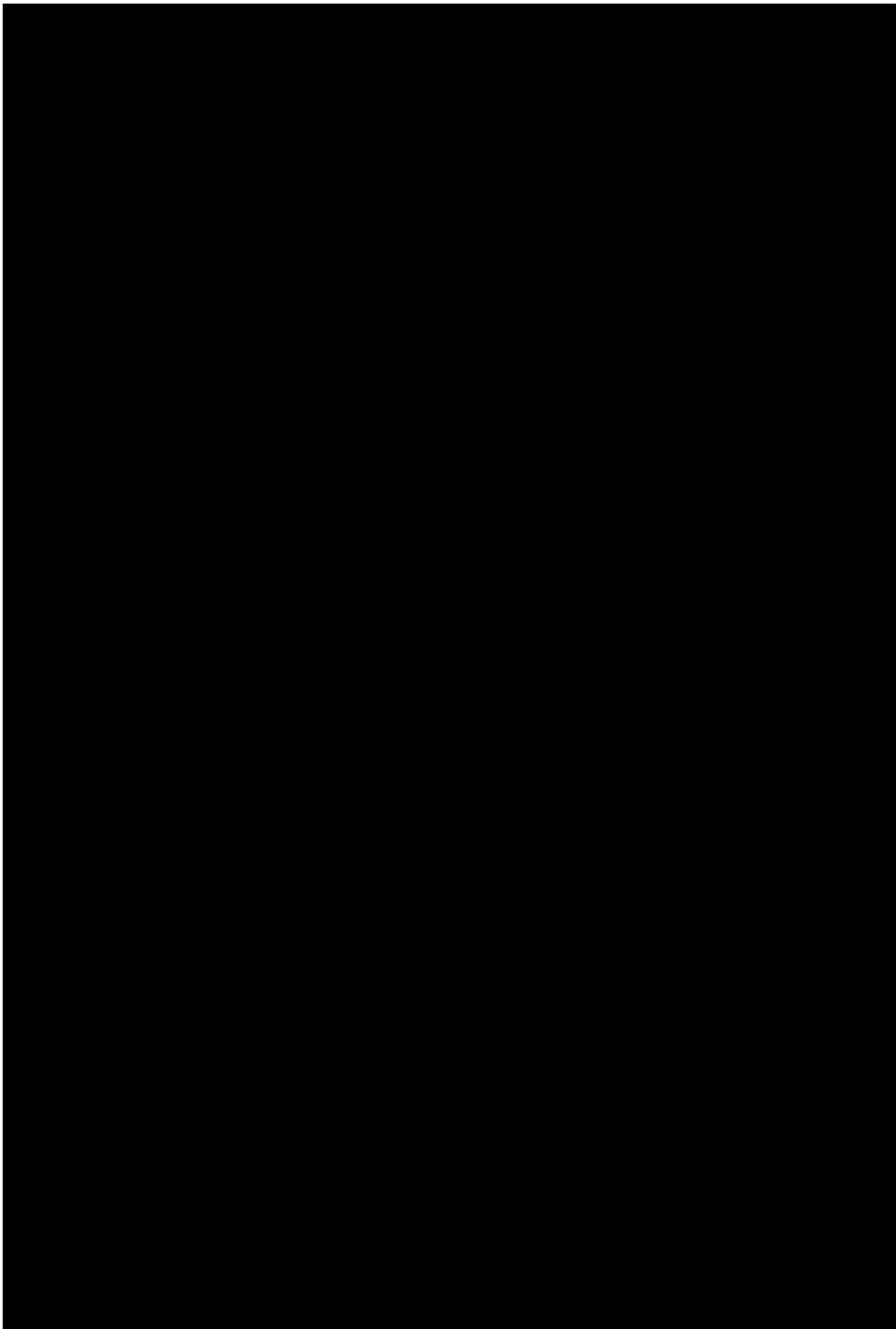
Figure 4.6: Proposed model of anti-inflammatory signalling pathway inhibition. 6-Bromoisatin in *D. orbita* hypobranchial gland (HGB) extracts prevent acute lung damage caused by inflammatory neutrophils by reducing the synthesis of pro-inflammatory cytokines. This may occur due to blocking the LPS-induced NFκB translocation into the nucleus and activation of macrophages and direct inhibition of inflammatory mediators, such as TNFα and nitric oxide (NO), as has been previously demonstrated *in vitro* for 6-bromoisatin (Ahmad et al., 2017b). Alternatively, it is possible that the HGB extracts and associated compounds also have an upstream effect by modulating the interaction of LPS with plasma membrane receptors. This figure was adapted from inflammatory pathways in lung macrophages previously reviewed by Hussell and Bell (2014).

Rodent models of ALI are well established and have been extensively used to explore the complex pathobiology of this syndrome. Activated neutrophils in the airways are the major risk that leads to weakened lung pathology (Birkedal-Hansen, 1995, Corteling et al., 2002). In this study, oral treatment with HBG extract and 6-bromoisatin showed significant suppression of neutrophil sequestration, although the full mode of action of these inflammatory antagonists needs further exploration. Furthermore, IL-1β is considered one of the most potent pro-inflammatory cytokines (Martinon and Tschopp, 2007). This cytokine has been shown to have more potent activity than TNFα in inducing fever, in addition to its many other effects on endothelial cells, such as promoting coagulation and

thrombosis, promoting infection-related and injury-related inflammation, pain hypersensitivity ([Samad et al., 2001](#)), and induction of collagenase production, which contributes to the induction of a range of inflammatory diseases ([Martinon and Tschopp, 2007](#)). IL-1 β is also known to play a significant role in the pathogenesis of type-1 diabetes ([Maedler et al., 2002](#)), acute neurodegeneration, stroke, tumour angiogenesis and invasiveness ([Voronov et al., 2003](#)), and destructive joint and bone diseases ([Martinon and Tschopp, 2007](#)). Thus, reduction of IL-1 β levels by the HBG extract could contribute to the broad range of traditional medicinal applications previously reported for Muricidae molluscs ([Benkendorff et al., 2015](#)), including preparations that have been used to treat asthma, cough and reduction of respiratory phlegm ([Guan and Wang, 2009](#), [Prabhakar and Roy, 2009](#), [Gopal et al., 2008](#), [Krishna and Singh, 2012](#), [Alves and Alves, 2011](#)). Both the HBG extract and 6-bromoisatin also significantly inhibited the leakage of proteins into the air spaces in the lungs, as supported by the normal protein concentrations in the collected BALF. These results were supported by the histopathological findings where clear healthy lung tissues were observed for mice pre-treated with HBG extract and 6-bromoisatin.

In conclusion, the anti-inflammatory activity of 6-bromoisatin and the HBG extract from *D. orbita* in a mouse model for ALI supports previous *in vitro* observations of the anti-inflammatory capability of these agents ([Ahmad et al., 2017b](#)). Although a number of brominated indoles have been characterised in the HBG extract from *D. orbita*, 6-bromoisatin is considered safer ([Esmaeelian et al., 2017](#)) and easier to synthesise ([Vine et al., 2007](#)). Several studies support the safety of oral consumption of this compound and the extracts in rodents, which suggests the potential for development as nutraceutical anti-inflammatory preparations. Further studies to elaborate the activity of HBG extract from *D. orbita* and 6-bromoisatin in particular seems warranted.





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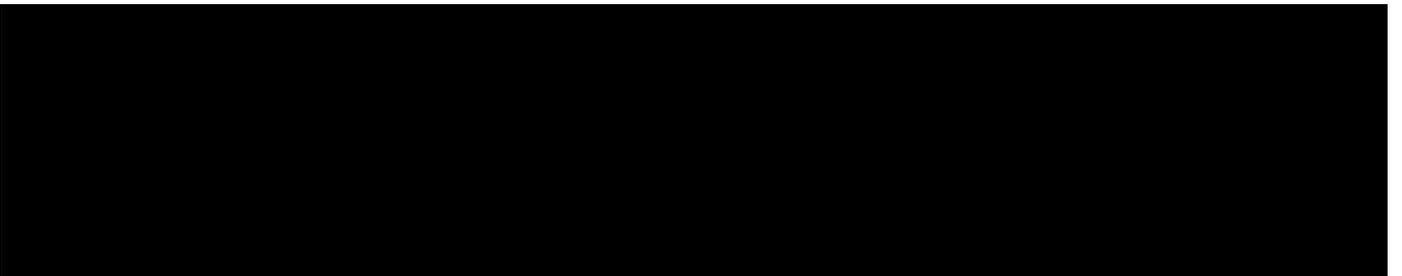
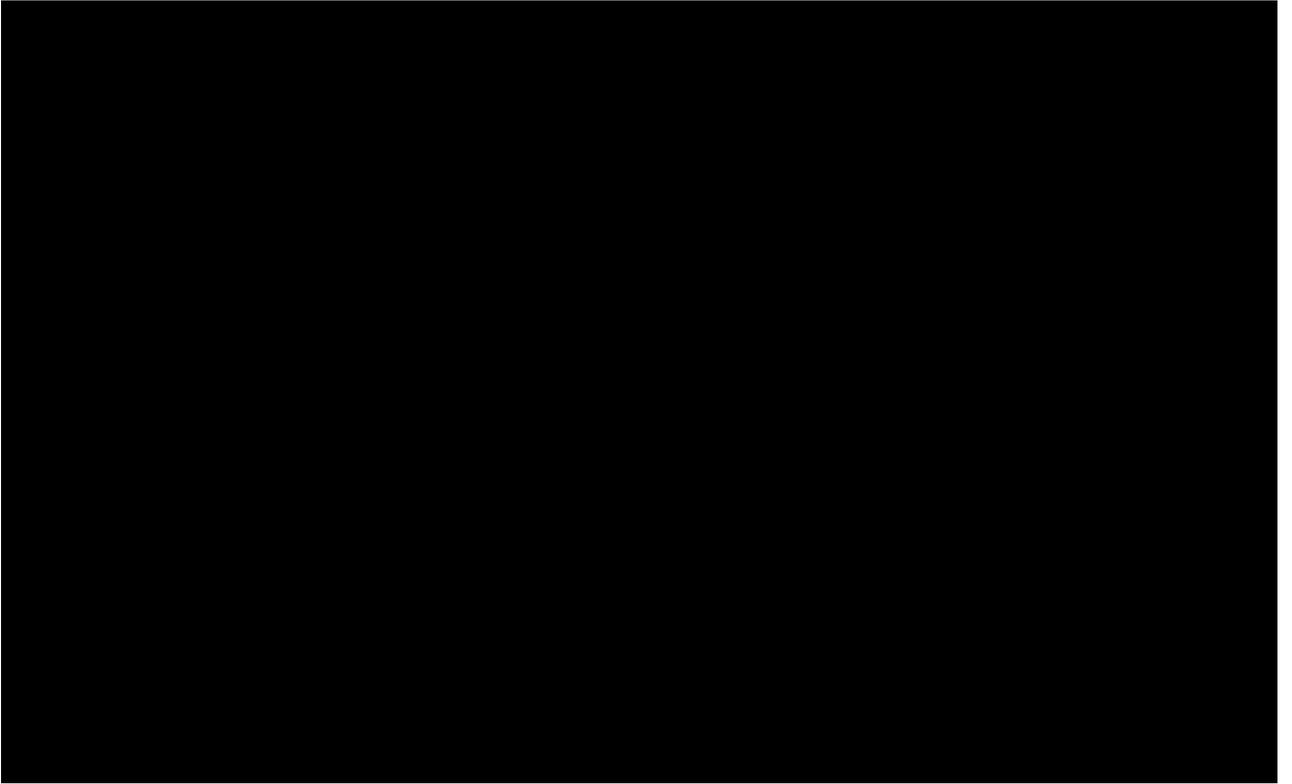
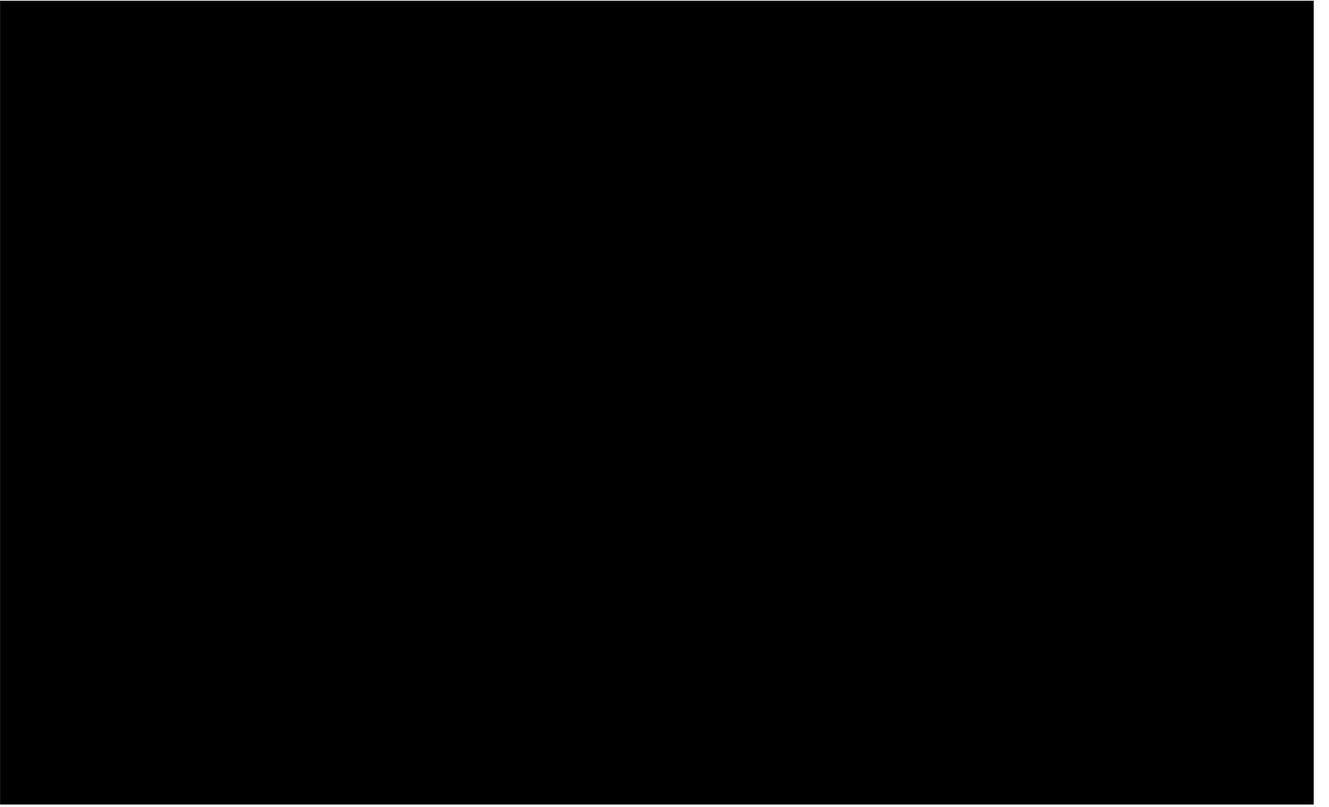
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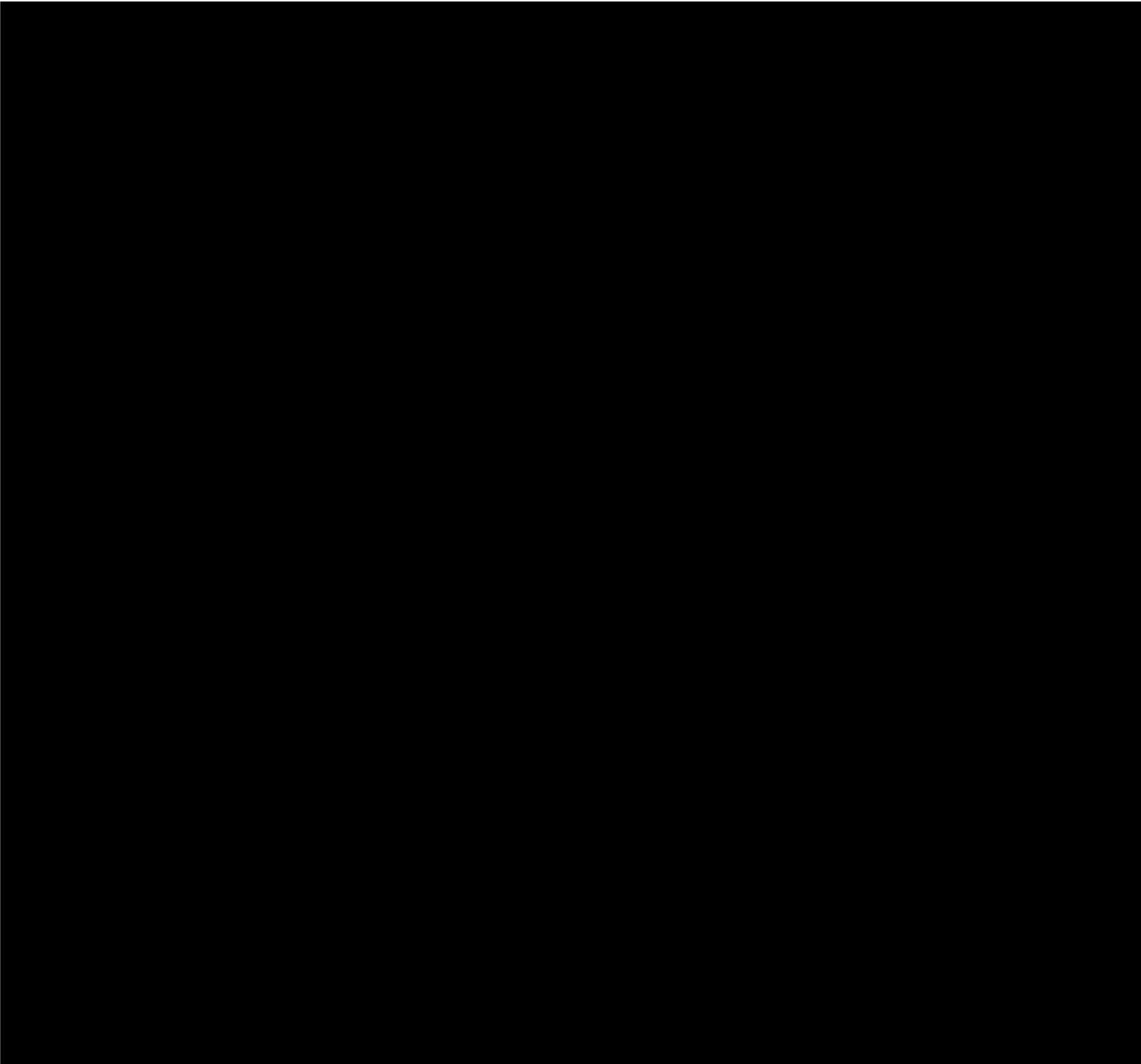
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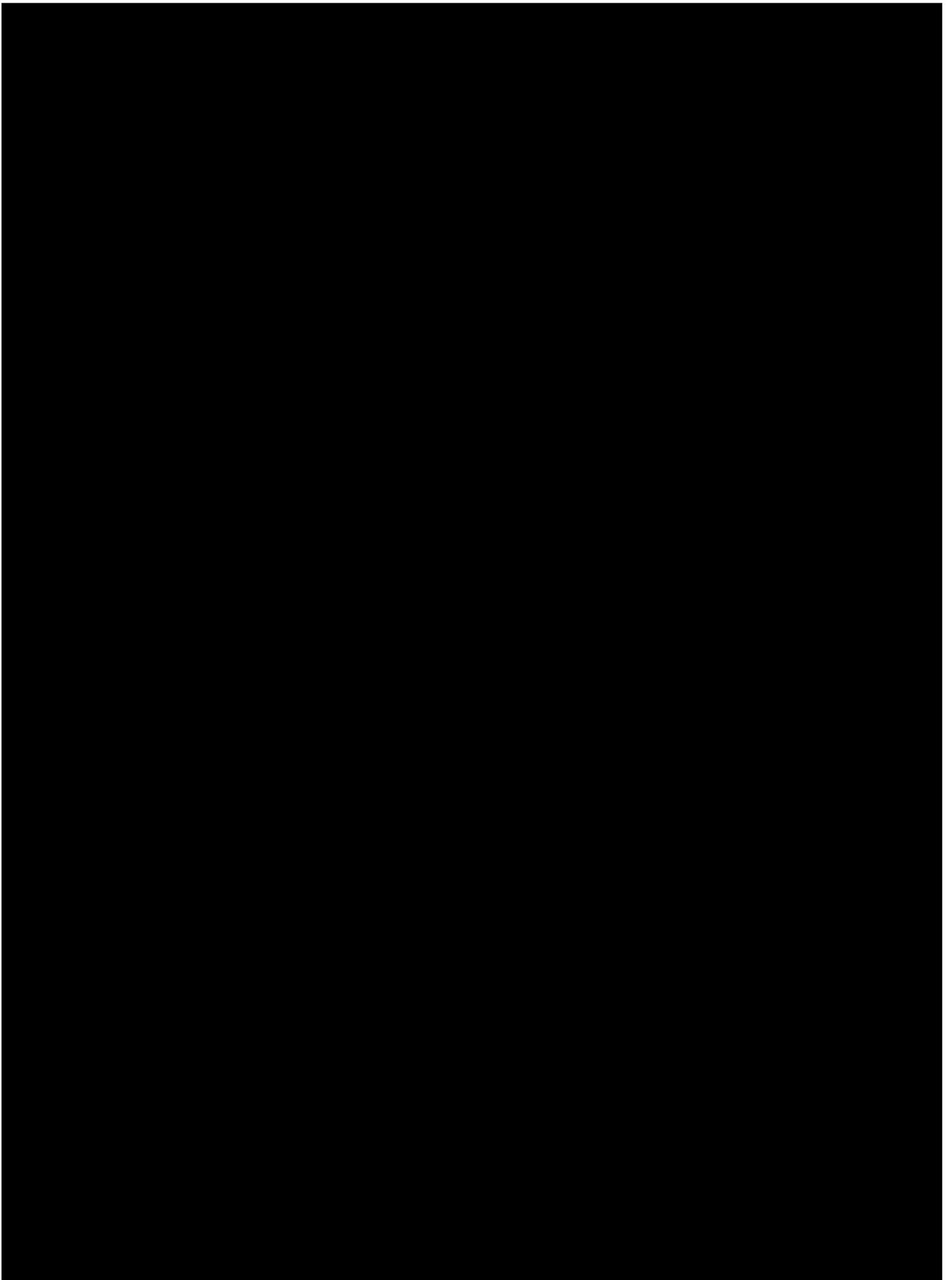
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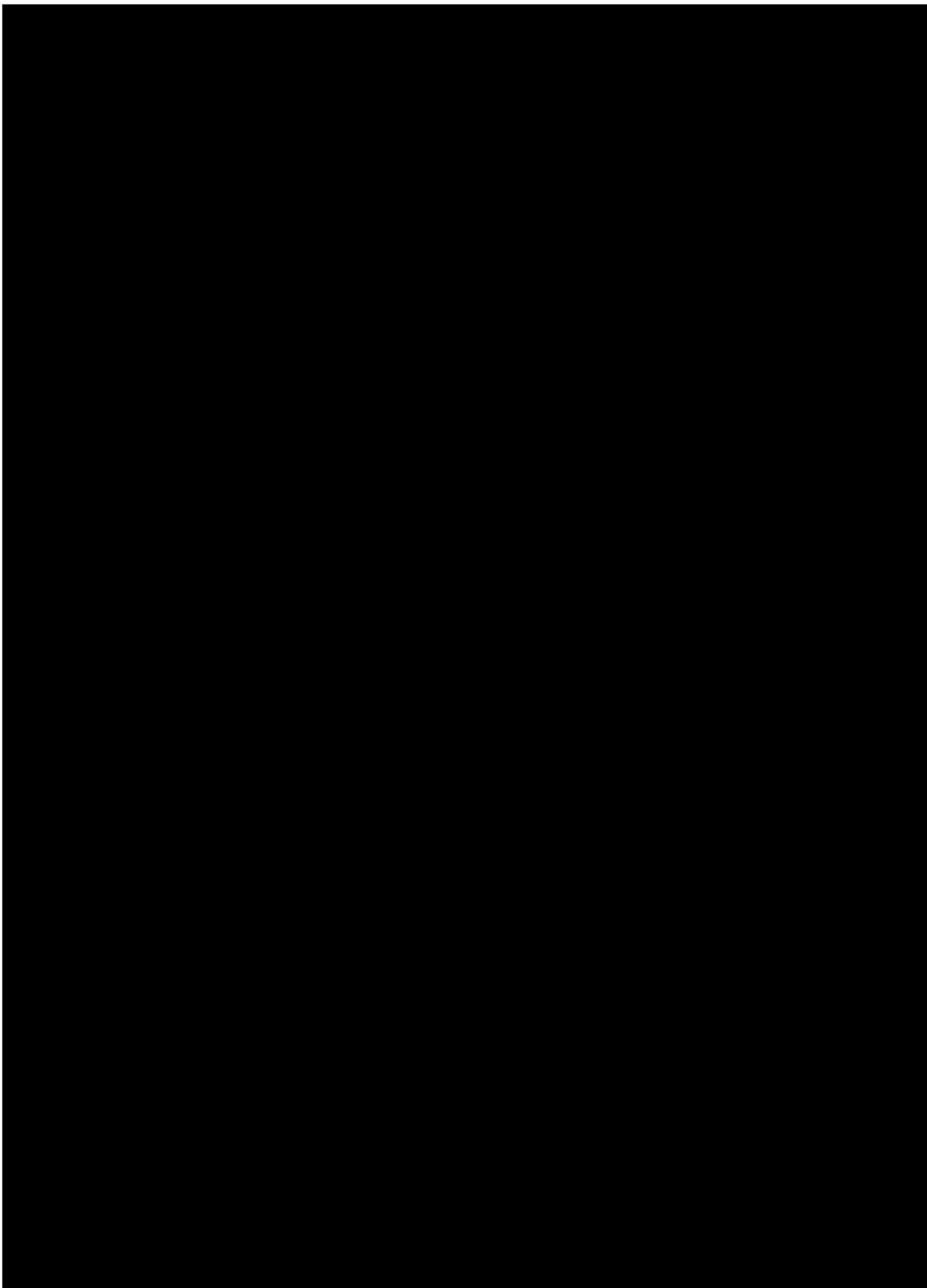
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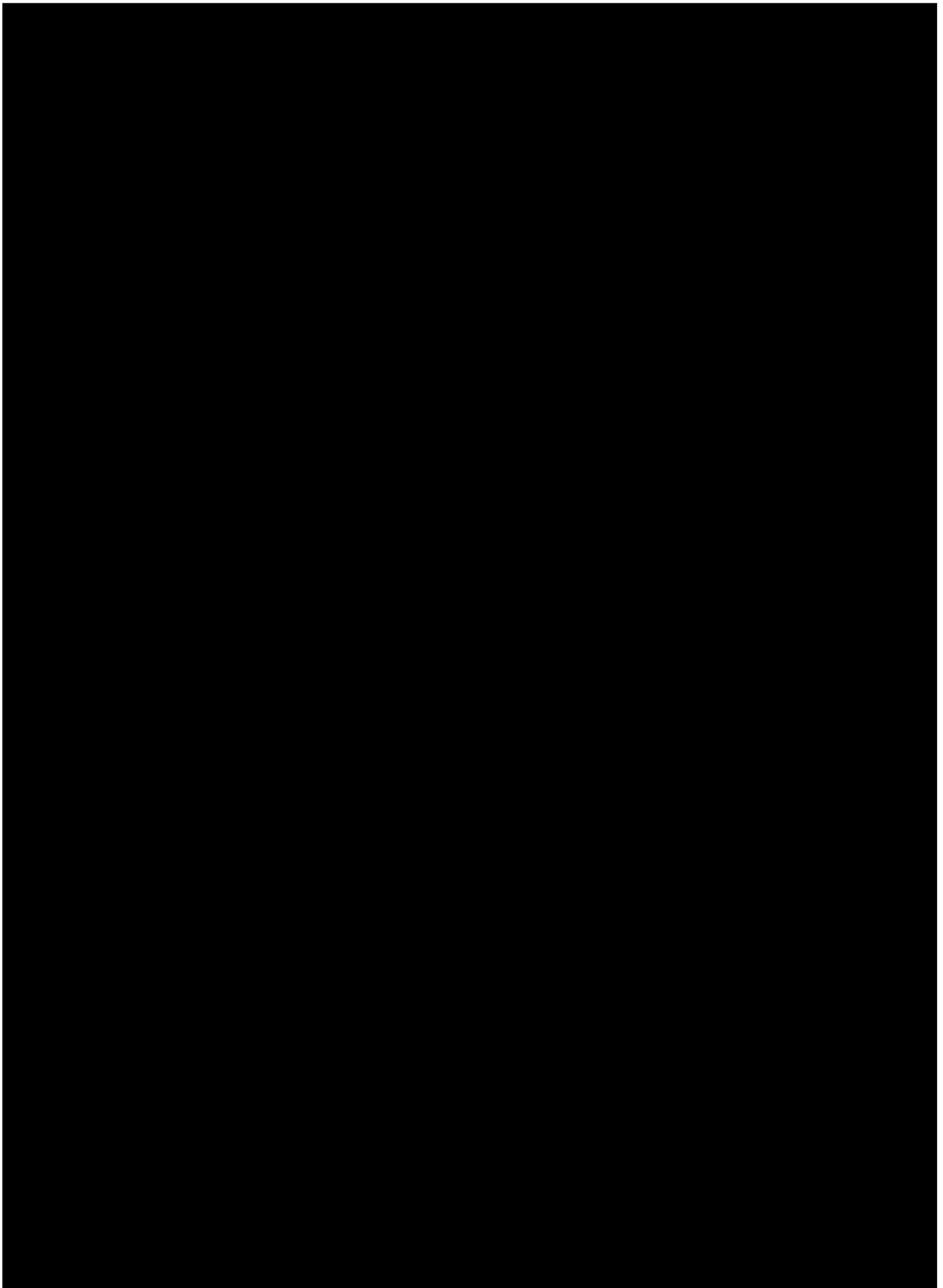
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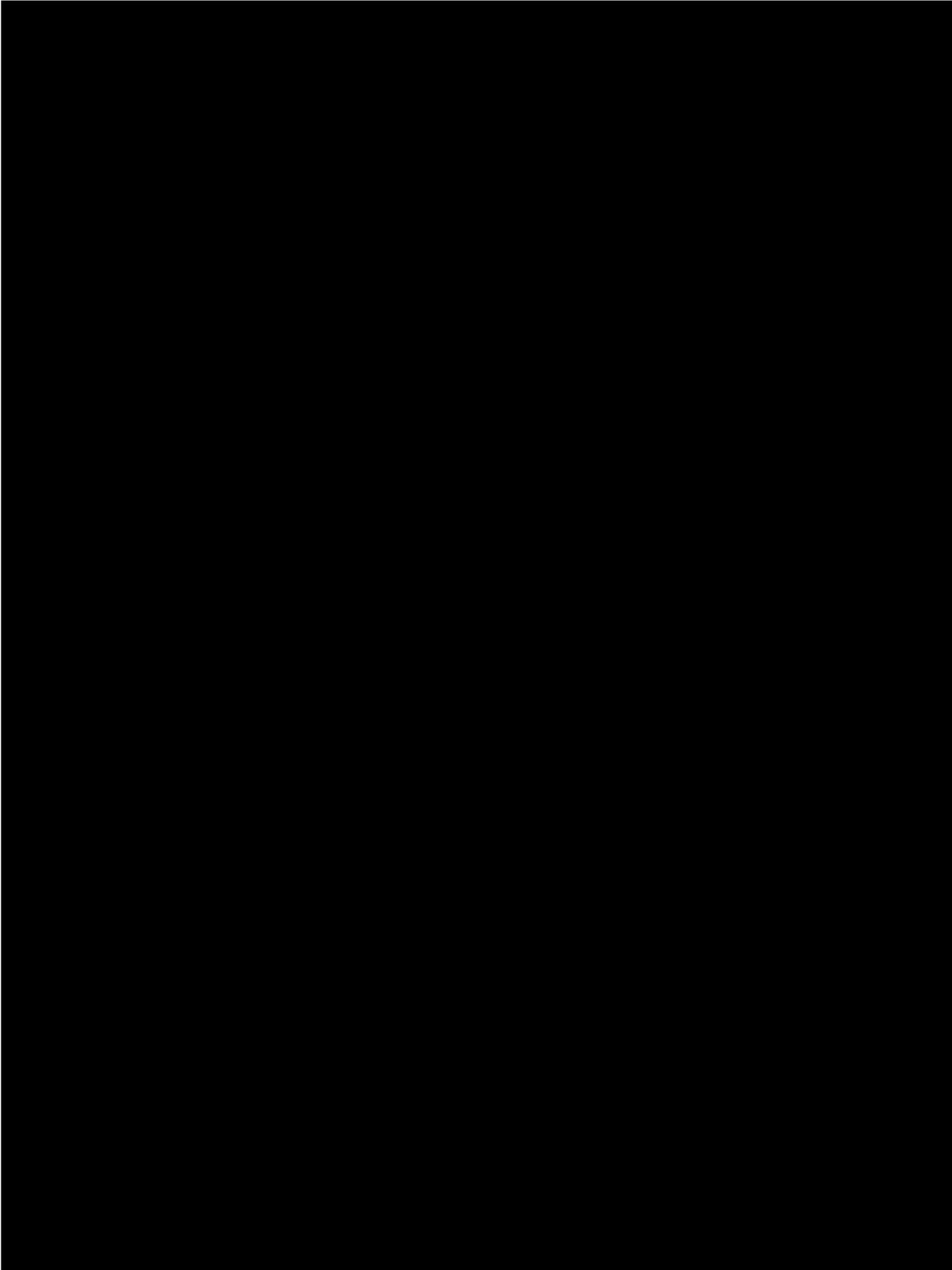


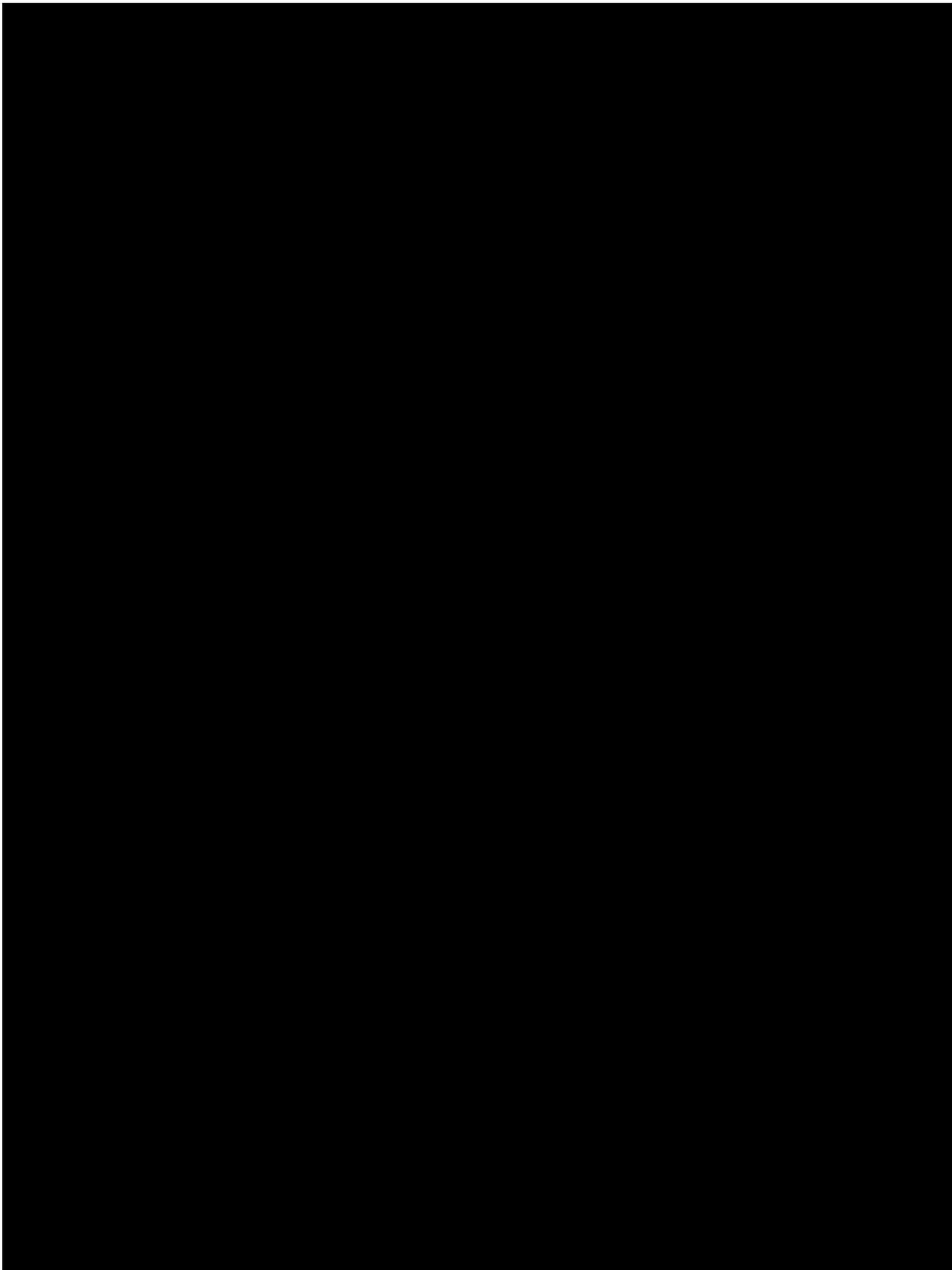




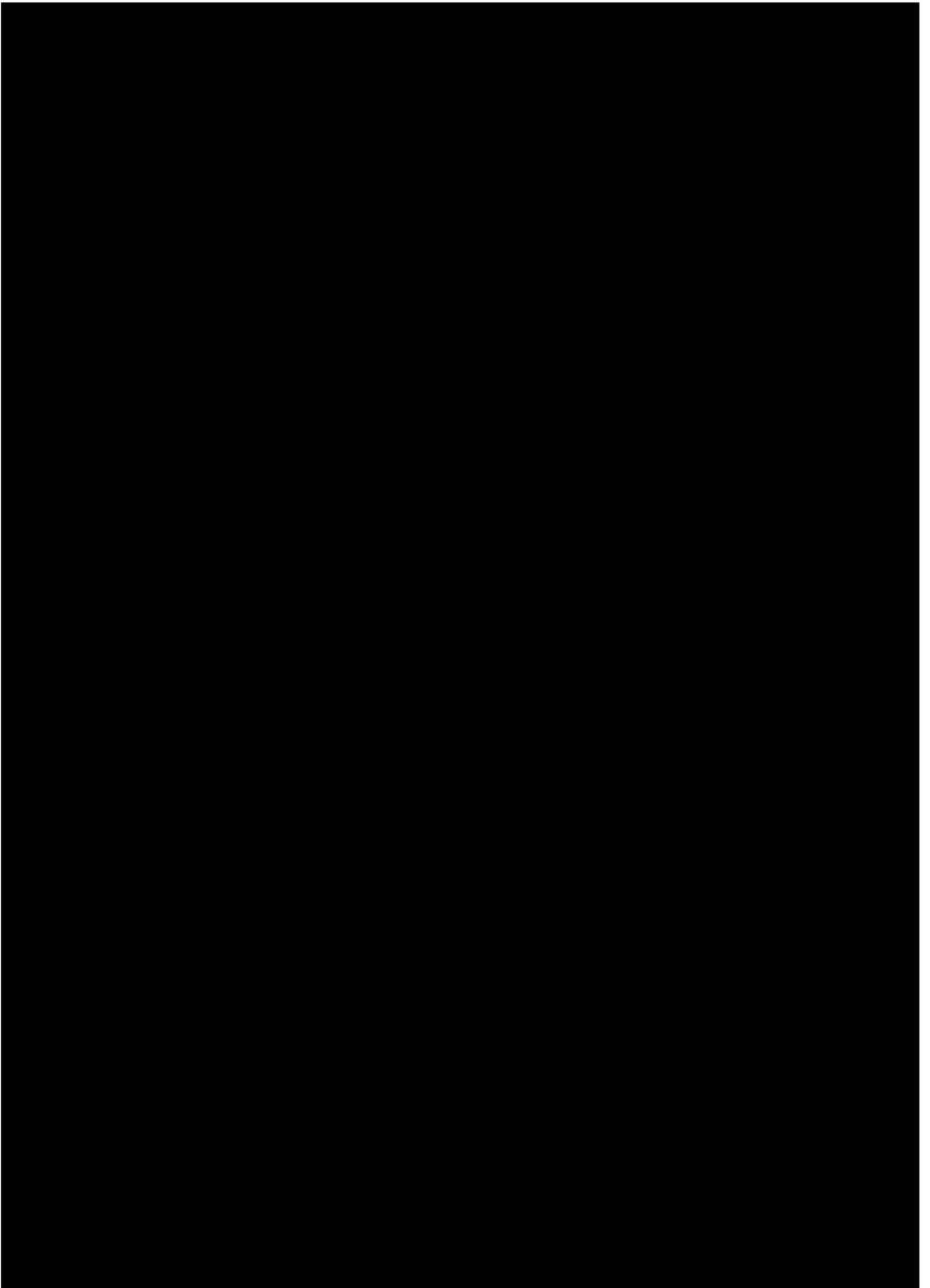


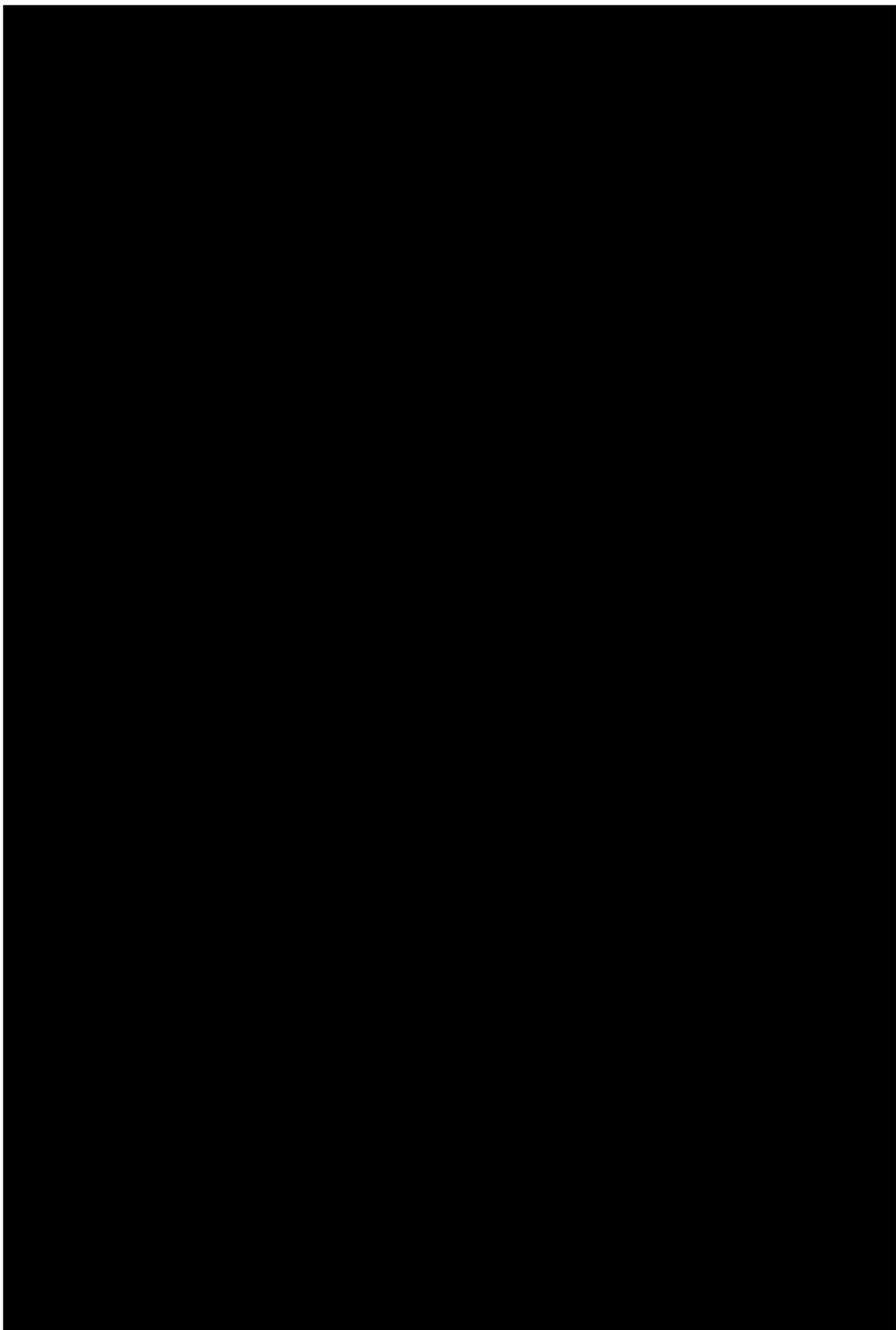






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Chapter 6:

General discussion and conclusion

6. Chapter 6: General discussion and conclusion

This study is the first reported investigation into the anti-inflammatory activity of extracts and compounds sourced from the Australian Muricidae mollusc *Dicathais orbita*. The overall results show significant anti-inflammatory activity in lipophilic extracts from different parts of the mollusc. The hypobranchial gland (HBG) extract was chemically dominated by the anti-cancer brominated indole derivatives 6-bromoisatin and tyrindoleninone. Moreover, the lipid extracts from the foot and viscera were dominated by the unusual long chain ω -3 PUFA DPA. In comparison to other seafood lipid extracts, which had weaker anti-inflammatory activity, the low IC₅₀ of *D. orbita* lipid extracts indicates that an increase in the amount of DPA in the lipid composition leads to an increase in the anti-inflammatory activity. Therefore, it may be concluded *D. orbita* is an excellent source of more than one anti-inflammatory agent, which may also have different modes of action.

6.1. *D. orbita* anti-inflammatory activity

Both the HBG extract from *D. orbita* and 6-bromoisatin demonstrated a significant anti-inflammatory activity, inhibiting the production of the pro-inflammatory mediator NO and down-regulating the expression of the pro-inflammatory cytokines such as TNF α . This activity was observed both *in vitro* (Chapter 3) and *in vivo* (Chapter 4) even in low concentrations (Table 6.1). 6-Bromoisatin showed promising anti-inflammatory activity *in vitro* by inhibiting the production of NO and down regulating the pro-inflammatory cytokine TNF α in LPS-stimulated RAW 264.7 macrophages and prostaglandin E2 (PGE2) in 3T3 ccl-92 fibroblasts. This compound was also active *in vivo* by preventing the development of LPS-induced acute lung injury in a mouse model of acute lung inflammation. 6-Bromoisatin contributes to activity of the crude lipophilic extract of the hypobranchial gland of the *D. orbita*.

Table 6.1: The significantly active concentrations of the chloroform extract of the HBG of the Australian mollusc and the dominant compound in this extract, 6-bromoisatin.

Inflammatory marker	<i>In vitro</i> ($\mu\text{g/mL}$)		<i>In vivo</i> (mg/g)	
	HBG extract	6-Bromoisatin	HBG extract	6-Bromoisatin
NO	50 and 10	50, 10, 2 and 0.4	Not tested	Not tested
TNFα	50, 10, 2 and 0.4	50, 10, 2, 0.4 and 0.08	0.5 and 0.1	0.1 and 0.05
PGE2	50, 10, 2, 0.4 and 0.08	50, 10, 2, 0.4 and 0.08	Not tested	Not tested
IL-1β	Not tested	Not tested	0.5 and 0.1	0.1 and 0.05
NFκB	40	40	Not tested	Not tested

All the extracts from *D. orbita* exhibited promising anti-inflammatory activity. In particular, the chloroform extract of the HBG and the lipids extracted from the viscera and the foot of *D. orbita* were among the most active anti-inflammatory extracts tested in this study with lipid extracts (Chapter 5) were more active than HBG and egg extracts (Chapter 3) for inhibiting the production of NO and downregulating TNF α as indicated by low IC₅₀s (Table 6.2). Stabilised foot lipid extracts showed the lowest IC₅₀ in regards to NO inhibition at 23.17 $\mu\text{g/mL}$. Stabilised viscera also inhibited TNF α with IC₅₀ as low as 10.38 $\mu\text{g/mL}$.

Table 6.2: Comparison of the IC₅₀s among all extracts from *D. orbita*.

Extract		NO inhibition IC ₅₀ ($\mu\text{g/mL}$)	TNF α inhibition IC ₅₀ ($\mu\text{g/mL}$)
HBG extract		30.81	43.03
Egg extract		44.031	Not tested
Foot lipids	Fresh	28.90	80.46
	Stabilised	23.17	23.85
Viscera lipids	Fresh	32.66	39.62
	Stabilised	10.38	27.82

6.2. Sustainability of *D. orbita* extract supply

The two sources of anti-inflammatory agents identified in this study seems promising. However, considering the low yield of the HBG extract, it may be impractical to produce higher amounts of this extract from wild caught *D. orbita*. Taking into consideration the over fishing of muricids around the

world ([González-Tizón et al., 2008](#), [Manríquez and Castilla, 2001](#)), in addition to the amount of data available on the reproductive activity and behaviour of *D. orbita* provide a valid reason to think about the aquaculture of this species. This could facilitate commercially sustainable production of this species to enable large scale extraction of the biologically active extracts from HBG and foot or viscera tissues and to create a regular supply of this muricid for seafood and further research. *D. orbita* is well-studied species and there have been studies investigating the possibility of aquaculturing this mollusc by attempts to close the life cycle ([Noble et al., 2015](#), [Benkendorff, 2013](#)). There is a significant molluscan aquaculture industry in Southern Australia that provides a regular supply of abalone, mussels and oysters. With the available intention of many farmers to expand their practice and include more species, *D. orbita* might be a good choice for commercial development because of its valuable biologically active compounds.

As an alternative to harvesting from the molluscs, 6-bromoisatin can be synthesised ([Vine et al., 2007](#)) and it is currently available in commercial amounts at a price of \$327.00 USD per 25 g from Tokyo Chemical Industry (Chuo-ku, Tokyo, Japan). This may be a better option for producing a basis for pharmaceutical drug development with high quality control. The synthetic compound can also facilitate further animal models to investigate the contribution of this pure compound to the bioactivity of the hypobranchial gland extracts from the snails.

On the other hand for nutraceutical production of the flesh fatty acids, chemical synthesis is not a viable option. Consumers purchasing natural health products expect them to be sustainably produced from a natural source. The yield of lipids from the foot and viscera extracts is about 0.3-0.6 % (~ 6 mg/g) of the foot/viscera tissue) from *D. orbita*. The yield of extract higher from the HBG (3-5 % of the gland tissue), however, the foot and viscera tissues form 7 % and 8 % of the whole snail including the shell respectively, while the HBG only forms about 1 % of the whole snail including the shell. Given that the lipid extracts are more potent anti-inflammatory agents, it seems more practical to produce higher amounts of lipid extracts from the whole bodies of *D. orbita*. This again emphasises the need to explore the fisheries and aquaculture potential for this medicinal species.

Considering the significant anti-inflammatory activity shown by lipophilic chloroform HBG extracts, viscera and the foot lipid extract, it could be worthwhile testing the whole body lipophilic extract from *D. orbita* including all these anti-inflammatory tissues. This would make it more practical to obtain high yields of biologically active extract which can be tested in future inflammatory animal models.

6.3. Priorities for future research

This thesis serves as a baseline for anti-inflammatory data of the muricidae mollusc *Dicathais orbita*. The mode of action of the HBG extract and 6-bromoisatin was investigated using an NFκB translocation experiment which demonstrated that both the HBG extract and the 6-bromoisatin prevented the translocation of NFκB into the nucleus of LPS-stimulated macrophages and subsequently they inhibit the downstream inflammatory pathway. It is not clear how they prevent the translocation of NFκB, hence, further investigations are required to reveal their exact mode of action. This could be facilitated by LPS-MD2 and TLR4-MD2 interference assays ([Park et al., 2009](#), [Kim et al., 2013](#)) to investigate the ability of HBG extract and 6-bromoisatin to interfere with LPS-MD2 and TLR4-MD2 binding and as a result inhibit the NFκB pathway from this point. This could be achieved by incubating macrophages treated by different concentrations of HBG extract or 6-bromoisatin with FITC-conjugated LPS (FITC-LPS) followed by flow cytometry analysis ([Wang et al., 2015](#)) whilst a competitive ELISA could be used to assess the effect of HBG extract and 6-bromoisatin on the LPS binding to rhMD2, MD2^{R90A/Y102A}, MD2^{R90A}, and MD2^{Y102A} proteins using biotin labelled LPS as outlined in ([Zhang et al., 2016](#)). These two assays would help confirm the anti-inflammatory mode of action of the HBG extract and 6-bromoisatin. MD2 inhibitors block the TLR4/MD2-downstream pro-inflammatory signalling pathway and the subsequent NFκB translocation into the nucleus.

The HBG extract and 6 brominated indole derivatives were also found to inhibit PGE₂ which is produced as a result of catalysing arachidonic acid by COX enzymes. These enzymes can be classified into two isoforms, COX-1 and COX-2 ([Vane and Botting, 1998](#)). COX-1 is constitutively expressed

in most normal tissues and COX-1 expression is not regularly controlled by extracellular stimulation ([Oneill and Fordhutchinson, 1993](#), [Fiebich et al., 2002](#)). COX-1 is believed to have homeostatic activity in the gastrointestinal tract and renal tract, and is involved in the platelet function and in macrophage differentiation ([Fiebich et al., 2002](#), [Evans et al., 2003](#)). In comparison, COX-2 is mostly specific to the sites of inflammation and is rapidly stimulated upon the inflammatory response in a variety of cells including macrophages, microglia and astrocytes ([Mizumura et al., 2003](#), [Fiebich et al., 2002](#)). Therefore, another useful *in vitro* study could be used to characterise the selectivity of HBG extract and 6-bromoisatin in the inhibition of COX-2 and could be achieved by testing the effect of HBG extract and 6-bromoisatin on the ability of COX-1 and COX-2 to catalyse arachidonic acid into prostaglandin. The HBG extract and 6-bromoisatin can be tested for the inhibition of COX-1 or COX-2 or both enzymes using the Cayman Chemical COX inhibitor screening Assay kit as described in previous studies e.g. ([Gautam et al., 2010](#), [Sheean et al., 2007](#)) and detailed in [Herrera-Salgado et al. \(2005\)](#).

It is also suggested that a more extensive animal model needs to be employed to investigate the effect of the long-term treatment with HBG extract and 6-bromoisatin on the inflammatory response *in vivo*. The biologically active lipid extracts from *D. orbita* also need more attention due to their potential therapeutic significance. Investigating the anti-inflammatory activity of the PUFAs-rich lipid extracts in *in vivo* models would be of a great importance. For example, adjuvant-induced arthritis in rats is a well-established inflammatory model which has been extensively used to screen anti-inflammatory agents *in vivo* ([Whiteley and Dalrymple, 2001](#)). This model is based on subcutaneous injection of 100 μ L of Complete Freund's adjuvant (CFA), containing heat killed *Mycobacterium tuberculosis*. The commonly used high responder strains include Lewis rats, Sprague-Dawley rats, Wistar rats and Brawn Norway rats. This animal model has been extensively used to assess the anti-inflammatory activity of lipid extracts obtained from many animals including molluscs (Chapter 2, Table 2.4).

6.4. Conclusion

The following conclusions are made based on the findings of this research:

Molluscs and molluscan products have been used in ethno-medical anti-inflammatory preparation in many traditional medical systems. In the past few decades a number of scientific studies have provided promising evidence to support the anti-inflammatory activity of mollusc derived material by both *in vitro* and *in vivo* assays, with a few human clinical trials also undertaken on the NZ green-lipped mussel extract, generating high value nutraceutical products.

Bioassay guided fractionation of the chloroform: methanol extraction from *D. orbita* revealed that methanol extract of HBG, as well as the egg capsules, contains polar compounds such as murexine and tyrindoxyl sulfate with no significant anti-inflammatory activity. The bioactive chloroform extract was dominated by brominated indoles including 6-bromoisatin and tyrindolinone, tyrindoleninone, tyriverdin and 6-bromoindole. These non-polar compounds in the chloroform extract of both HBG and egg capsules demonstrated significant anti-inflammatory activity. The HBG extract from *D. orbita* along with the dominant compound, 6-bromoisatin significantly inhibited the inflammatory response in lungs in a murine acute lungs inflammation. The structure-activity relationships (in terms of anti-inflammatory activity) of the brominated indoles have been established as follows:

- The monomer indoles are more active than dimers.
- Bromine atom addition to the indole compound significantly increases the anti-inflammatory activity.
- The position of the bromine atom on the indole rings also affects the anti-inflammatory activity with the bromine on C5 is more active than C6 which is more active than on C7.

All lipid extracts from *D. orbita* and other seafood organisms examined contained significant amounts of PUFAs, even in the under-utilised parts like heads and viscera. Mollusc species showed the highest amounts of PUFAs and the biologically active ω -3 PUFAs in *D. orbita* include an unusual

lipid composition with the highest percent composition of DPA. All the lipid extracts from *D. orbita* and other seafoods showed no cytotoxic effects on RAW 264.7 and 3T3 ccl-92 fibroblasts, and their inhibitory impact on the NO and TNF α production in LPS-stimulated RAW 264.7 was significant, with superior anti-inflammatory activity in *D. orbita*.

This thesis demonstrated that the under-valued Australian Muricidae *Dicathais orbita* has good potential for future development as a source of anti-inflammatory nutraceuticals and the bioactive brominated indoles in this species provide potential leads for pharmaceutical development.

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APPENDICES:

Appendix 1: Data from the preliminary review of the literature on the previous research on marine invertebrate anti-inflammatory compounds.

Table A.1: The examples of previous research on anti-inflammatory compounds from marine animals.

Species	Compounds	Anti-inflammatory Activity	Reference
Porifera			
not-specified	Girolline	Inhibits signalling through both MyD88-dependent and -independent TLRs (i.e., TLR2, 3, 4, 5, and 7). Downregulates production of IL-6 and IL-8 in human PBMC and macrophages	(Fung et al., 2014)
<i>Aplysina fistularis</i>	11-oxoaerothionin	Suppresses NO ₂ and iNOS; inflammatory cytokines and PGE ₂	(de Medeiros et al., 2012)
<i>Aplysina caissara</i>	extract	reduced hind paw swelling	(Azevedo et al., 2008)
<i>Geodia barretti</i>	barettin	inhibit IL-1 and TNF α	(Lind et al., 2013)
<i>Cacospongia mollior</i>	Scalaradial (SLD), (1,4-dialdehyde terpenoid)	Inhibits Secretory phospholipases A ₂ (sPLA ₂ s)	(Margarucci et al., 2012)
<i>Theonella swinhoei</i>	Perthamide C	Reduces carrageenan-induced paw oedema in mouse, Promotes: TNF α down-regulation IL-8 release in primary human keratinocyte cell lines. selectively interfere with the NO release triggered by eNOS or iNOS without affecting COX-1 or COX-2, leading to reduction in:vascular	(Vilasi et al., 2013)

		permeability, infiltration proliferation	neutrophile lymphocyte	
<i>Coscinoderma mathewsi</i>	Sesterterpene Suvanine	Inhibits HSP60 (important in the inflammatory process)		(Cassiano et al., 2012)
<i>Spongia officinalis</i>	methanol extract/fractions	significant reduction of the oedema		(Dellai et al., 2012)
<i>Plakortis angulospiculatus</i>	Spiculoic acid. zyggomphic acid	Inhibition of iNOS		(Ankisetty et al., 2010)
<i>Hyrtios sp.</i>	heteronemin	Inhibits TNF α -induced NF κ B activation through proteasome inhibition		(Schumacher et al., 2010)
<i>Dysidea frondosa</i>	frondosins	not-specified		(Reiter et al., 2010)
<i>Hymeniacidon sinapium</i>	spongin	Inhibitory effect on production TNF α , IL-1 β , and PGE2 in macrophage, RAW264.7 cells		(Kim et al., 2009)
<i>Petrosaspongia sp.</i>	Petrosa-spongiolides	Selective sPLA(2) inhibition.		(Monti et al., 2009)
<i>Luffariella variabilis</i>	manoalide	Covalently inactivates secretory phospholipase A(2) (sPLA(2)).		(Ettinger-Epstein et al., 2008)
<i>Cacospongia mollior</i>	Scalaradial, a 1,4-dialdehyde marine terpenoid	Selective sPLA(2) inhibition.		(Monti et al., 2007)
not specified	Ethylsmenoquinone, smenospongiarine, smenospongidine and ilimaquinone	Inhibit PLA activity		(Motti et al., 2007)
<i>Stylissa flabellata</i>	stylissadines A and B	a novel class of P2X (7) receptor inhibitors inhibit BzATP-mediated pore formation in THP-1 cells		(Buchanan et al., 2007)
	Cacospongionolide and B	Phospholipase A2 inhibition. Regulates the activation of NF κ B.		(Alcaraz and Paya, 2006)

	petrosaspongiolide M		
<i>Ircinia spinosula</i>	1 polyprenyl chromenols 3(pentaprenyl chromeno) and 4 and 1,4-hydroquinone	Inhibitory Effects on Leukotriene Formation.	(Tziveleka et al., 2005)
	hymenialdisine	Inhibits the production of interleukin-2 and TNF-alpha.	(Sharma et al., 2004)
<i>Petrosaspongia nigra,</i>	g-hydroxybutenolide-containing sesterterpenoid (petrosaspongiolide s M-R)	PLA2 inhibition	(Monti et al., 2004)
<i>Chelonaplysilla violacea</i>	Pourewic acid A (4) and 15-methoxypourewic acid B	Inhibits the production of superoxide by stimulated human peripheral blood neutrophils	(Keyzers et al., 2004)
<i>Haliclona sp.</i>	halipeptin A	carrageenan induced oedema reduction	(Randazzo et al., 2001)
<i>Cacospongia linteiformis</i>	Cyclolinteinone, a sesterterpene	prevents iNOS and inducible COX expression via inhibiting the NFκB translocation in J774 cells.	(D'Acquisto et al., 2000)
	(-)-ilimaquinone		(Radeke et al., 1999)
<i>Ircinia spinosula</i>	prenylated hydroquinone	Suppression of leukotriene B4 and TNFα release in acute inflammatory responses	(Terencio et al., 1998)
<i>Luffariella variabilis</i>	manoalide (MLD)	Inactivates phospholipase A (2) (PLA2) from several sources Inhibit synthesis of eicosanoids in human polymorphonuclear leukocytes (HPMNL). Reduces chemically-induced inflammation <i>in vivo</i> .	(Cabre et al., 1996)

Mediterranean sponge <i>Dysidea avara</i>	avarol	Potent inhibitors of superoxide generation. Inhibit human recombinant synovial phospholipase A (2) . Activity effectively control acute inflammation in experimental models after either oral or topical administration.	(Ferrandiz et al., 1994)
Mediterranean sponge <i>Dysidea avara</i>	avarone	Potent inhibitors of superoxide generation in rat. Peritoneal leukocytes effectively control acute inflammation in experimental models.	(Ferrandiz et al., 1994)
<i>Phyllospongia foliascens</i>	Scalarane-type bishomosesterterpene, foliaspongin		(Kikuchi et al., 1983)
Dictyoceratid sponge	dendalone 3-hydroxybutyrate	Potent inhibitor of prostaglandin synthetase activity and Inhibits platelet aggregation <i>in vitro</i> .	(Buckle et al., 1980)
<i>Stylissa carteri</i>	Carteramine A	inhibitor of neutrophil chemotaxis	(Kobayashi et al., 2007)
<i>Hyrtios erecta</i>	aplysinopsintype	selective inhibitor for neuronal nitric oxide synthase (nNOS)	(Aoki et al., 2001)
Acanthostrongylophora	Manzamines A-F	inhibit the generation of thromboxane (TXB2) in brain microglia	(Sayed et al., 2008)
<i>Plakortis angulospiculatus</i>	plakortide P	strongly inhibits thromboxane B2 release from activated rat brain microglia	(Kossuga et al., 2008)
<i>Gracilaria caudata</i>	sulfated polysaccharide fraction	reduces neutrophil migration and cytokines concentration	(Chaves Lde et al., 2013)
<i>Dichotomaria obtusata</i>	methanol extract	Inhibits mouse ear oedema	(Delgado et al., 2013)
<i>Bryothamnion triquetrum</i>	Bryothamnion triquetrum (BT-MeOH)	peripheral anti-inflammatory activities	(Cavalcante-Silva et al., 2012)

<i>Lithothamnion corallioides</i>	Aquamin (multi-mineral)	Modulating NFκB signalling pathway.	(O'Gorman et al., 2012)
<i>Laurencia glandulifera</i>	Neorogioltriol (tricyclic brominated diterpenoid)	Inhibits the LPS-induced NFκB activation and TNFα production.	(Chatter et al., 2011)
<i>Laurencia okamurae</i> ,	Ethyl acetate extracts	Inhibits the generation of NO, PGE2, IL-6, and TNFα.	(Yang et al., 2010)
<i>Grateloupia elliptica</i> ,	Ethyl acetate extracts	Inhibits the generation of NO, PGE2, IL-6, and TNFα.	(Yang et al., 2010)
<i>Gloiopeltis furcata</i> ,	Ethyl acetate extracts	Inhibits the generation of NO, PGE2, IL-6, and TNFα.	(Yang et al., 2010)
Cnidaria			
<i>lobophytum crassum</i>	cembranoids, lobocrasols A-D (1-4)	Significantly inhibits TNFα - induced NFκB activation in HepG2 cells.	(Thao et al., 2014)
<i>Pseudopterogorgia acerosa</i>	pseudopterane diterpene	Inhibited the expression and secretion of: TNFα, IL-6, IL-1β, NO, interferon gamma-induced protein 10 (IP-10), COX-2, iNOS, monocyte chemoattractant protein-1 (MCP-1) induced by LPS in primary murine macrophages.	(Gonzalez et al., 2013)
soft corals	Diterpenoids and Sesquiterpenoids	Inhibits expression of iNOS and COX-2 in LPS-Stimulated RAW264.7 macrophage. Inhibits the production of superoxide anion.	(Wei et al., 2013)
<i>Sinularia flexibilis</i>	11-Episinulariolide Acetate	Inhibits the accumulation of the iNOS and COX-2 in RAW264.7 macrophages.	(Hsu et al., 2013)
<i>Pseudopterogorgia elisabethae</i>	pseudopterosins	Blocks PLA2.	(Onumah, 2013)
<i>Eunicea fusca</i>	dilophol diterpene (eunicidiol)	reduces oedema	(Marchbank et al., 2012)

<i>Cladocora cespitosa</i>	cladocoran A (CLD A)	Human group IIA phospholipase A2 inactivation	(Monti et al., 2011)
<i>Eunicea fusca</i>	diterpene (fuscoside E)	Inhibition of oedema	(Reina et al., 2011)
<i>Menella sp</i>	menellsteroid A. Menellin A	Inhibition of iNOS	(Chai et al., 2010)
<i>Nephthea chabroli</i>	New 19-oxygenated and 4-methylated steroids Nebrosteroid I,J,L,M	Nebrosteroid I,J,L and M reduces the expression of iNOS and COX-2 in RAW 264.7 macrophages.	(Cheng et al., 2009a)
the gorgonian <i>Pseudopterogorgia elisabethae</i>	F-1 (pseudopterოსins PsQ, PsS and PsU) and F-2 (amphilectosins A and B, PsG, PsK, PsP and PsT and seco-pseudopterოსins seco-PsJ and seco-PsK)	Inhibition of MPO levels by all extracts and fractions, stronger than indomethacin and dexamethasone.	(Correa et al., 2009)
<i>Nephthea erecta</i>	oxygenated ergostanoids ((3b,23S)-Ergosta-5,24(28)-diene-3,23-diol and (3b,22S)-ergosta-5,24(28)-diene-3,17,22-triol)	Reduce the expression of iNOS and COX-2 enzymes.	(Cheng et al., 2009b)
<i>Pseudopterogorgia elisabethae</i>	Pseudopterოსin A	Inhibits phagocytosis. Adjusts intracellular calcium turnover in a pertussis toxin sensitive site in <i>Tetrahymena thermophila</i>	(Moya and Jacobs, 2006)
<i>Sinularia crassa</i>	sphingosine derivative	Reduces the carrageenin-induced rat hind paw edema.	(Radhika et al., 2005)
<i>Lobophytum species</i>	cembrenoid diterpene (lobohedleolide)	Reduces the carrageenin-induced rat hind paw edema.	(Radhika et al., 2005)

<i>Eunicea fusca</i>	diterpene (fucoside E)	Selectively inhibits leukotriene biosynthesis.	(Jacobson and Jacobs, 1992)
a soft coral	flexibilide	Anti-inflammatory activity <i>in vitro</i> .	(Buckle et al., 1980)
gorgonian <i>Junceella fragilis</i> ,	Frajunolides B and C	Significantly inhibit superoxide anion. Inhibit the generation of elastase by human neutrophils <i>in vitro</i>	(Shen et al., 2007)
Chordata			
<i>Aplidium orthium</i>	1,14-sperminedihomovanillamide (orthidine F, 7), Orthidines A-E, tubastrine, 3,4-dimethoxyphenethyl-beta-guanidine	Inhibits the production of superoxide by PMA-stimulated human neutrophils <i>in vitro</i> .	(Pearce et al., 2008)
<i>Cystodytes dellechiaiei</i>	ascididemin (marine alkaloid)	Downregulates COX-2 and PLA2.	(Morvan, 2013)
<i>Clavelina moluccensis</i>	Lepadiformines A and B		(Sauviat et al., 2006)
The New Zealand ascidian <i>Aplidium spp.</i>	Asciathiazones A and B	Both inhibit the generation of superoxide by neutrophils <i>in vitro</i> .	(Pearce et al., 2007a)
<i>Aplidium conicum</i>	conicamin,	Histamine-antagonistic activity.	(Aiello et al., 2003)
<i>Synoicum sp.</i> ,	A halogenated furanone rubrolide O	Inhibited superoxide anion production in human neutrophils	(Pearce et al., 2007b)
Echinodermata			
<i>Marthasterias glacialis</i>	Unsaturated Fatty Acids and Ergosta-7,22-dien-3-ol	Prevention of CHOP-Mediated ER-Stress and NFκB Activation.	(Pereira et al., 2014)
<i>Heliocidaris erythrogramma</i>	Dichloromethane (DCM) methanol (MeOH) extract	Inhibition of COX-1 and COX-2. Aqueous extract caused reduction of leukotriene production by stimulated porcine neutrophils was affected only by the.	(Sheean et al., 2007)

	aqueous extracts of male gonadal tissue	The DCM and aqueous extracts, showed anti-inflammatory activity in rats developing adjuvant-induced polyarthritis.	
<i>Marthasterias glacialis</i> (spiny sea-star)	Unsaturated Fatty Acids and Ergosta-7,22-dien-3-ol	Prevention of: CHOP-Mediated ER-Stress. NFκB translocation.	(Pereira et al., 2014)
Mollusca			
<i>Arca subcrenata</i>	a novel polypeptide fraction (P2)	Suppresses the production of NO in LPS-induced RAW264.7 macrophage and the secretion of inflammatory cytokines IL-6 and TNFα in human cervical cancer HeLa cells. Downregulate the IL-8 Inhibit the COX-2 and iNOS-related pathways in HeLa cells. Inhibit gene expression of proinflammatory cytokine IL-6.	(Wu et al., 2014)

Appendix 2: Mass spectral fragmentation patterns of the brominated indoles detected by GC/MS in an extract from the hypobranchial glands of *Dicathais orbita*.

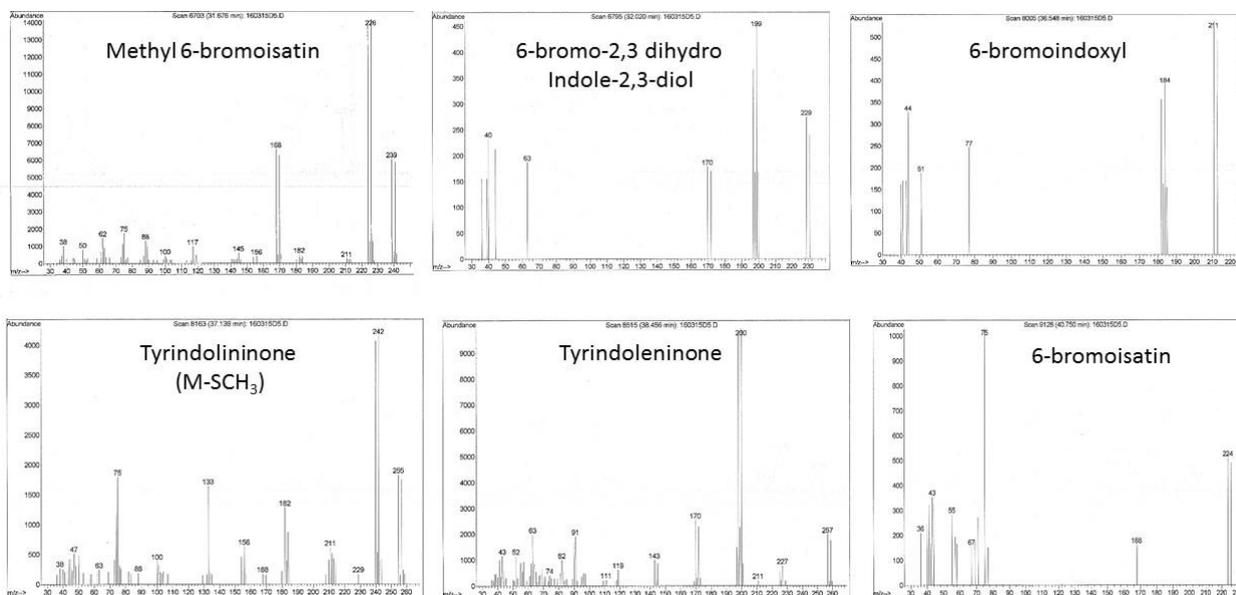


Figure A.1: Mass spectral fragmentation patterns of the brominated indoles detected by GC/MS in an extract from the hypobranchial glands of *Dicathais orbita*.

Appendix 3: Mass spectral fragmentation patterns of the brominated indoles detected by LC/MS in an extract from the hypobranchial glands of *Dicathais orbita*.

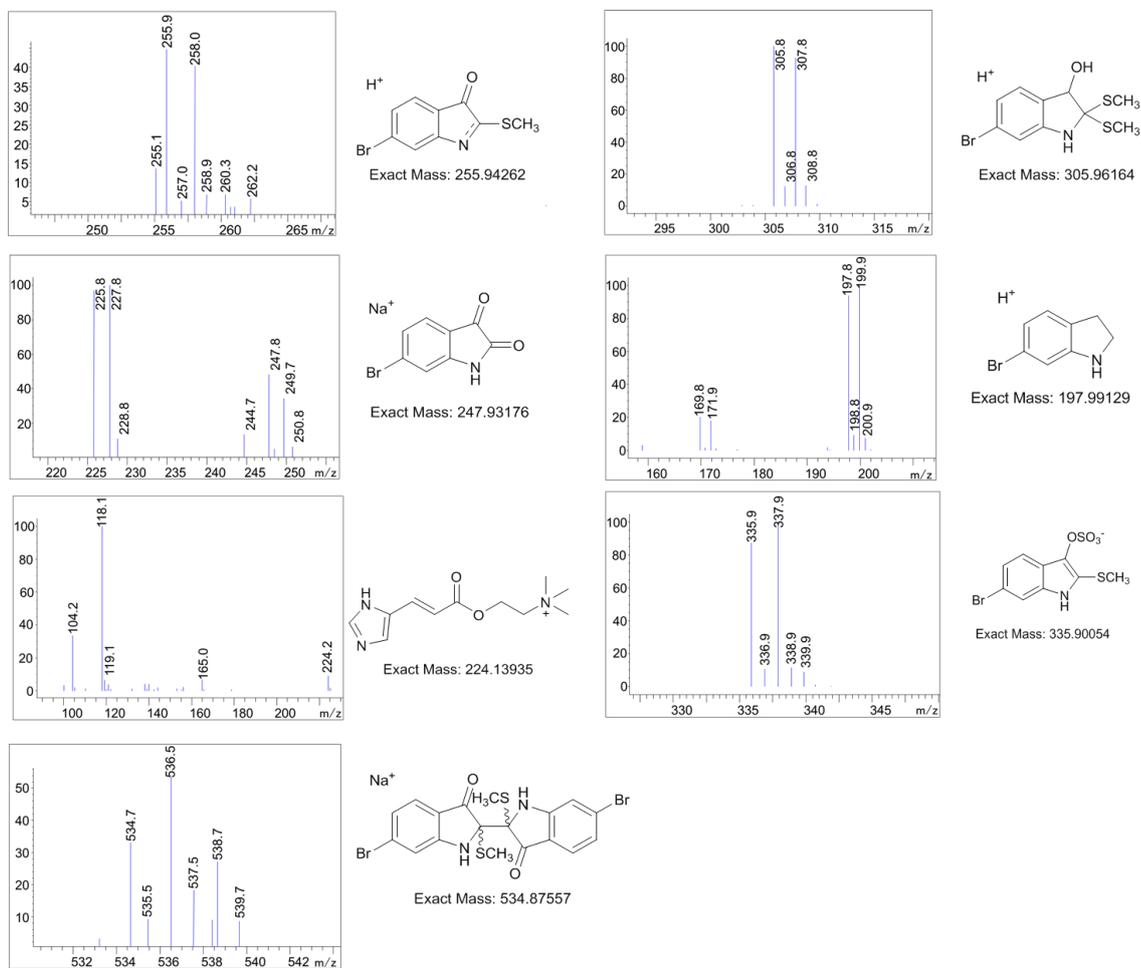


Figure A.2: Mass spectral fragmentation patterns of the brominated indoles detected by LC/MS in an extract from the hypobranchial glands of *Dicathais orbita*.

Appendix 4: Composition and the morphology of the cellularity in BALF.

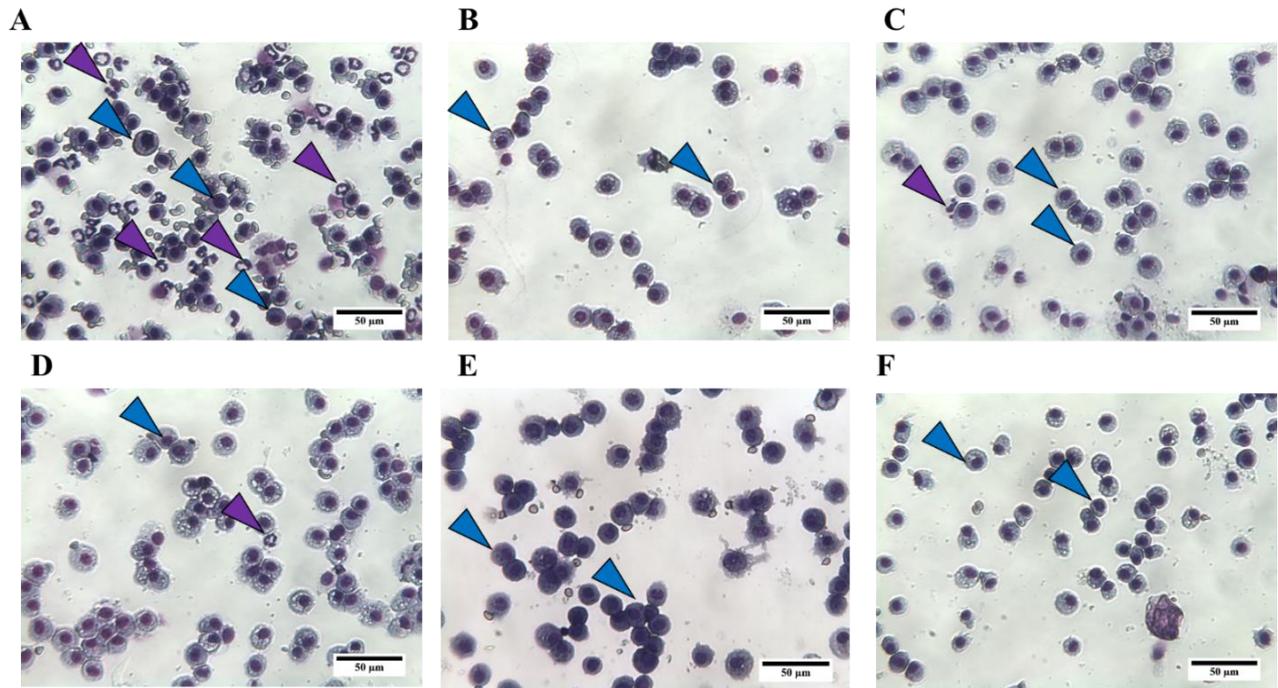


Figure A.3: Composition and the morphology of the cellularity in BALF from A) LPS stimulated untreated mice; B) Unstimulated untreated mice; C) mice stimulated and treated with 0.5 mg/g HBG extract; D) mice treated with 0.1 mg/g HBG extract; E) mice treated with 0.1 mg/g 6-bromoisatin; F) mice treated with 0.05 mg/g 6-bromoisatin. Alveolar macrophages (blue arrows) are the dominant cells with unsegmented nucleus and the neutrophils (purple arrows) are plenty in the LPS stimulated positive control while very scanty or absent in the other groups. Images are representative of three different fields on the slide from total of 6 slides per group except for the PBS negative group n= 5. Scale bars set to 50 µm.

Appendix 5: Microscopic examination of the H&E stained lung sections.

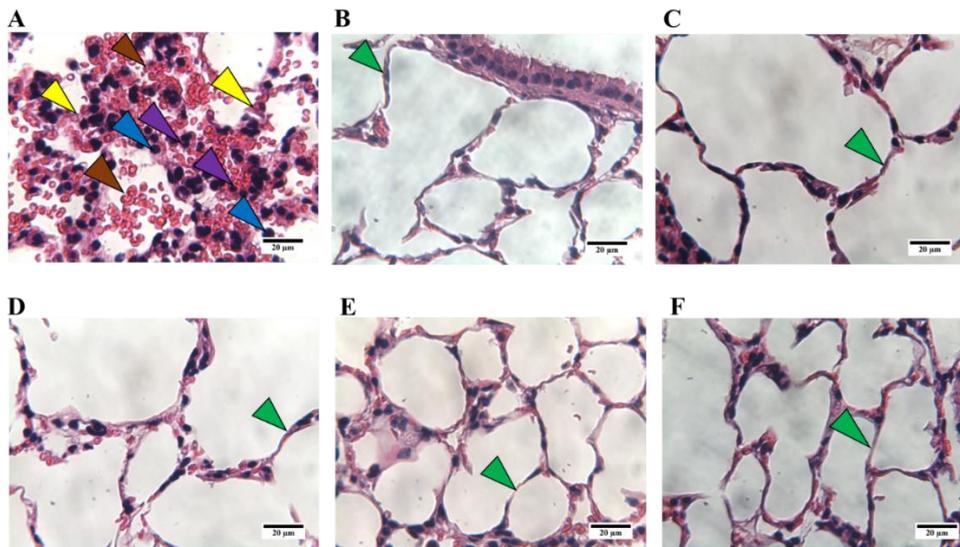


Figure A.4: Microscopic examination of the H&E stained lung sections from A) LPS positive control showing the areas severe haemorrhage as indicated by large amount of red blood cells in the air space (brown arrows), thickened and damaged alveolar walls (yellow arrows), infiltration of alveolar macrophages (blue arrows) and neutrophils (purple arrows) into the air space; B) PBS negative control group received 50 µL of PBS intranasally; C) 0.5 mg/g HBG extract treated mice; D) 0.1 mg/g HBG extract treated mice with undamaged alveolar space and parenchymal architecture; E) mice treated with 0.1 mg/g of 6-bromoisatin, and; F) mice treated with 0.05 mg/g 6-bromoistain. All HBG extract and 6-bromoisatin treated mice showing preserved alveolar space and parenchymal architecture and all showed thin alveolar wall (green arrow heads) and lack of signs of haemorrhage or macrophage and neutrophil infiltrations. Images are representative of 3 different sections on each slide out of 6 slides per group magnified X 400 and scale bars set to 20 µm.

Appendix 6: Correlations between histological parameters in the lungs of mice.

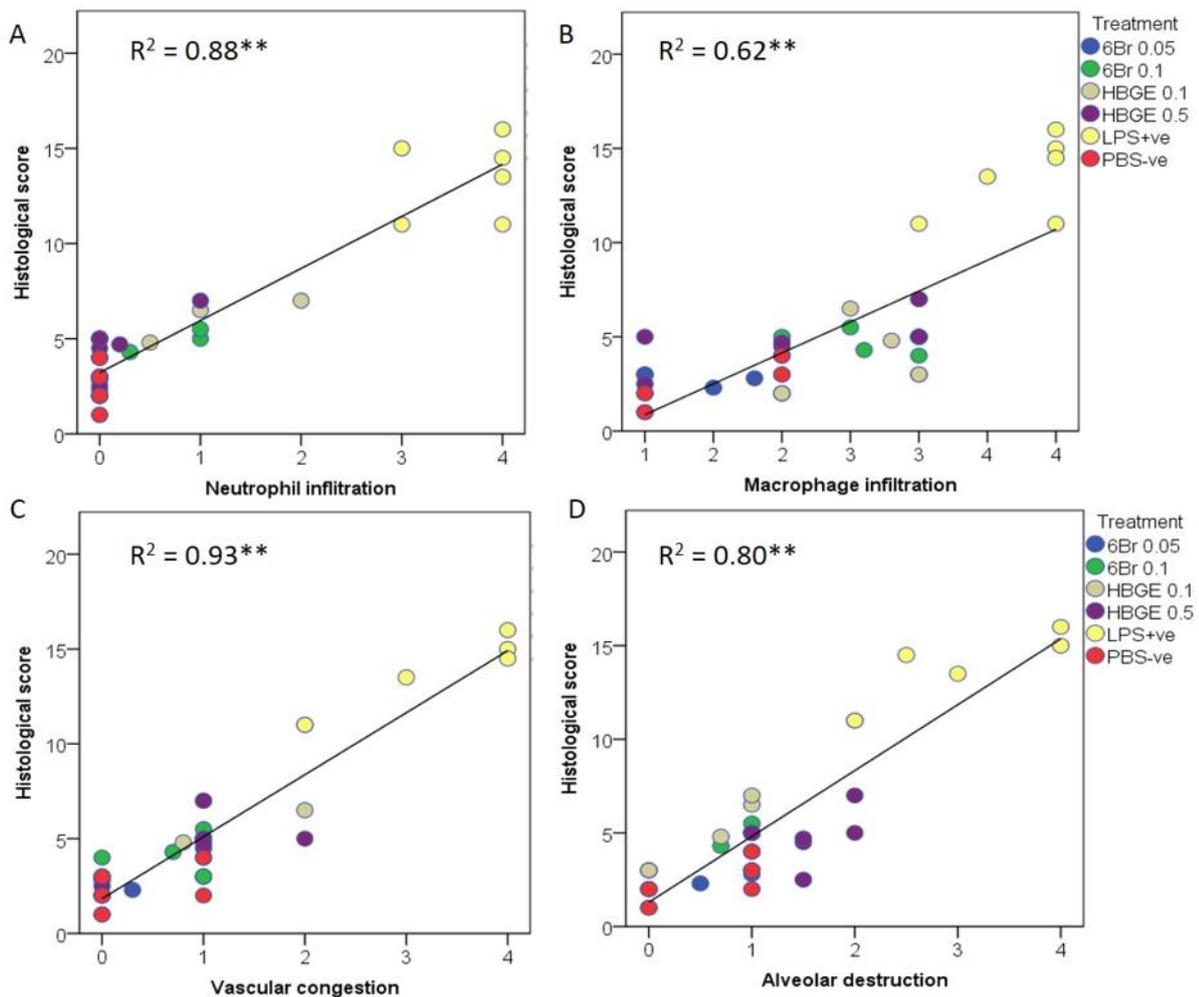


Figure A.5: Correlations between histological parameters in the lungs of mice. The relationship between the overall histopathological score and A) neutrophil infiltration; B), macrophage infiltration; C) vascular congestion; D) alveolar destruction. The linear relationship and R2 values are determined from all samples pooled across all mice in the LPS-stimulated acute lung inflammation model: PBS-ve negative control; LPS +ve lipopolysaccharide stimulated positive control; HBGE Hypobranchial gland extract at 0.1 and 0.5 mg/g and; 6Br 6 bromoisatin at 0.1 and 0.05 mg/g. ****** significant relationship at $p < 0.01$.

Appendix 7: Correlations between neutrophil infiltration and inflammatory markers in the lungs of mice.

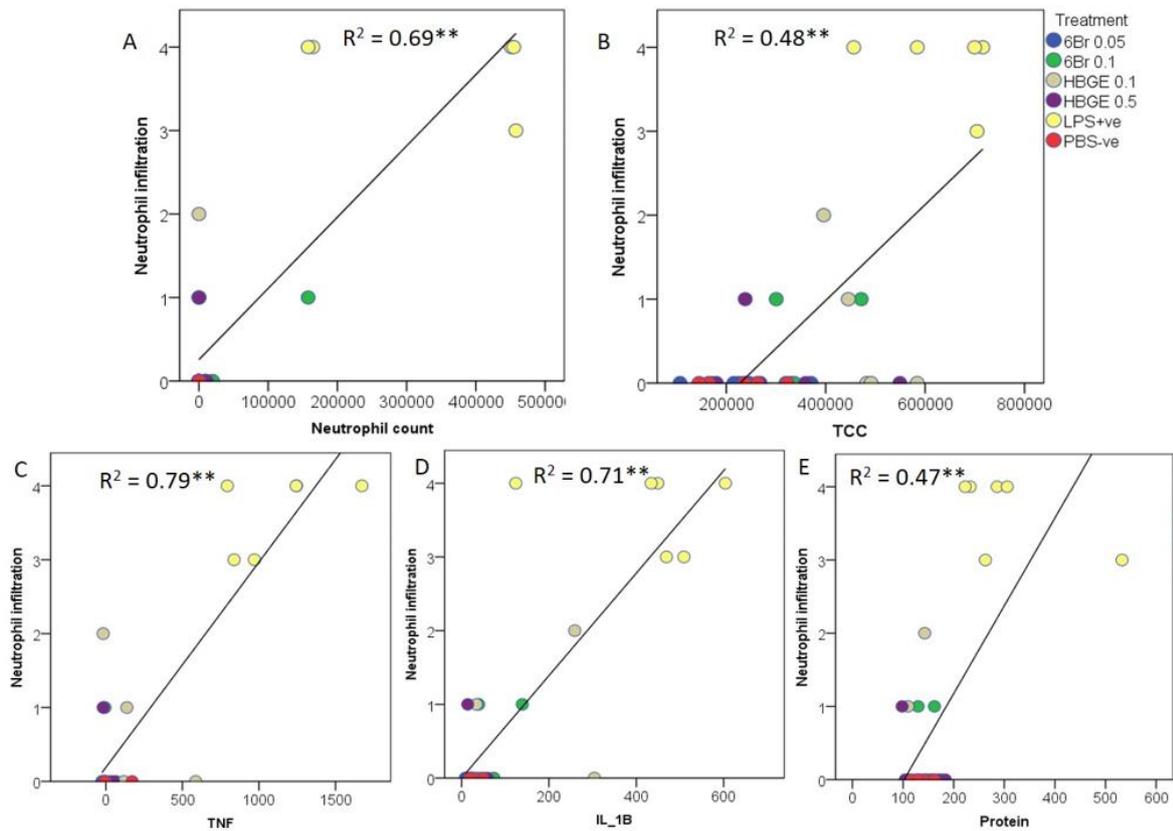


Figure A.6: Correlations between neutrophil infiltration and inflammatory markers in the lungs of mice. The relationship between the histopathological score for neutrophils and parameters in the bronchoalveolar lavage fluids: A) Neutrophil count; B), total cell count (TCC); C) Tumor necrosis factor (TNF) alpha; D) interleukin I-1B; and E) protein. The linear relationship and R2 values are determined from all samples pooled across all mice in the LPS-stimulated acute lung inflammation model: PBS-ve negative control; LPS +ve lipopolysaccharide stimulated positive control; HBGE Hypobranchial gland extract at 0.1 and 0.5 mg/g and; 6Br 6 bromoisatin at 0.1 and 0.05 mg/g. ****** significant relationship at $p < 0.01$.

Appendix 8: Correlations between macrophages and inflammatory markers in the lungs of mice.

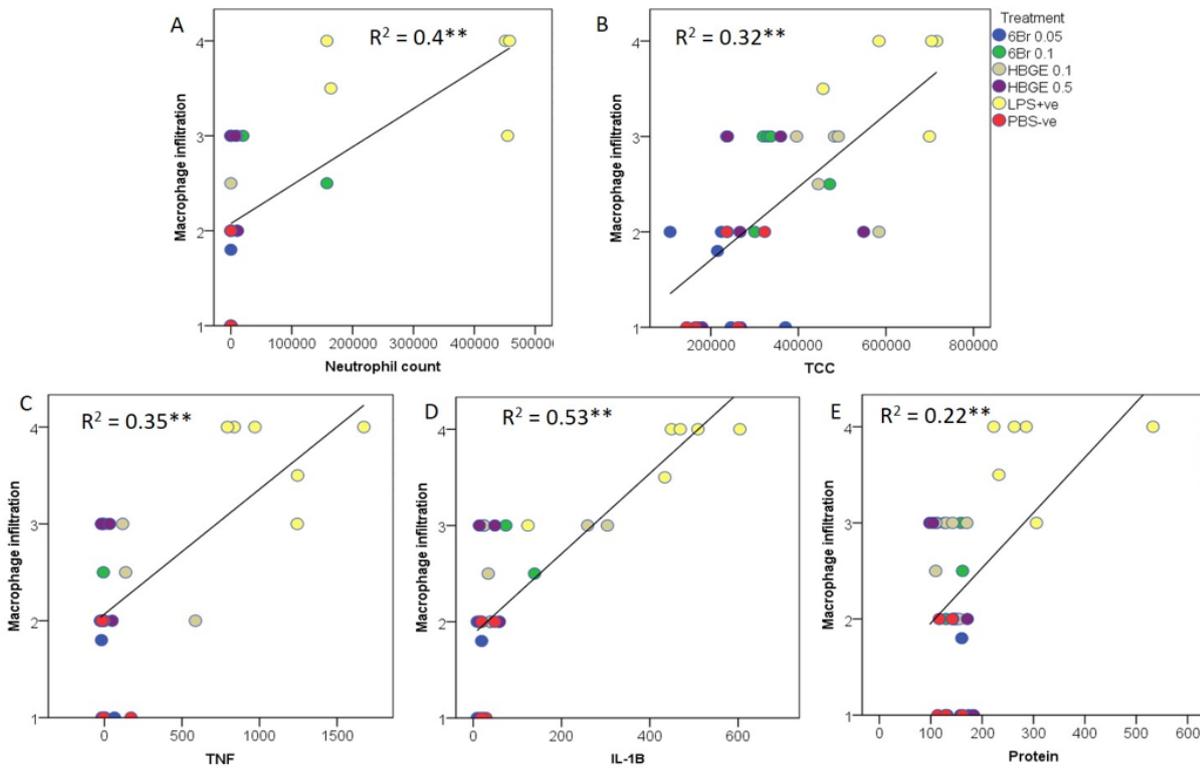


Figure A.7: Correlations between macrophages and inflammatory markers in the lungs of mice. The relationship between the histopathological score for macrophages infiltration and parameters in the bronchoalveolar lavage fluids: A) Neutrophil count; B), total cell count (TCC); C) Tumor necrosis factor (TNF) alpha; D) interleukin I-1B; and E) protein. The linear relationship and R2 values are determined from all samples pooled across all mice in the LPS-stimulated acute lung inflammation model: PBS-ve negative control; LPS +ve lipopolysaccharide stimulated positive control; HBGE Hypobranhial gland extract at 0.1 and 0.5 mg/g and; 6Br 6 bromoisatin at 0.1 and 0.05 mg/g. ****** significant relationship at $p < 0.01$.

Appendix 9: Correlations between alveolar destruction and inflammatory markers in the lungs of mice.

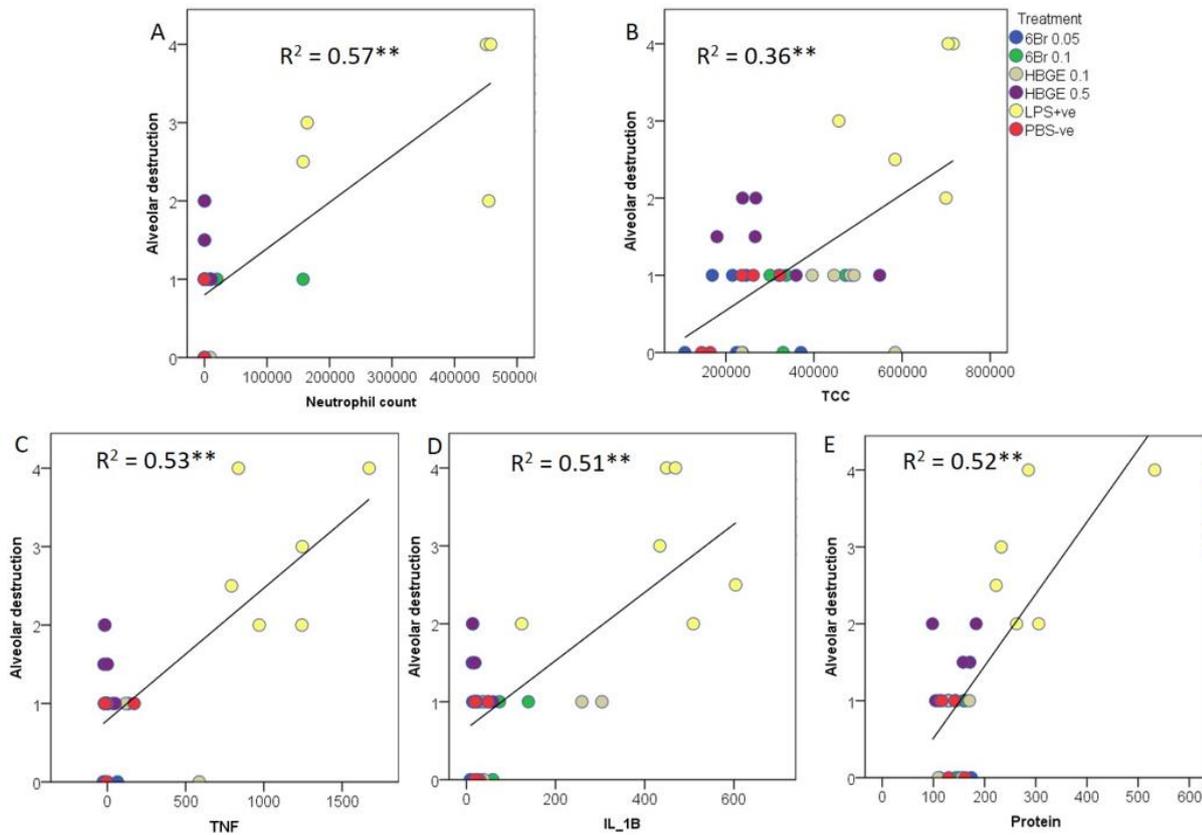


Figure A.8: Correlations between alveolar destruction and inflammatory markers in the lungs of mice. The relationship between the histopathological score for alveolar destruction and parameters in the bronchoalveolar lavage fluids: A) Neutrophil count; B), total cell count (TCC); C) Tumor necrosis factor (TNF) alpha; D) interleukin I-1B; and E) protein. The linear relationship and R2 values are determined from all samples pooled across all mice in the LPS-stimulated acute lung inflammation model: PBS-ve negative control; LPS +ve lipopolysaccharide stimulated positive control; HBGE Hypobranhial gland extract at 0.1 and 0.5 mg/g and; 6Br 6 bromoisatin at 0.1 and 0.05 mg/g. ** significant relationship at $p < 0.01$.

Appendix 10: Correlations between vascular congestion and inflammatory markers in the lungs of mice.

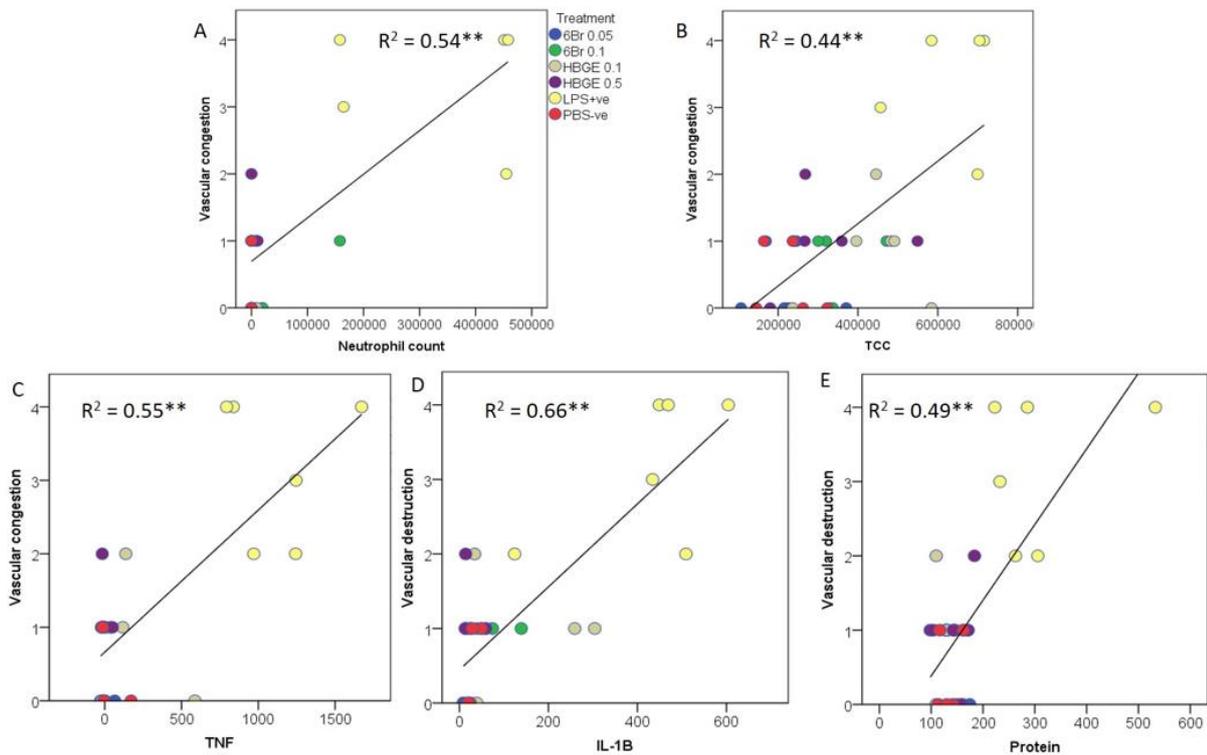


Figure A.9: Correlations between vascular congestion and inflammatory markers in the lungs of mice. The relationship between the histopathological score for vascular congestion and parameters in the bronchoalveolar lavage fluids: A) Neutrophil count; B), total cell count (TCC); C) Tumor necrosis factor (TNF) alpha; D) interleukin I-1B; and E) protein. The linear relationship and R2 values are determined from all samples pooled across all mice in the LPS-stimulated acute lung inflammation model: PBS-ve negative control; LPS +ve lipopolysaccharide stimulated positive control; HBGE Hypobranchial gland extract at 0.1 and 0.5 mg/g and; 6Br 6 bromoisatin at 0.1 and 0.05 mg/g. ****** significant relationship at $p < 0.01$.

Appendix 11: Correlations between cell counts and inflammatory markers in the bronchoalveolar lavage fluids of mice.

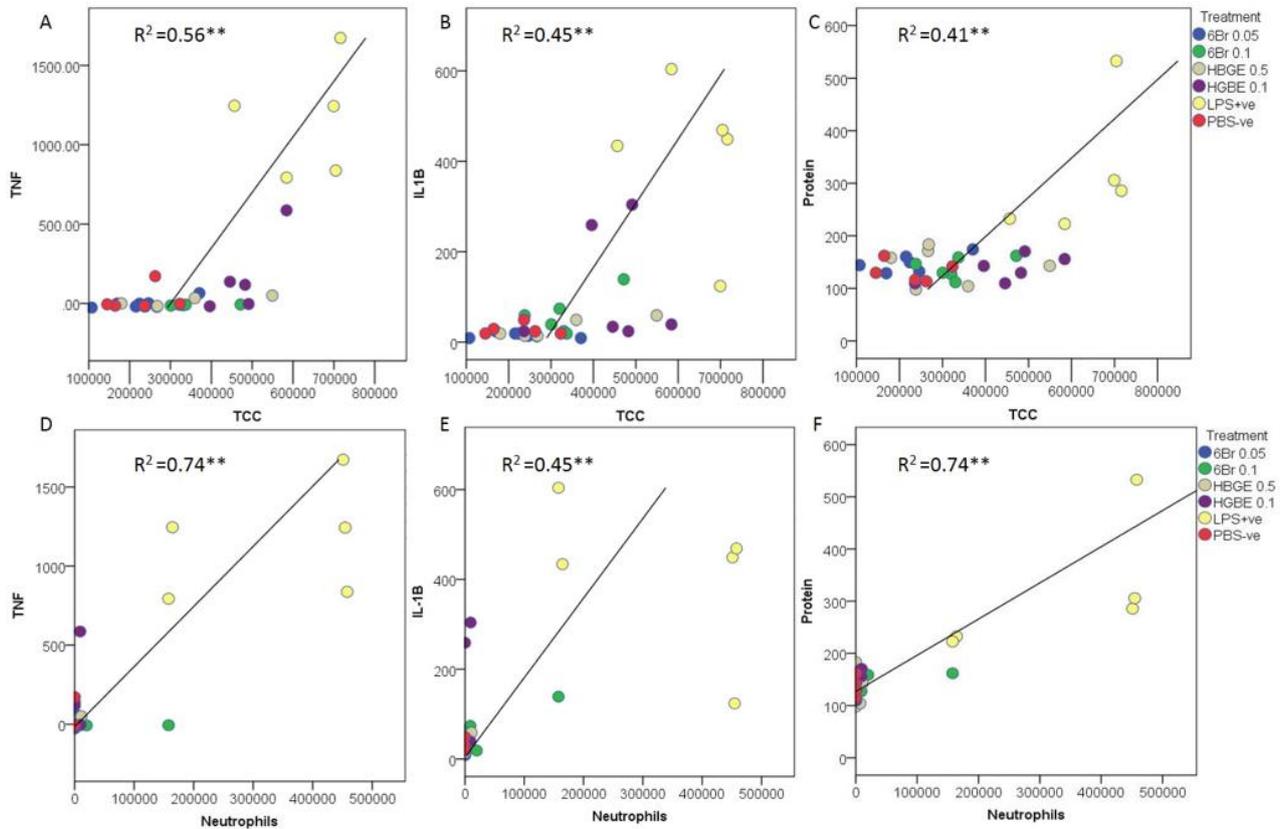


Figure A.10: Correlations between cell counts and inflammatory markers in the bronchoalveolar lavage fluids of mice. The relationship between total cell count (top panels) or neutrophils (bottom panels) and: A & D) Tumor necrosis factor alpha; B & E) interleukin 1B; and C & F) protein. The linear relationship and R2 values are determined from all samples pooled across all mice in the LPS-stimulated acute lung inflammation model: PBS-ve negative control; LPS +ve lipopolysaccharide stimulated positive control; HBGE Hypobranchial gland extract at 0.1 and 0.5 mg/g and; 6Br 6 bromoisatin at 0.1 and 0.05 mg/g. ** significant relationship at $p < 0.01$.

Appendix 12A: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from marine test mix.

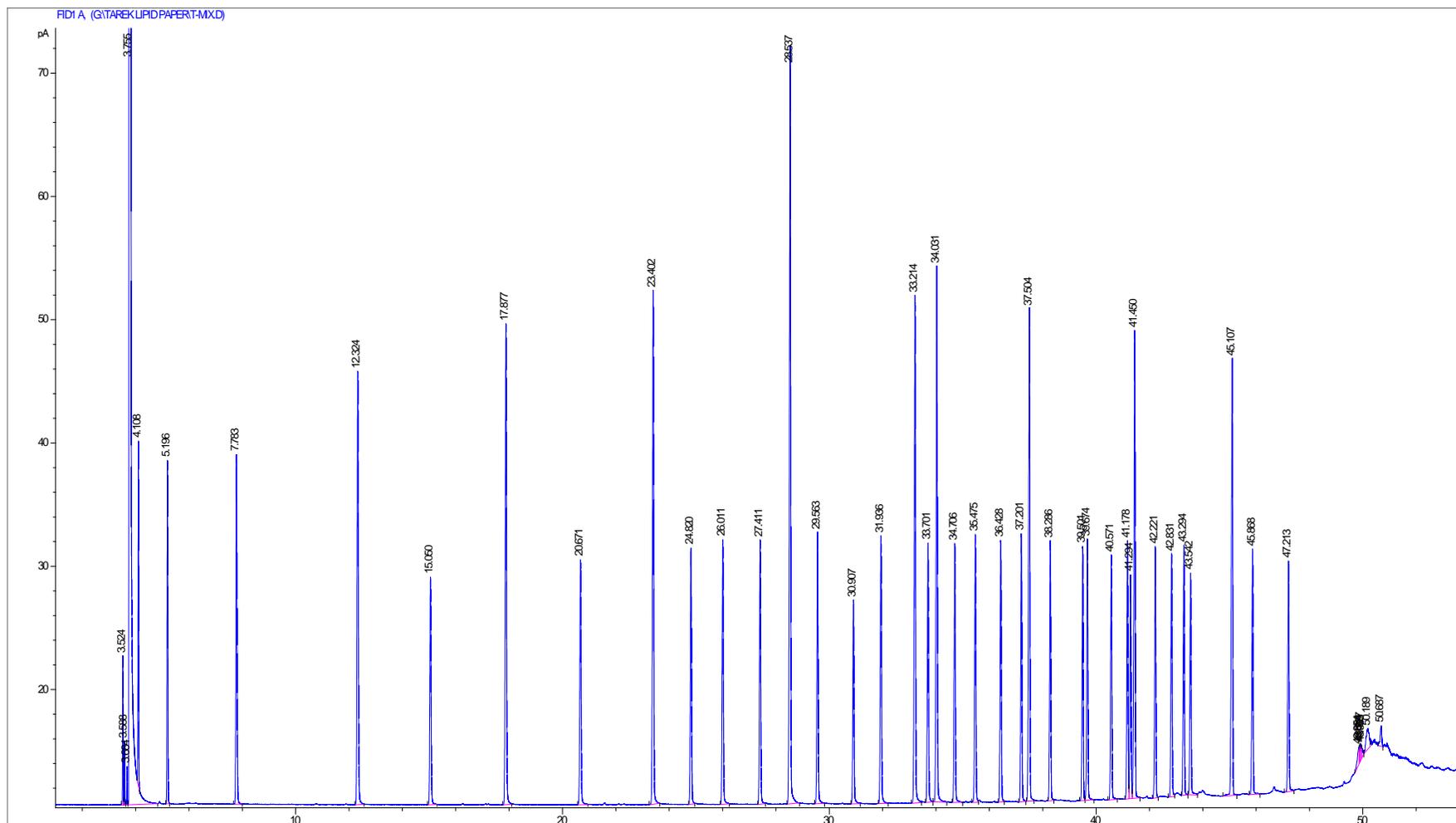


Figure A.11A: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from marine test mix.

Appendix 12B: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from octopus tentacles.

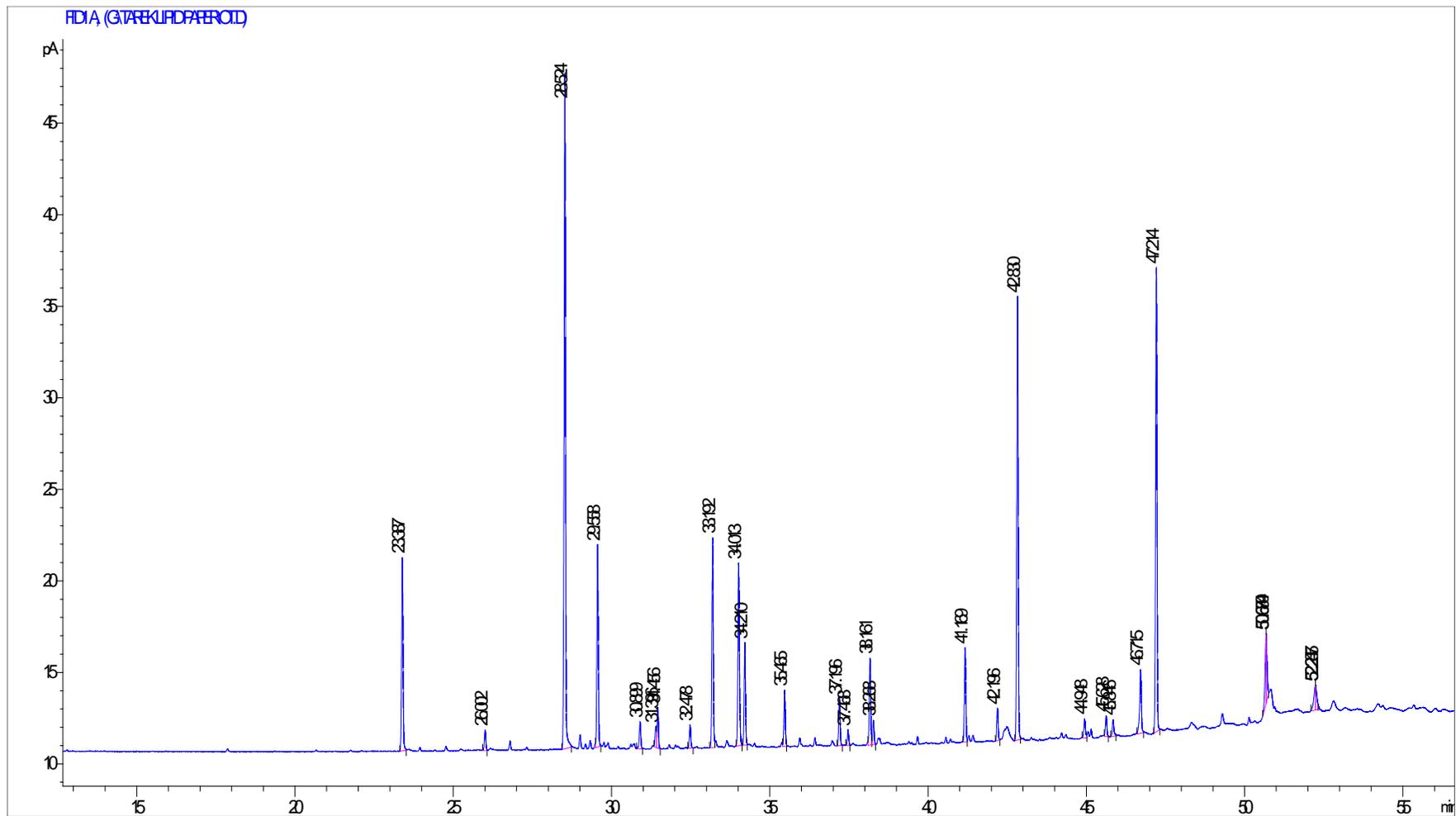


Figure A.11B: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from octopus tentacles.

Appendix 12D: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from Australian sardine fillet.

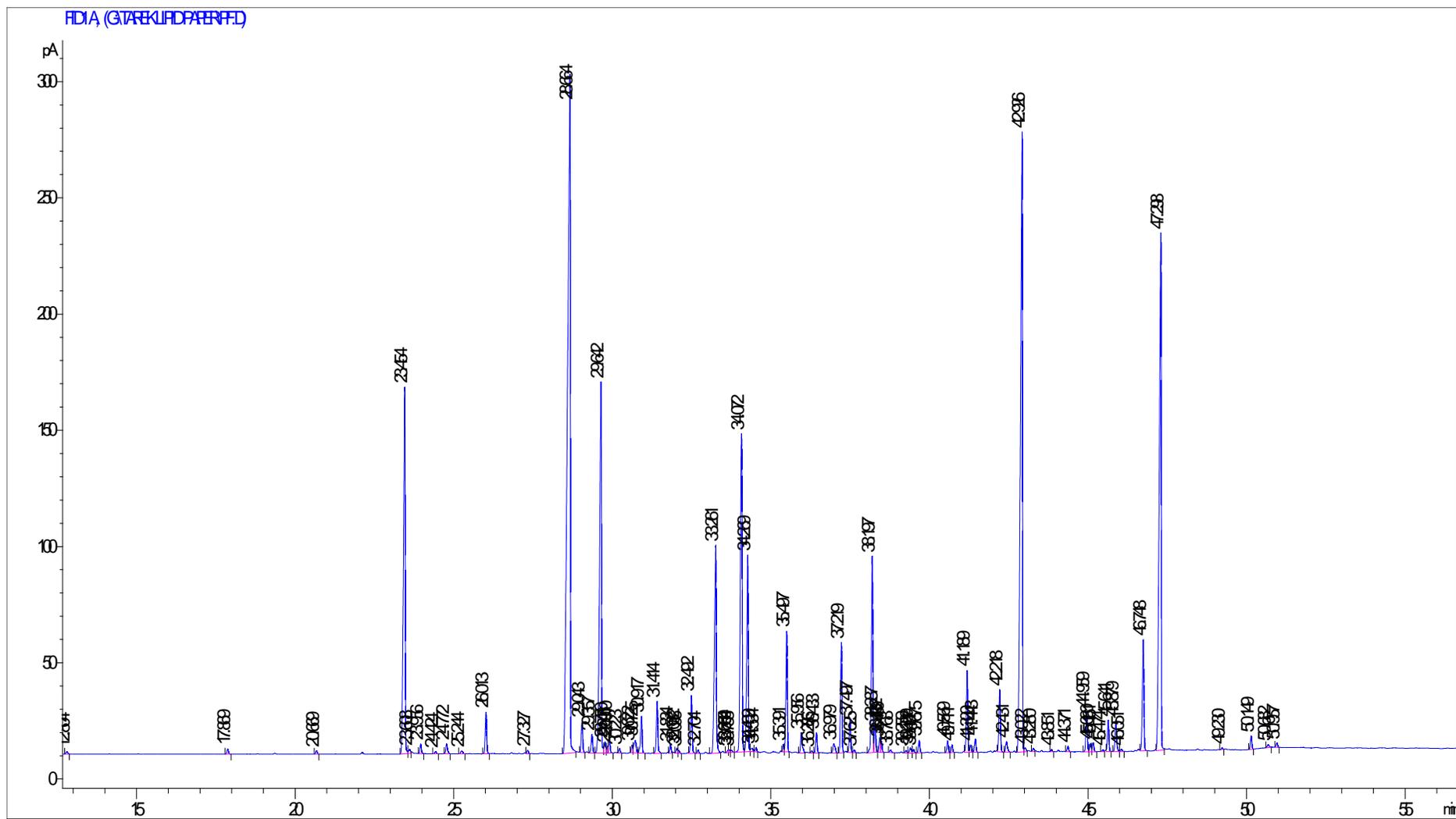
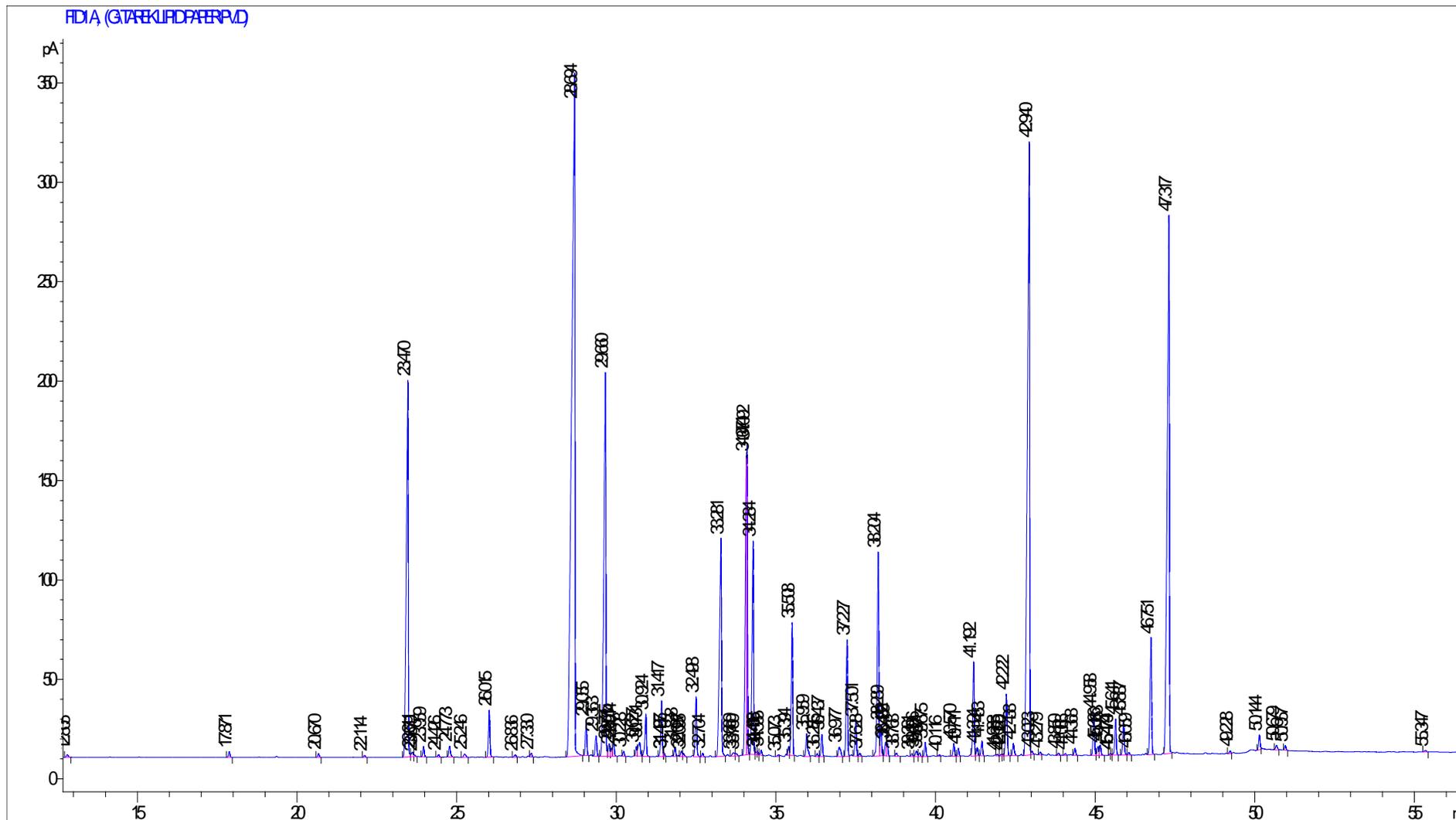


Figure A.11D: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from Australian sardine fillet.

Appendix 12E: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from Australian sardine viscera.



Appendix 12F: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from salmon fillet.

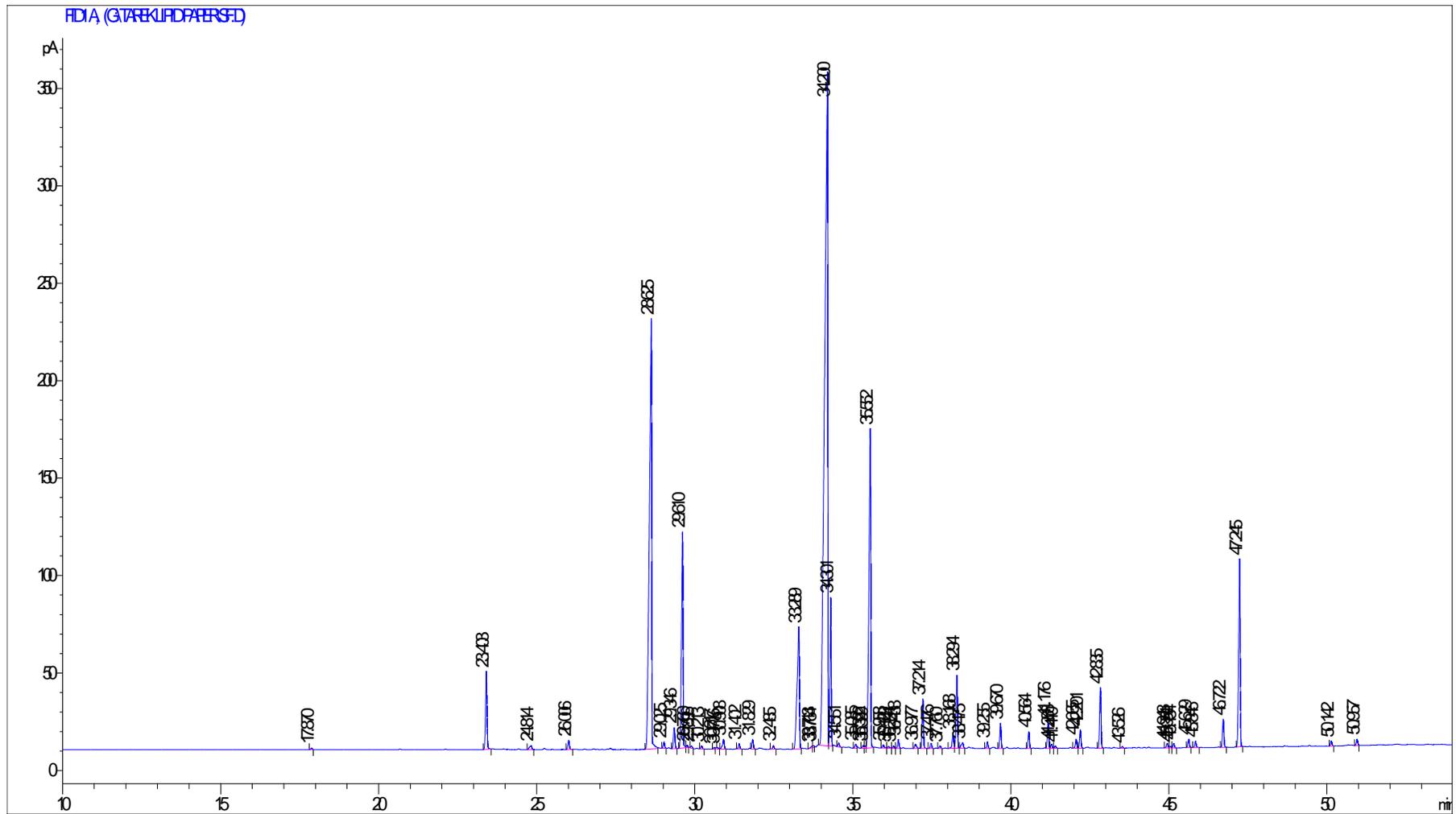


Figure A.11F: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from salmon fillet.

Appendix 12G: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from salmon head.

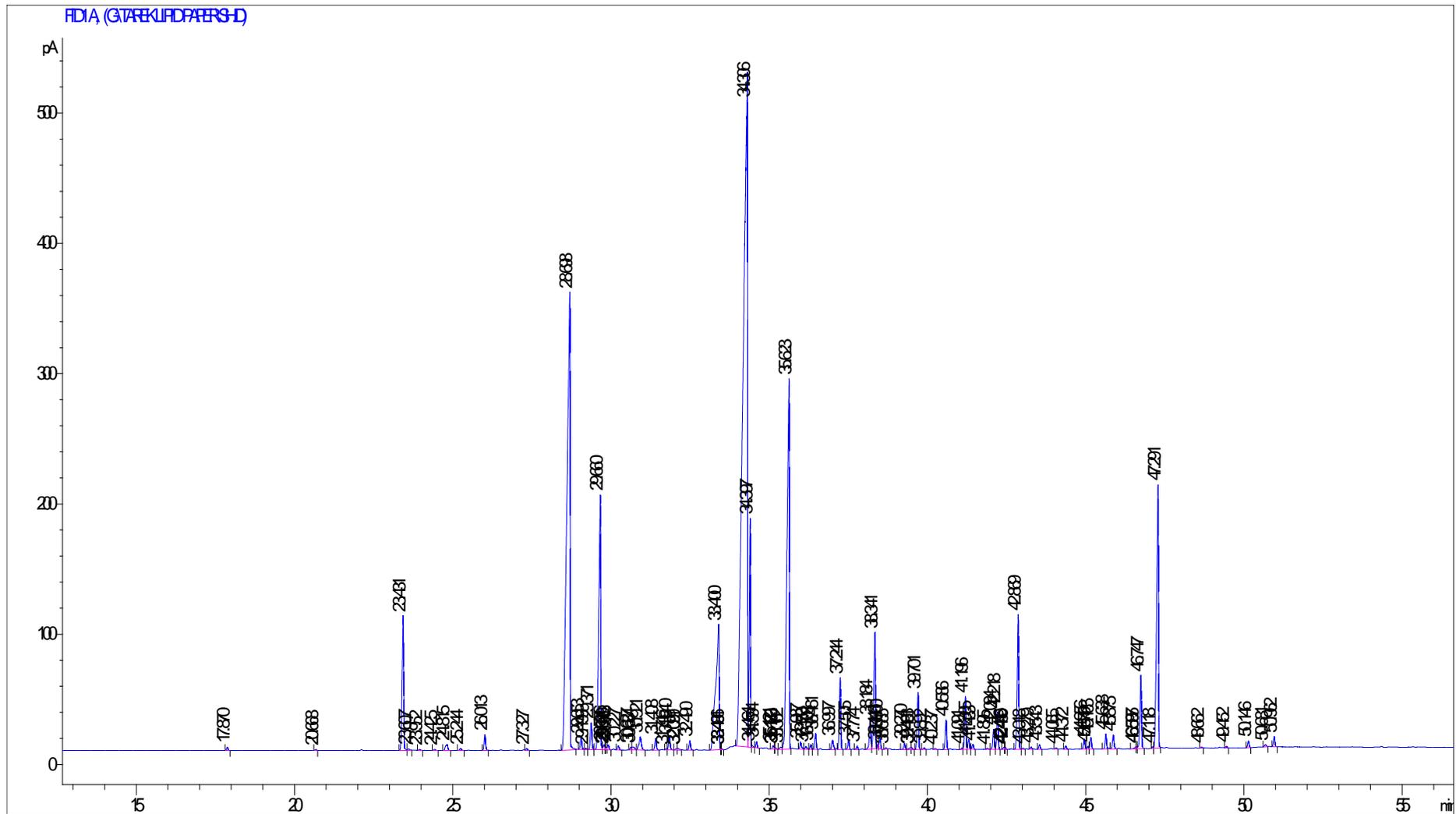


Figure A.11G: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from salmon head.

Appendix 12H: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from school prawn head.

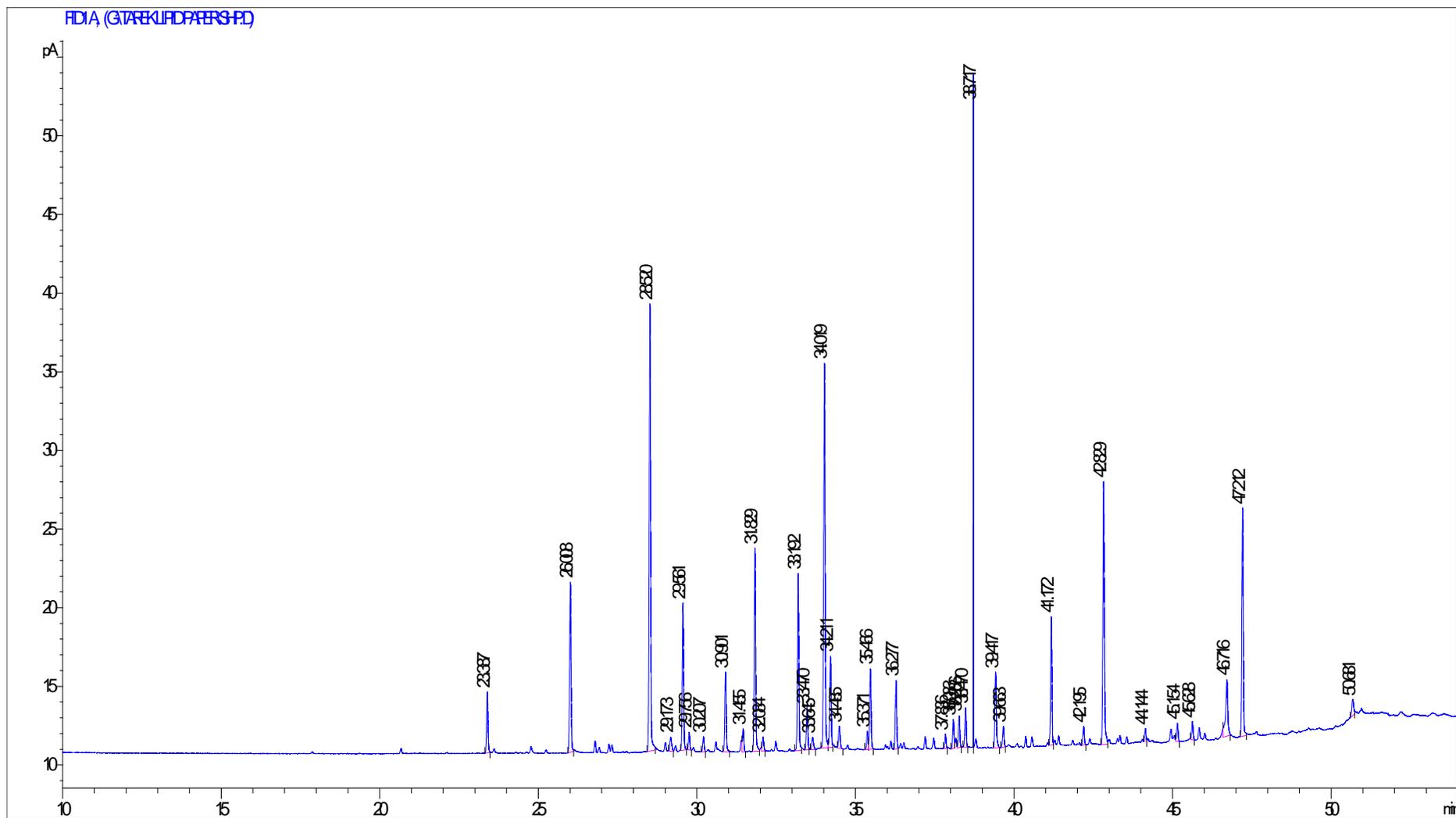


Figure A.11H: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from school prawn head.

Appendix 12I: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from school prawn head.

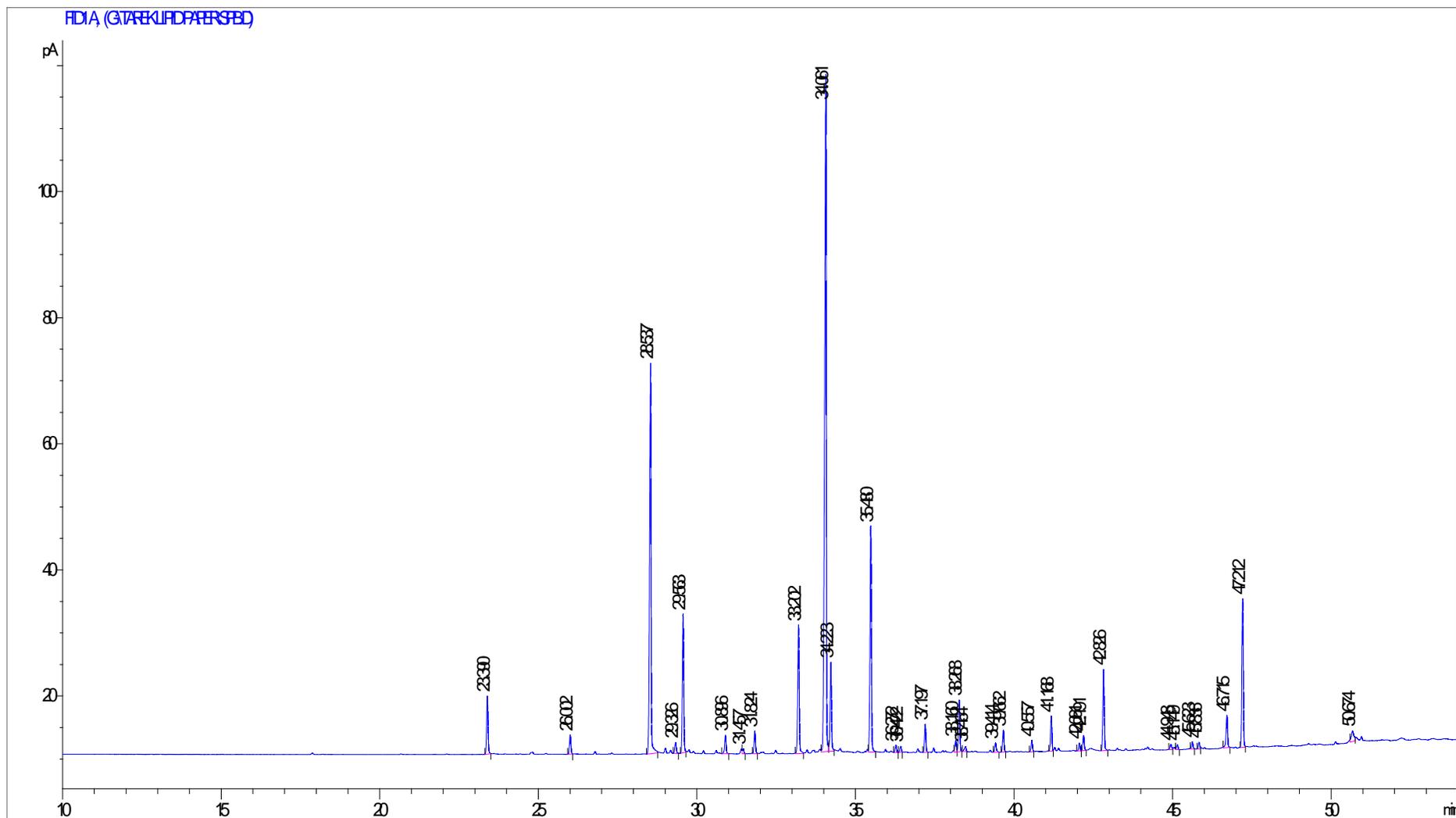


Figure A.11I: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from school prawn head.

Appendix 12J: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from squid head.

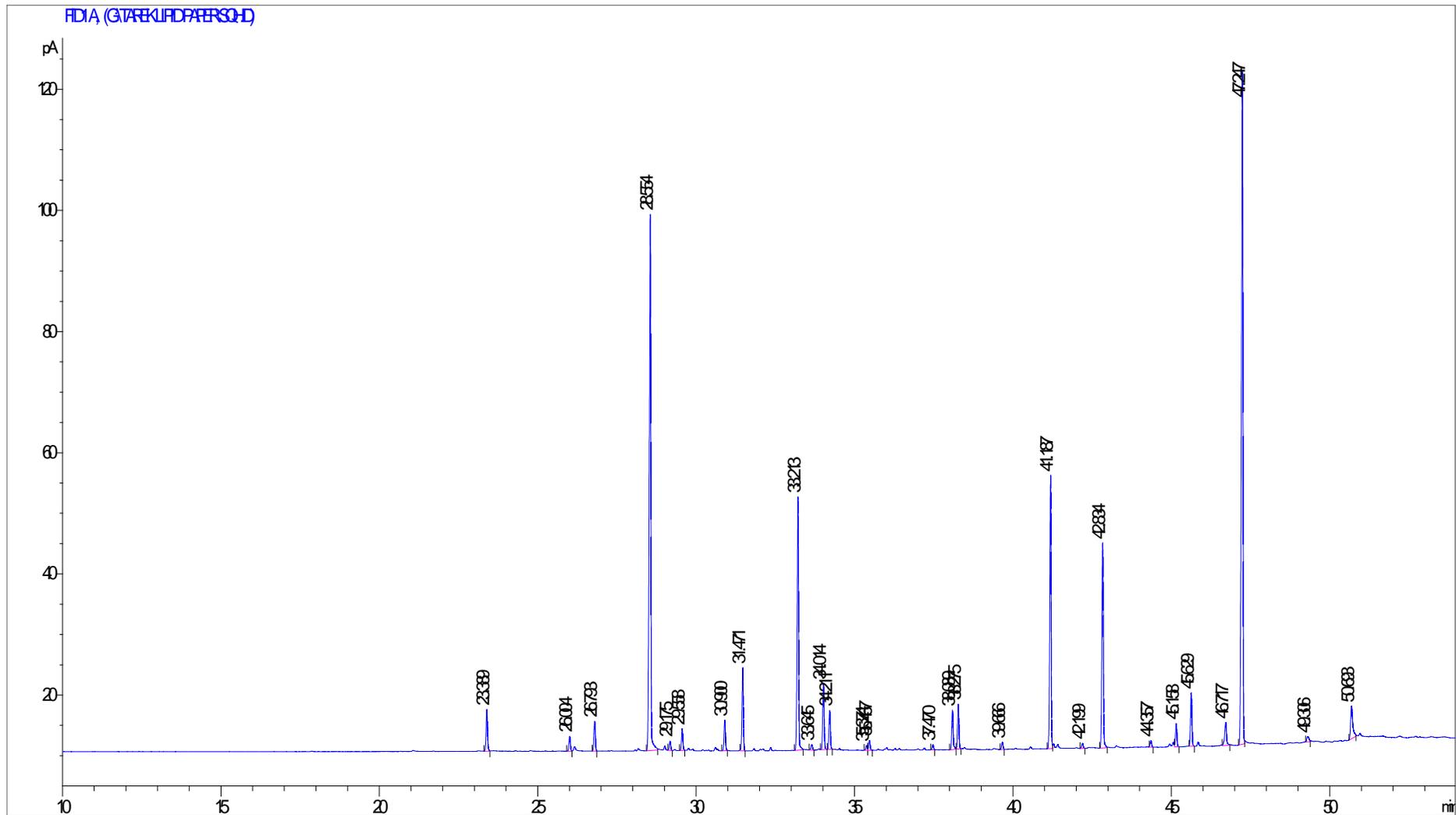


Figure A.11J: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from squid head.

Appendix 12K: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from squid tentacles.

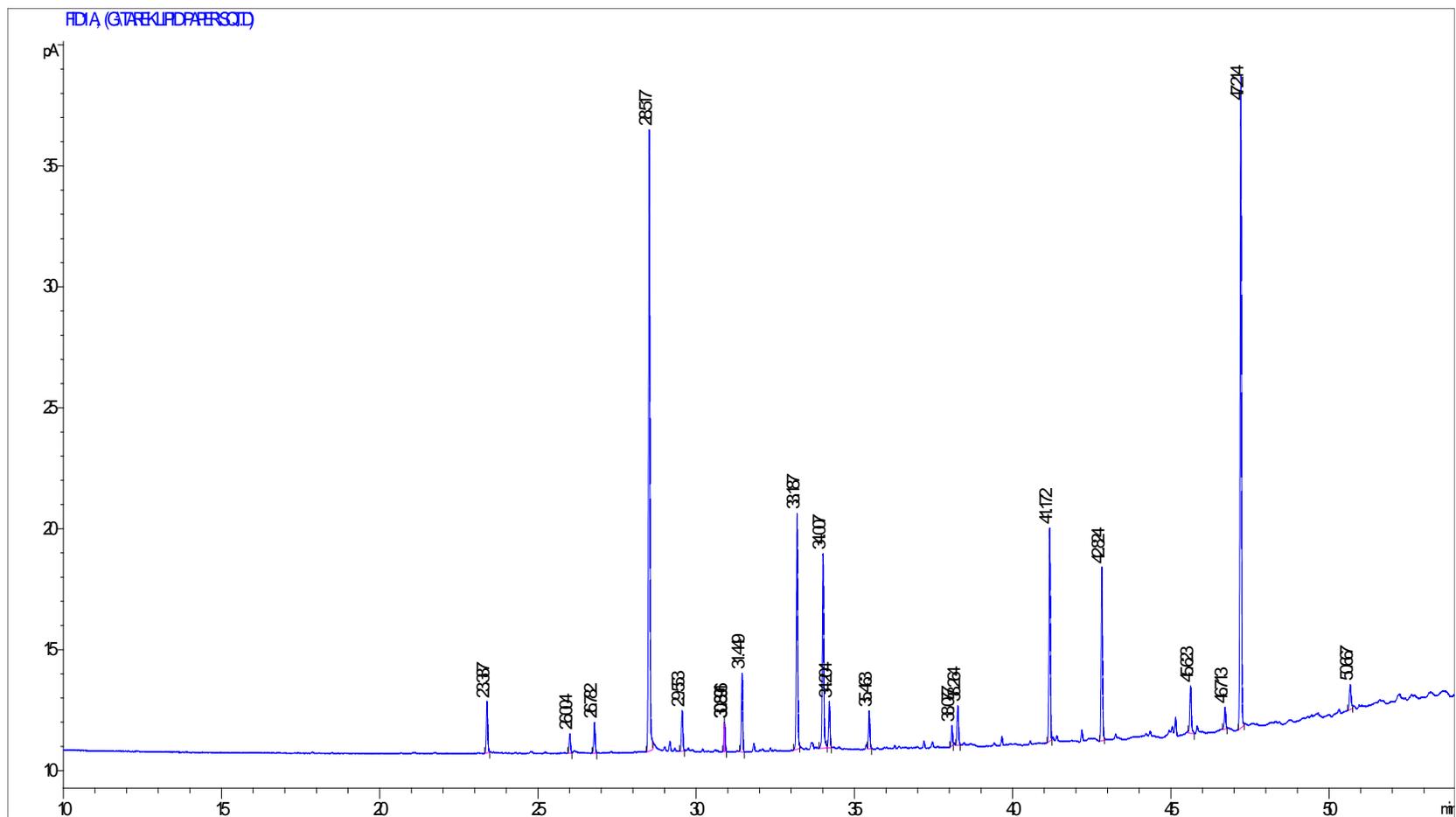


Figure A.11K: Fatty acid methyl esters (FAMES) chromatograms of lipid extract from squid tentacles.

Appendix 13: Two factor PERMANOVAs data for chapter 5.

Table A.2: Summary of the statistical data for all the Two factor PERMANOVAs performed to analyse the effect of organisms and tissue (flesh vs. viscera and head). Multivariate analysis was used for fatty acid composition and the proportion of main fatty acids classes, whereas univariate analysis was used for each individual class of fatty acid and the ratio of ω -3: ω -6 PUFAs. Univariate analysis was also used for anti-inflammatory assays for nitric oxide (NO) inhibition and Tumor Necrosis Factor alpha (TNF α). Significant values are underlined.

	Organism		Tissue		Organism x Tissue	
	Pseudo <i>F</i>	<i>p</i> Value	Pseudo <i>F</i>	<i>p</i> Value	Pseudo <i>F</i>	<i>p</i> Value
FA composition	21.276	<u>0.000</u>	2.730	0.103	1.800	0.197
NO inhibition	573.320	<u>0.021</u>	315.140	<u>0.045</u>	165.880	0.078
TNF inhibition	1.919	0.559	2.097	0.513	0.762	0.705
Fatty acid classes	19.414	<u>0.003</u>	2.155	0.226	1.207	0.424
MUFAs	561.960	<u>0.001</u>	52.136	<u>0.017</u>	18.543	0.055
PUFAs	7.550	<u>0.056</u>	0.592	0.598	0.152	0.960
SFA	8.260	<u>0.045</u>	0.248	0.788	1.870	0.241
ω -3	11.390	<u>0.042</u>	1.333	0.367	0.334	0.843
ω -6	6.140	0.097	0.299	0.751	1.716	0.345
ω -9	1226.500	<u>0.002</u>	196.540	<u>0.012</u>	89.029	<u>0.030</u>
ω -3: ω -6	57.326	<u>0.017</u>	9.600	0.161	13.935	0.107
DMAAs	3.431	0.217	0.034	0.967	0.521	0.712

Appendix 14: Statements of co-authorship for all papers incorporated into this thesis.

Statement of Co-Authorship-1

Chapter 2 of this thesis incorporates the following publication:

AHMAD, T. B., LIU, L., KOTIW, M. & BENKENDORFF, K. 2018. Review of anti-inflammatory, immune-modulatory and wound healing properties of molluscs. *Journal of Ethnopharmacology*, 210, 156-178.

The following people and institutions contributed for the publication of work undertaken as part of this thesis:

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undertook database searched for the literature on molluscan anti-inflammatory and wound healing activity. Author L.L. translated and interpreted the application of molluscs from the Chinese *Materia Medica*. Author M.K. assisted with the interpretation of anti-inflammatory assays and *in vivo* models. The primary author wrote the first draft of the paper and all authors provided feedback and approved the final version of the manuscript.

I, the undersigned, agree with the stated proportion of work undertaken for the published peer reviewed manuscript contributing to this thesis.

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Statement of Co-Authorship-2

Chapter 3 of this thesis has been published as follows:

AHMAD, T. B., RUDD, D., SMITH, J., KOTIW, M., MOUATT, P., SEYMOUR, L. M., LIU, L. & BENKENDORFF, K. 2017. Anti-inflammatory activity and structure-activity relationships of brominated indoles from a marine mollusc. *Mar Drugs*, 15, 133.

The following people and institutions contributed for the publication of work undertaken as part of this thesis:

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Chapter 4 of this thesis incorporates the following publication:

AHMAD, T. B., RUDD, D., BENKENDORFF, K., MAHDI, L. K., PRATT, K. A., DOOLEY, L., WEI, C. & KOTIW, M. 2017. Brominated indoles from a marine mollusc inhibit inflammation in a murine model of acute lung injury. *PLoS One*, 12, e0186904.

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T.B.A., M.K. and K.B. conceived and designed the experiments, with some input from all authors; T.B.A. ran the animal model, performed the anti-inflammatory experiments and analysed the data, with support from D.R.; L.K.M, K.A.P. and C.W. also assisted with the animal model and processing of samples. L.D. assisted with the histology of lung tissue. D.R. assisted with the extraction, ran the LC-MS and H^1 NMR analysis and assisted with the interpretation of chemical data, with additional support from K.B. T.B.A. wrote the first draft with significant contributions from M.K. and K.B. All authors read, commented on and approved the final manuscript.

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Chapter 5 of this thesis has been prepared for submission

AHMAD, T. B., RUDD, D., KOTIW, M. & BENKENDORFF, K. 2018. Fatty acid profile and anti-inflammatory activity of underutilised seafood. Prepared for *Journal of Functional food*.

The following people and institutions contributed for the publication of work undertaken as part of this thesis:

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Candidate was the primary author. The Co-authors (M.K. & K.B.), the supervisors contributed to this work. The primary author and author K.B. conceptualised, conceived and designed the experiments, with some input from D.R. D.R. assisted with the extraction, ran the LC-MS and GC-MS analysis and assisted with the interpretation of chemical data, with additional support from K.B. M.K. revised the anti-inflammatory experiments. The primary author T.B.A. wrote the first draft of the paper and all authors provided feedback and approved the final version of the manuscript.

I, the undersigned, agree with the stated proportion of work undertaken for the published peer reviewed manuscript contributing to this thesis.

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