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Phytochemistry and pharmacology of plants from the ginger family, Zingiberaceae

Hans Wohlmuth

Southern Cross University

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PHYTOCHEMISTRY AND PHARMACOLOGY OF PLANTS FROM THE GINGER FAMILY, ZINGIBERACEAE

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Submitted for the Degree of Doctor of Philosophy

Department of Natural and Complementary Medicine
Southern Cross University
Lismore, Australia

April 2008
Flowering *Curcuma australasica*, an endemic Australian Zingiberaceae with potential anti-inflammatory activity, cultivated at Southern Cross University, Lismore, Australia.
Declaration

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.

I acknowledge that I have read and understood the University's rules, requirements, procedures and policy relating to my higher degree research award and to my thesis. I certify that I have complied with the rules, requirements, procedures and policy of the University (as they may be from time to time).

Name: Hans Wohlmuth

Signature: .................................................................

Date: ........................................................................
SYNOPSIS

This thesis reports on a series of investigations into the phytochemistry and pharmacology of plants belonging to the ginger family, Zingiberaceae (incl. Costaceae). The work falls into two main parts. The first part examines the pungent compounds and essential oil in 17 clones of ginger (*Zingiber officinale*) with a view to identify one or more with unique chemistry and consequent particular therapeutic (or flavouring) prospects. The second part comprises the screening of 41 taxa for inhibition of PGE2 and other biological activities, with the primary aim of identifying species with potential anti-inflammatory activity. This part tested the hypothesis that the combination of ethnobotanical and taxonomic information is a productive strategy to identify previously unrecognised plant species with therapeutic potential.

Chapter 1 provides a general introduction to plants as medicines and the field of ethnopharmacology. It also provides an overview of the process of inflammation, in particular arachidonic acid metabolism.

The main literature review is presented in Chapter 2. It reviews the literature relating to the chemistry and pharmacology of 15 genera in the Zingiberaceae, with the focus on species included in the experimental work. The Zingiberaceae is rich in species used as traditional medicines or spices, but extensive information about their chemistry and pharmacology is available only for a few species, most notably ginger and turmeric (*Curcuma longa*). These attributes make the family an ideal target for a screening project, since phylogenetically related plant species usually display a significant degree of similarity in the kinds of secondary metabolites they produce.

Chapter 3 describes the preliminary experimental work with ginger. This work aimed at determining a suitable extraction solvent and method, guided by the activity of the extracts in a cyclooxygenase-1 (COX-1) bioassay. It also established an HPLC method suitable for the quantification of gingerols and shogaols in the extracts.

Seventeen ginger clones, including commercial cultivars and 12 experimental clones, were analysed by HPLC for their content of pungent compounds. The result of this work is reported in Chapter 4. Because ginger is a sterile cultigen, there is an increased likelihood that chemically distinct and genetically stable clones may exist. This work identified one cultivar that when compared with other clones contained a significantly higher level of
pungent gingerols. The analysis included 12 tetraploid clones, but these did not display elevated gingerol production compared with their diploid parent cultivar.

The essential oils obtained from the same 17 ginger clones by steam distillation were analysed by GC-MS. These results are presented in Chapter 5. The oil from one particular clone was distinctly different from the others; this was the same clone that had a very high gingerol content. The essential oil of this clone differed from the others by having a lower citral content and higher levels of sesquiterpene hydrocarbons. The unique chemistry of this clone in terms of aroma, pungency and flavour should make it of interest to the flavour, fragrance and pharmaceutical industries.

Chapter 6 presents the results of the screening of 41 taxa in an in vitro cell-based bioassay for inhibition of PGE2 production. A number of the samples were also tested for antioxidant activity in the oxygen radical absorbance capacity (ORAC) assay, for inhibition of nitric oxide production and for modulation of natural killer cell activity. Known medicinal plants, in particular ginger and turmeric, emerged as the most active in these assays. Included in the work were seven native Australian species not previously investigated for pharmacological activity. Two of these species showed good activity in the PGE2 assay and were selected for further investigations.

Chapter 7 reports on the bioactivity-guided fractionation of these two native Australian species, Curcuma australasica and Pleuranthodium racemigerum. Inhibition of PGE2 was used as the primary bioassay in this process, but fractions with high activity in that assay were also tested for cytotoxic properties. This work succeeded in isolating and structurally characterising a novel curcuminoid compound with potent PGE2 inhibitory activity from P. racemigerum as well as two known compounds from C. australasica.

The final chapter (Chapter 8) provides a short summary and concluding remarks, and identifies areas for future research arising from the present work.
PUBLICATIONS ARISING FROM THE WORK IN THIS THESIS


ACKNOWLEDGMENTS

Many individuals have supported the work presented in this thesis in a variety of ways, and without their assistance, guidance, help and support the project would not have been possible. I am grateful to them all.

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Most of the work presented in this thesis was carried out in the laboratories of the Centre for Phytochemistry and Pharmacology at Southern Cross University, and in addition to my supervisors I wish to thank staff and fellow students of the Centre for their invaluable assistance during the project. No one deserves more thanks and credit than Don Brushett, who tirelessly and patiently introduced me to the intricacies of analytical chemistry, and I am also deeply grateful to Dr Myrna Deseo (NMR), Paul Connellan (flow cytometry), Ashley Dowell (analytical chemistry) and Dion Thompson (pharmacology) for their generous assistance. I also thank Linda Banbury, Karen Beattie, Bill Eickhoff, Yen Lin Ho, Dr Denise Hunter, Dr Gloria Karagianis, Kate MacFarlane, Aaron Pollack, Kelly Shepherd, Emeritus Professor Peter Waterman and Kelly Winter for their support.

The plant materials I worked on in this project came from a variety of sources. I am indebted to Mike Smith, who so generously made available ginger cultivars and experimental clones arising from his work at the Maroochy Research Station (Queensland Department of Primary Industries and Fisheries). I am equally grateful to Dr Greg Leach of the Northern Territory’s Department of Natural Resources, Environment and the Arts, who facilitated access to the living collection of Zingiberaceae in the George Brown Darwin Botanic Gardens. Similarly, through the generosity of Curator David Warmington, I was able to access the extensive living collection of Zingiberaceae in the Flecker Botanic Gardens in Cairns. I wish to thank Rigel Jensen of Malanda, whose impressive botanical and local knowledge of the North Queensland Wet Tropics made the task of collecting native Australian species in the wild a surprisingly easy one.
Many of the plants were grown for several years at Southern Cross University, and I owe the Curator of the Medicinal Plant Garden, Geoff Callan, and his staff much thanks for the construction of purpose build beds and for general support and friendship. I also gratefully acknowledge the support of the university’s Ground Staff who let me have a large number of plants growing in pots in their nursery for several years.

I acknowledge and appreciate the support of my employer, Southern Cross University, in particular that of Professor Iain Graham, Head of the School of Health and Human Sciences. I also thank my colleagues in the Department of Natural and Complementary Medicine, in particular Sue Evans and Holly Muggleston, who shouldered most of the burden during the times I was away working on my research and thesis.

I also thank Dr Myrna Deseo and Associate Professor John Stevens who provided valuable comments on the final draft.

Finally, thanks to Catherine for her love and support, and for never resenting me being busy with my gingers.
**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-HETE</td>
<td>5-hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>12-HETE</td>
<td>12-hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>12-HTT</td>
<td>12-hydroxy-5,8,10-heptadecatrienoic acid</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>BCE</td>
<td>Before current era (= B.C.)</td>
</tr>
<tr>
<td>B.P.</td>
<td>British Pharmacopoeia</td>
</tr>
<tr>
<td>CE</td>
<td>Current era (= A.D.)</td>
</tr>
<tr>
<td>CONSORT</td>
<td>Consolidated Standards of Reporting Trials</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>ED₅₀</td>
<td>Median effective dose</td>
</tr>
<tr>
<td>ELAM-1</td>
<td>Endothelial leukocyte adhesion molecule-1</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography – mass spectrometry</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HETEs</td>
<td>Hydroxyeicosatetraenoic acids</td>
</tr>
<tr>
<td>HPETEs</td>
<td>Hydroperoxyeicosatetraenoic acids</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Median inhibition concentration</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFN-β</td>
<td>Interferon-beta</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1-beta</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
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</table>
IL-6    Interleukin-6
IL-9    Interleukin-9
iNOS    Inducible nitric oxide synthase
JNK     c-Jun N-terminal kinase
LC-MS   Liquid chromatography – mass spectrometry
LDL     Low density lipoprotein
IP-10   Interferon-γ activated protein
L       Litre
LD₅₀    Median lethal dose
LOX     Lipoxygenase
5-LOX   5-Lipoxygenase
LPS     Lipopolysaccharide
LTC₄    Leukotriene C4
LTD₄    Leukotriene D4
LTE₄    Leukotriene E4
M       Molar
MAPK, MAP kinase Mitogen-activated protein kinase
MIC     Minimum inhibitory concentration
MCP-1   Macrophage (monocyte) chemotactic protein-1
MCSF    Macrophage colony-stimulating factor
mg      Milligram
MIP-1α  Monocyte inflammatory protein-1-alpha
mL      Millilitre
MMPs    Metalloproteinases
MS      Mass spectrometry
NFκB    Nuclear factor kappa B
NK cells Natural killer cells
NMR     Nuclear magnetic resonance
NO      Nitric oxide
NSAIDs  Non-steroidal anti-inflammatory drugs
ORAC    Oxygen radical absorption capacity
PAF     Platelet-activating factor
PBS     Phosphate-buffered saline
PG      Prostaglandin
<table>
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<th>Abbreviation</th>
<th>Description</th>
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</thead>
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<tr>
<td>PGD&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin D2</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PGF&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>Prostaglandin F2-alpha</td>
</tr>
<tr>
<td>PGH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin H2</td>
</tr>
<tr>
<td>PGI&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin I2 (prostacyclin)</td>
</tr>
<tr>
<td>PLA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PMNs</td>
<td>Polymorphonuclear neutrophils</td>
</tr>
<tr>
<td>q.i.d.</td>
<td><em>quarter in die</em> (four times a day)</td>
</tr>
<tr>
<td>RCF</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>t.i.d.</td>
<td><em>ter in die</em> (three times a day)</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>TxA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Thromboxane A&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>TxB&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Thromboxane B&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UV-VIS</td>
<td>Ultraviolet-visible</td>
</tr>
<tr>
<td>UVB</td>
<td>Ultraviolet B</td>
</tr>
<tr>
<td>VAS</td>
<td>Visual analogue scale</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>VR1</td>
<td>Vanilloid receptor-1</td>
</tr>
<tr>
<td>WOMAC</td>
<td>Western Ontario and McMaster Osteoarthritis Index</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
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1. **GENERAL INTRODUCTION**

This thesis reports on research into the chemistry and pharmacology of plants from the Zingiberaceae family, in particular in terms of their potential use as anti-inflammatory agents.

The experimental work presented in this thesis is divided into two main parts. The first part focuses on the common ginger (*Zingiber officinale*), which in recent years has attracted attention as a potential anti-inflammatory agent. Seventeen clones of ginger were analysed in terms of their content of pungent compounds with a view to identify any with a profile that might be of particular interest from a pharmacological point of view. Because ginger is also an important flavouring commodity, profiling of the essential oil was also carried out.

The second part of the work comprises the screening of some 43 Zingiberaceae taxa, representing 14 genera, for potential anti-inflammatory activity in a whole cell assay. Simple chemical profiling of these extracts was carried out, and two were subjected to activity-guided fractionation.

This chapter provides a brief introduction to the use of plants as medicines and the process of inflammation. Chapter 2 provides a review of the plant species investigated during the course of this work.

### 1.1 Plants as medicines

Plants have provided humans with medicines since time immemorial. The oldest known document concerning medicinal plants and their uses is the Chinese *Pen Ts’ao*, which was written 4800 years ago and describes no less than 360 plants, suggesting that herbal medicine was already at an advanced stage in China at this time (Mann, 1992). In Mesopotamia (part of present-day Iraq), 4600-year old clay tablets inscribed with cuneiform characters have been found that contain references to familiar medicinal plants such as myrrh, licorice and the opium poppy (Cragg & Newman, 2002). Another famous early document detailing the use of plants as medicines is the Ebers papyrus from Egypt, which was written about 3500 years ago (Mann, 1992).
Much more ancient, albeit less conclusive, evidence suggests that humans might have employed the pharmacological properties of plants much earlier. At the famous burial site in the Shanidar Cave in the northern part of Iraq, a Neanderthal (*Homo neanderthalensis*) was laid to rest with bunches of flowers about 60,000 years ago (Solecki, 1975). Of the eight plants identified in the grave from preserved pollen, seven are considered medicinal plants today. There is of course no way of knowing with certainty whether they were placed in the grave because of their medicinal properties, to serve the dead man on his final journey, or whether they simply were used for decorative purposes.

The more recent discovery of the ‘Iceman’ on the Italian-Austrian border in the Alps provides intriguing evidence of early use of medicinal fungi in Europe. This hunter, who had been lying well preserved in the ice for about 5300 years, was found to be in possession of a fungus, the birch polypore (*Piptoporus betulinus*), which is known to have purgative and antibiotic properties, and which he might well have been using to treat the whipworm infestation of his intestines (Heinrich *et al*., 2004).

Plants play a key role in sophisticated ancient traditional medical systems such as traditional Chinese medicine and Ayurveda of India, and have also been central in the Greco-Roman medical tradition, which developed into modern biomedicine. Hippocrates (468-377 BCE), used more than 400 plant species for therapeutic purposes, and it was a Roman army surgeon by the name of Dioscorides (c. 40-80 CE) who wrote the most influential early European manual of medicinal plants, *De Materia Medica*, in the first century CE (Griggs, 1997). This comprehensive work included illustrations and descriptions of about 600 plant species, along with text detailing their uses, doses and potential toxic effects. The writings of Galen (c. 129-199 CE), who classified herbs according to their humoral properties, had a profound and almost unimaginable impact on medical thought in Europe for about 1500 years (Griggs, 1997). The English apothecary Nicholas Culpeper (1616-1654) wrote many herbal books, the most famous being *The English Physician* (1653), in which he presented herbal medicine in an astrological framework (Griggs, 1997).

Although modern biomedicine to a significant degree employs synthetic drugs as therapeutic agents, plants still occupy a prominent place in contemporary pharmacy, either as sources of pharmaceutical drugs in the form of isolated plant compounds, as sources of precursors to drugs, or as sources of compounds that have served as models for synthetic or semisynthetic drugs. It has been estimated that about one-half of all drugs in current use are natural
compounds or derivatives thereof (Iwu, 2002). It is however important to realise that despite the many advances of biomedicine, the progress afforded residents of first world countries is beyond the reach of the majority of the world’s population. For the majority of people, many of whom live in miserable poverty, crude plants preparations are still the main form of medicine. In acknowledgment of this situation, the World Health Organization (WHO) is actively promoting the development of traditional medicine (Anonymous, 2002).

1.2 Ethnobotany and ethnopharmacology

1.2.1 Ethnobotany

The term ethnobotany lacks a singular, uniformly agreed definition. The term was coined by J. W. Harshberger, who defined it as “...the use of plants by aboriginal peoples” (Harshberger, 1896). Since then, ethnobotany has been redefined and reinterpreted by many scholars in the area (for an overview, see Cotton, 1996). One of the broadest definitions of ethnobotany is that provided by Martin, who described it as the subdiscipline of ethnoecology that is concerned with local people’s interaction with plants (Martin, 1995).

Early ethnobotany was focused on plants of economic significance or potential, while contemporary ethnobotany tends to have a far broader scope and include, for example, traditional agricultural knowledge and traditional vegetation management (Cotton, 1996). Throughout the history of formal ethnobotany, medicinal plants have been an area of keen interest to many ethnobotanists.

1.2.2 Ethnopharmacology

Ethnopharmacology is a multidisciplinary field devoted to the study of pharmacologically active agents traditionally used by humans. The term ethnopharmacology was coined as recently as 1967 by Efron, who used the term in the context of hallucinogenic substances (Heinrich & Gibbons, 2001).
More recently, ethnopharmacology has been defined as “… the interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by man” (Bruhn & Holmstedt, 1981).

Ethnopharmacology applies conventional chemical and pharmacological analysis to traditional medicines and in doing so differs from two related disciplines: medical anthropology, which examines health and disease from a cultural perspective, and medical ethnobotany, which is concerned with the use of plants within traditional medical systems (Cotton, 1996).

Although ethnopharmacology is not exclusively concerned with plants or plant products, the plant kingdom is the major focus of the discipline, because this kingdom has provided humans with the greatest number of pharmacologically active substances throughout history.

The multidisciplinary nature of ethnopharmacology is evidenced by the important roles played by fields such as botany, pharmacognosy, natural product chemistry, pharmacology, toxicology, anthropology and others (Heinrich & Gibbons, 2001; Houghton, 2002).

1.2.3 Ethnopharmacology and bioprospecting

Heinrich and Gibbons (2001) have noted the differences between ethnopharmacology and bioprospecting, while acknowledging that the two approaches are not mutually exclusive. In brief, ethnopharmacology aims to develop (through increased knowledge and understanding) the use of crude plant preparations in local communities, whereas the goal of bioprospecting is the identification and development of compounds from nature as pharmaceutical drugs in the international market place (Heinrich & Gibbons, 2001). Although the aims of these two approaches to natural products research are vastly different from a socio-economic viewpoint, many of the methodologies will often be the same, and work focussed on one approach might yield results that are relevant to the other. For example, phytochemical and pharmacological investigations of a traditional medicine might lead to the identification of a compound that can be developed into a pharmaceutical drug. Well known examples of this include ephedrine (from Ephedra spp.), atropine (from Atropa bella-donna and other Solanaceae) and more recently the development of the anti-malarial drug artemether from the
lead compound artemisinin in the traditional Chinese herb *Artemisia annua* (Evans, 2002; Wright, 2002).

### 1.2.4 Ethnobotany and ethnopharmacology as strategies in bioprospecting

Different approaches can be employed in the process of bioprospecting. Cotton (1996) outlined three main approaches to the collection of plants for screening: the *random method*, where every species in a given area is included; *phylogenetic targeting*, where a particular taxon (such as a family) is targeted, because it is already known to be a good source of pharmacologically active metabolites; and the *ethno-directed sampling*, which is guided by traditional plant use. The latter approach is based on the notion that initial screening and selection has already been conducted effectively by the owners of the traditional knowledge.

The ethno-directed approach to identifying plants with biological activity has been shown in a number of studies to be more efficient than the random method at identifying plants with promising pharmacological activity. Such studies include one aimed at identifying plants with anti-HIV activity from Central America (Balick, 1990) and another that showed that plants used ethnomedically to treat viral infections were more than 100 times more likely to yield compounds with anti-viral activity than randomly collected plants (Carlson, 2002). However, it has been argued that the successful development of drugs from traditional medicines is most likely for conditions such as inflammation, gastrointestinal or nervous system disorders, because these pathologies are widely recognised and treated in indigenous systems of medicine (Cox, 1994).

### 1.2.5 Ethnopharmacology and phytotherapy

The term phytotherapy is used to describe the use of plant-based, chemically complex therapeutic agents in contemporary, mostly industrialised societies. Phytotherapy is usually based on a history of traditional use, but it differs from traditional indigenous herbal medicine by employing industrialised extraction and manufacturing methods and by being cosmopolitan in scope. Hence phytomedicines made from plants from around the globe are
available in most industrialised countries. Ethnopharmacology has the potential to increase our knowledge and understanding of traditional herbal medicines, how they work, how they are best prepared, and how they can be applied in a safe and efficacious manner. Due to the chemical complexity of both traditional herbal medicines and modern phytomedicines, the task of elucidating their pharmacology is a complex one indeed. A full understanding of how complex mixtures of plant compounds interact with the human body and with each other is probably not achievable, and pharmacological investigations of plant extract almost always focus on one or a few ‘active’ constituents, i.e. compounds with profound biological activity. It should always be borne in mind that many other compounds present in a plant extract could potentially play a role in the overall activity of that extract, for example by modulating the pharmacokinetic and/or pharmacodynamic properties of the ‘actives’.

Despite this caveat, ethnopharmacological investigations clearly have much to offer modern phytotherapy, and the long-term success of the ‘herbal renaissance’ currently experienced in most of the industrialised world undoubtedly depends on the scientific underpinning of traditional or anecdotal uses.

1.3 Inflammation

Inflammation is being implicated in the pathophysiology of an increasing number of diseases. In addition to conditions traditionally considered to be inflammatory in nature, inflammation is now considered to have a role in a wide range of pathologies, including cardiovascular disease (Hansson, 2005; Kaperonis et al., 2006), cancer (Zhang & Rigas, 2006), diabetes (Deans & Sattar, 2006; Duncan & Schmidt, 2006), age-related macular degeneration (Rodrigues, 2007), Parkinson’s disease (Hald et al., 2007), Alzheimer’s disease (Eikelenboom et al., 2006), and possibly depression (Kulmatycki & Jamali, 2006).

1.3.1 Overview over the inflammatory process

Inflammation is a rapid and non-specific response to cellular injury in vascularised tissues. The inflammatory response is produced and controlled by complex interactions between
cellular and plasma protein components. The cellular component involves intercellular communication effected by a range of cytokines.

The inflammatory response commences with a brief constriction of arterioles followed by vasodilation and exudation of protein-containing plasma and blood cells into the injured tissue. This causes swelling and oedema. Meanwhile leukocytes adhere to vessel walls and cause the endothelial cells to contract, creating enough space between these cells for the leukocytes to enter the extravascular tissue. Increased vascular permeability is maintained until the inflammatory state is resolved, and it is the interplay between blood cells and plasma proteins in the affected tissue that controls the inflammatory response and interacts with part of the immune response.

The principal cell types involved in inflammation are mast cells, endothelial cells, phagocytic leukocytes (polymorphonuclear neutrophils, macrophages, and eosinophils), and platelets.

Mast cells play a key role in the initiation of the inflammatory response. Degranulation leads to the release of stored chemicals such as histamine, which causes increased vascular permeability, and mast cells also synthesise pro-inflammatory mediators such as prostaglandins, leukotrienes and platelet-activating factor (PAF).

Endothelial cells express adhesion molecules (selectins) for leukocytes and platelets, and also produce nitric oxide (NO), which causes vasodilation but also may play a regulatory role by suppressing mast cell and platelet function. Endothelial cells also produce two prostaglandin derivatives with opposite action: the vasoconstrictor thromboxane A₂ (TxA₂) and the vasodilator prostacyclin (PGI₂), and it is the interplay between these two regulatory compounds that allows for platelet aggregation to occur only at the site of injury (McCance & Huether, 2002).

1.3.2 Cellular products as inflammatory mediators

The various cells involved in the inflammatory response produce a range of compounds that act as inflammatory mediators, including cytokines and products of arachidonic acid metabolism, i.e. prostaglandins and leukotrienes.
Cytokines are proteins produced by a range of different cell types. The major types of cytokines are the interleukins and the interferons, but the class also includes tumour necrosis factors, colony-stimulating factors, transforming growth factor, and others (McCance & Huether, 2002).

Table 1-1 lists cytokines that play an important role in inflammation.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Source</th>
<th>Main actions relevant to inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>Mainly macrophages</td>
<td>Inflammatory mediator; increases prostaglandin production</td>
</tr>
<tr>
<td>IL-6</td>
<td>Monocytes, macrophages, T and helper T cells</td>
<td>Stimulates inflammatory response</td>
</tr>
<tr>
<td>IL-9</td>
<td>Helper T cells</td>
<td>T cell and mast cell growth factor</td>
</tr>
<tr>
<td>IFN-γ (IL-18)</td>
<td>T and helper T cells, NK cells</td>
<td>Activates macrophages</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Macrophages, mast cells, lymphocytes</td>
<td>Increases other cytokines; increases inflammatory and immune responses</td>
</tr>
<tr>
<td>Macrophage colony-stimulating factor</td>
<td>Inflamed endothelial cells, monocytes, lymphocytes, fibroblasts</td>
<td>Macrophage growth factor</td>
</tr>
<tr>
<td>Transforming growth factor-β</td>
<td>Macrophages, platelets, lymphocytes</td>
<td>Chemotactic for macrophages; increases IL-1 production</td>
</tr>
</tbody>
</table>

1.3.3 Arachidonic acid metabolism

Products of arachidonic acid metabolism such as prostaglandins and leukotrienes play key roles in inflammation, and their syntheses are well established targets in the pharmacological treatment of inflammation.

Arachidonic acid (5Z,8Z,11Z,14Z-eicosatetraenoic acid) is an unsaturated, 20-carbon, omega-6 fatty acid found in cell membranes. It can be obtained from the diet or be derived
from linoleic acid. In the cell membrane, arachidonic acid is esterified to phospholipid, and arachidonate must be liberated from phospholipid before it can act as a substrate for enzymatic modification. These modifications, catalysed by various enzymes, are known as arachidonic acid metabolism and can lead to the formation of inflammatory mediators collectively known as eicosanoids, i.e. prostaglandins (PGs), thromboxanes (TXs), hydroxyeicosatetraenoic acids (HETEs) and leukotrienes (LTs) (Calder, 2005; Eberhart & Dubois, 1995).

Arachidonic acid is released from the cell membrane by phospholipase enzymes, in particular phospholipase A2, and the free acid can be metabolised to eicosanoids by cyclo-oxygenase (COX) and lipoxygenase (LOX) enzymes. Metabolism catalysed by COX enzymes gives rise to prostaglandins of the 2-series as well as thromboxanes, while LOX metabolism leads to the formation of leukotrienes (Fig. 1-1).

COX is also known as prostaglandin endoperoxide synthase. This protein possesses two discrete activities: the cyclo-oxygenase activity first inserts two oxygen molecules into arachidonic acid resulting in PGG2; this is followed by the reduction of PGG2 to PGH2, a result of the protein’s peroxidase activity (Needleman et al., 1986). Since prostaglandins of the 2-series, including PGE2, are involved in many inflammatory processes, the inhibition of the COX pathway of arachidonic acid metabolism is a prime pharmacological target and one that has been exploited long before the pathway was known. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the cyclo-oxygenase activity (but not the peroxidase activity) of prostaglandin endoperoxide synthase (Marnett et al., 1999). Acetylsalicylic acid (aspirin) does so in an irreversible fashion, by acetyllating the enzyme (Foster, 1996; Needleman et al., 1986).

Two isoforms of the COX enzyme are known; these are known as COX-1 and COX-2 and their respective protein products show differential distribution (Marnett et al., 1999). COX-1 is constitutively expressed in most tissues, where it catalyses the biosynthesis of eicosanoids (prostaglandins and thromboxanes) that regulate numerous cellular processes. In contrast, COX-2 activity is generally undetectable in most tissues, but the expression of COX-2 can be rapidly induced in inflammatory cells in response to stimulation by pro-inflammatory cytokines or by growth factors (Calder, 2005). A new class of anti-inflammatory and analgesic agents that are selective COX-2 inhibitors were developed in the 1990s (e.g. celecoxib, refecoxib). The rationale for this drug development was that selective COX-2
inhibitors were not expected to interfere with homoeostatic physiological processes and should therefore be less likely than non-selective COX inhibitors to cause the unwanted side-effects typical of traditional NSAIDs (Lipsky, 1999; Sautebin, 2000; Simon et al., 1999).

Fig. 1-1. Schematic representation of the metabolism of arachidonic acid catalysed by cyclo-oxygenase (COX) and lipoxygenase (LOX) enzymes.

Already in the late 1990s there was growing evidence, however, that viewing COX-2 as solely pro-inflammatory and COX-1 as essentially benign was too simplistic. COX-2 has now been shown to be involved in normal physiology such as the regulation of vascular and renal blood flow (Brater et al., 2001), and Sautebin reviewed work suggesting that COX-2 could have anti-inflammatory action, while COX-1 could contribute to inflammation under certain circumstances (Sautebin, 2000). Nevertheless, COX-2 inhibitory drugs (coxibs) were aggressively marketed and became very widely prescribed for inflammatory conditions in industrialised countries in the early 2000s, only for some of them to be withdrawn from the
market a few years later (2004) due to the increased cardiovascular risks associated with their use (rofecoxib [Vioxx®] was withdrawn; celecoxib [Celebrex®] is still used, but in a far more discriminate manner) (Fitzgerald, 2004; Yoon & Baek, 2005).

A so-called splice variant of COX-1, known as COX-3, COX-1b or COX-1v, is also known, but after initial speculation that it might be a target for paracetamol, medical interest in it has waned (Hersh et al., 2005).

The alternative metabolic pathway of arachidonic acid is controlled by the lipoxygenase (LOX) enzymes (Fig. 1-1). They catalyse the insertion of molecular oxygen into polyunsaturated fatty acids with a 1Z,4Z-pentadiene system. Depending on the location of the oxygen insertion into arachidonic acid, mammalian lipoxygenase enzymes are classified as 5-, 12- or 15-lipoxygenases. The primary products are 5-, 12- and 15-hydroperoxyeicosatetraenoic acids (HPETEs), which are subsequently reduced to the corresponding hydroxyeicosatetraenoic acids (HETEs) or, in case of the 5-LOX pathway, converted to the eicosanoids known as leukotrienes (Calder, 2005; Needleman et al., 1986; Schneider & Bucar, 2005).

1.3.3.1 Prostaglandins

The prostaglandins are a group of cyclic, 20-C unsaturated fatty acids, which all share a double-bond at C_{13}-C_{14}. Based on structural differences, the prostaglandins (PGs) are divided into 9 groups, named A-I. A subscript numeral indicates the number of unsaturated carbon bonds in the compound, and the subscripts α- and β- denotes the orientation of a hydroxyl-group on C_{9} below or above the molecular plane (e.g. PGF_{2α}) (Foster, 1996).

Originally discovered in seminal fluid from the prostate (giving rise to their name), prostaglandins are known to be synthesised and released in virtually all body tissues. No tissue (except seminal fluid) appears to have the capacity to store prostaglandins, so rate of release reflects the rate of biosynthesis. Prostaglandins are produced in response to a variety of stimuli, including inflammation, allergic responses and trauma, and they usually exert their action locally, close to their site of release. Several prostaglandins are known to be metabolised rapidly in the liver, kidneys and lungs (Foster, 1996).
Five prostanoid receptors named DP, EP, FP, IP and TP have been identified that show some degree of selectivity for PGD$_2$, PGE$_2$, PGF$_{2\alpha}$, PGI$_2$ and thromboxane A$_2$ (TxA$_2$), respectively. The existence of several subtypes of the EP receptor has been proposed and it is believed that PGE$_2$ exerts different actions at these (Foster, 1996).

The formation of different PGs from the unstable metabolite PGH$_2$ is cell-specific. For example, PGI$_2$ (prostacyclin) is the predominant prostaglandin in the vascular endothelium, where it inhibits platelet aggregation and causes vasodilation. In mast cells, PGD$_2$ is the major COX product, while PGE$_2$ is the prevailing prostaglandin in the kidney (Needleman et al., 1986). PGE$_2$ has several pro-inflammatory activities, including vasodilation and increasing vascular permeability, inducing fever, and enhancing pain and oedema caused by other mediators such as bradykinin and histamine. Large amounts of PGE$_2$ and PGF$_2$ can be produced by stimulated monocytes and macrophages, whereas stimulated neutrophils produce moderate amounts of PGE$_2$ (Calder, 2005).

1.3.3.2 Thromboxanes

The thromboxanes are eicosanoids arising from the COX metabolic pathway like the prostaglandins. Thromboxane A$_2$ (TxA$_2$) is the major metabolite of PGH$_2$ in platelets. Unlike its metabolite thromboxane B$_2$ (TxB$_2$), which is inactive, TxA$_2$ contracts vascular smooth muscle, induces platelet aggregation and causes serotonin release (Needleman et al., 1986).

1.3.3.3 Leukotrienes

Leukotrienes (LTs) are potent biologically active compounds produced by the 5-LOX pathway via 5-HPETE. LTC$_4$, LTD$_4$, and LTE$_4$ are produced mainly by mast cells, basophils, eosinophils and endothelial cells from the precursor LTA$_4$, while LTB$_4$ is produced in neutrophils, monocytes and macrophages (Calder, 2005; Jala & Haribabu, 2004).

LTB$_4$ is a potent chemotactic agent for leucocytes, it increases vascular permeability, causes the release of lysosomal enzymes, and promotes the generation of reactive oxygen species
and the production of inflammatory cytokines including tumour necrosis factor α (TNFα), interleukin-1 (IL-1) and interleukin-6 (IL-6). LTC₄, LTD₄, and LTE₄ increase vascular permeability, but also cause bronchoconstriction and promote hypersensitivity reactions (Calder, 2005).

Leukotrienes have been associated with a range of inflammatory conditions including asthma, colitis, dermatitis, rheumatoid arthritis and septic peritonitis, and recent work has suggested an important role for leukotrienes in the development and progression of atherosclerosis (Jala & Haribabu, 2004). The modification of leukotrienes as a pharmacological target has only been achieved relatively recently, but several drugs (inhibitors of leukotrienes biosynthesis and receptor antagonists) are now approved for the treatment of asthma and allergic rhinitis (Jala & Haribabu, 2004; Valli, 2003).

### 1.4 Plants as anti-inflammatory agents

*When you examine a man with an irregular wound...and that wound is inflamed...[there is] a concentration of heat; the lips of that wound are reddened and that man is hot in consequence...Then you must make cooling substances for him to draw the heat out...leaves of willow.*

(Translated entry from the Ebers papyrus, as quoted in Mann 1992)

The bark and leaves of willow (*Salix* spp.) are among the most famous plants with anti-inflammatory properties. A source of a range of salicylates, mostly in glycoside form, willow has been used medicinally for millennia. The parent compound of these salicylates, salicylic acid, was shown to be highly effective in the treatment of rheumatic fever in one of the earliest clinical trials in history. Its corrosive effects in the gastrointestinal tract lead to the development of a derivative, acetylsalicylic acid, which was marketed by the German pharmaceutical company Bayer in 1899 under the name Aspirin (Mann, 1992). The most successful pharmaceutical drug of all time, acetylsalicylic acid became the prototype for the class of pharmaceuticals known as non-steroidal anti-inflammatory drugs (NSAIDs). However, it was not until 1971 that the main mechanism of action for Aspirin was elucidated. In groundbreaking work led by John Vane, who was later rewarded with a Nobel
Prize, the key to Aspirin’s anti-inflammatory action was shown to be its potent ability to inhibit cyclooxygenase (Mann, 1992).

Over the past two decades, numerous plant extracts and plant compounds have been investigated for their ability to modulate inflammation. Most of these investigations have been conducted in vitro or in animal models, while only a relatively small number of human trials have been conducted in this area. Plant compounds with anti-inflammatory activity have been reviewed and arachidonic acid metabolism, nitric oxide and nuclear factor kappa B (NFkB) identified as major targets (Bremner & Heinrich, 2002; Calixto et al., 2003). In many cases such anti-inflammatory activity appears to be the result of the ability of a compound to inhibit the action and/or biosynthesis of pro-inflammatory cytokines, chemokines or adhesion molecules involved in the inflammatory process, for example by activating transcription factors (incl. NFkB) and protein kinases (Calixto et al., 2004). Table 1-2 provides examples of anti-inflammatory plant compounds and their targets.
<table>
<thead>
<tr>
<th>Compound(s)</th>
<th>Plant source</th>
<th>Molecular target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isocoumarins</td>
<td>Hydrangea dulcis</td>
<td>Mast cells</td>
<td>(Matsuda et al., 1999)</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Vitis vinifera</td>
<td>Mast cells</td>
<td>(Baolin et al., 2004)</td>
</tr>
<tr>
<td>Orixalone A</td>
<td>Orix a japonica</td>
<td>Nitric oxide</td>
<td>(Ito et al., 2004)</td>
</tr>
<tr>
<td>Eugenol</td>
<td>Ocimum sanctum</td>
<td>COX-1</td>
<td>(Kelm et al., 2000)</td>
</tr>
<tr>
<td>Ursolic acid</td>
<td>Plantago major</td>
<td>COX-2</td>
<td>(Ringbom et al., 1998)</td>
</tr>
<tr>
<td>Wogonin</td>
<td>Scutellaria baicalensis</td>
<td>COX-2 expression</td>
<td>(Chen et al., 2001)</td>
</tr>
<tr>
<td>Rhamnetin</td>
<td>Guiera senegalensis</td>
<td>5-lipoxygenase</td>
<td>(Bucar et al., 1998)</td>
</tr>
<tr>
<td>Maesanin</td>
<td>Maesa lanceolata</td>
<td>5-lipoxygenase</td>
<td>(Abourashed et al., 2001)</td>
</tr>
<tr>
<td>Ugandensidial</td>
<td>Warburgia ugandensis</td>
<td>5-lipoxygenase</td>
<td>(Wube et al., 2006)</td>
</tr>
</tbody>
</table>

In terms of arachidonic acid metabolism, it is noteworthy that a number of plant compounds and extracts have been shown to be dual inhibitors of cyclo-oxygenase and 5-lipoxygenase enzymes *in vitro* (Liu et al., 1998; Resch et al., 1998).
2. LITERATURE REVIEW

2.1 Introduction

Many plants belonging to the ginger family, Zingiberaceae, have a history of medicinal use in systems of traditional medicine. Best known are ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*), both of which has been the subject of substantial pharmacological and clinical investigations over the last three decades, but many lesser known species are also used, mostly in tropical Asia, where the majority are native. Several species in the family are also important spices.

This chapter provides background information on the genera included in the present work, with emphasis on chemistry and pharmacology, in particular as it relates to potential anti-inflammatory activity. Ginger has been given special attention in this review. Zingiberaceae genera not included in the present study have not been reviewed.

2.2 The ginger family, Zingiberaceae Martinov

The ginger family, Zingiberaceae, is a monocotyledenous family in the order Zingiberales. The family comprises some 52 genera with a total about 1100 species. The family is essentially tropical in distribution, with few species occurring in temperate climates, and is particularly richly represented in the Indomalesian flora, i.e. from India to New Guinea. Zingiberaceae species typically have thickened rhizomes with secretory cells producing essential oil (Mabberley, 1997). The taxonomy of the Zingiberaceae according to K. Kubitzki’s *The Families and Genera of Vascular Plants* as presented by Mabberley (1997) is outlined in Table 2-1.
Table 2-1. Taxonomy of the family Zingiberaceae.  
(After Mabberley 1997).

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Angiospermae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class</td>
<td>Monocotyledonae (Liliopsida)</td>
</tr>
<tr>
<td>Subclass</td>
<td>Zingiberidae</td>
</tr>
<tr>
<td>Order</td>
<td>Zingiberales</td>
</tr>
<tr>
<td>Family</td>
<td>Zingiberaceae</td>
</tr>
</tbody>
</table>

The Zingiberaceae is divided into two subfamilies (Table 2-2). The subfamily Zingiberoideae comprises four tribes. The subfamily Costoideae is commonly treated as a separate family, Costaceae (Meissner) Nakai (Mabberley, 1997).

Table 2-2. Subfamilies, tribes and representative genera of the Zingiberaceae.  
(After Mabberley 1997.) Genera in bold were included in the present work.

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Tribe</th>
<th>Representative genera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zingiberoideae</td>
<td>Hedychieae</td>
<td><em>Boesenbergia, Curcuma, Hedychium, Kaempferia, Scaphochlamys</em></td>
</tr>
<tr>
<td></td>
<td>Zingibereae</td>
<td><em>Zingiber</em></td>
</tr>
<tr>
<td></td>
<td>Alpinieae</td>
<td><em>Aframomum, Alpinia, Amomum, Elettaria, Etlingera, Hornstedtiia</em></td>
</tr>
<tr>
<td></td>
<td>Globbeae</td>
<td><em>Globba</em></td>
</tr>
<tr>
<td>Costoideae</td>
<td></td>
<td><em>Costus, Tapeinochilus</em></td>
</tr>
</tbody>
</table>

2.2.1 Zingiberaceae in Australia

In Australia, the Zingiberaceae is represented by 14 native species from 8 genera. In addition, 7 introduced species from 5 genera also occur (Smith, 1987) (Table 2-3).
Table 2-3. Zingiberaceae species occurring in Australia.

Native species in bold were included in the present study. (After Smith 1987).

<table>
<thead>
<tr>
<th>Genus</th>
<th>Native species</th>
<th>Introduced and naturalised species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpinia</td>
<td>A. arctiflora (F. Muell.) Benth.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A. arundelliana (Bailey) Schumann</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A. caerulea (R. Br.) Benth.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A. hylandii R. M. Smith</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A. modesta F. Muell. ex Schumann</td>
<td></td>
</tr>
<tr>
<td>Amomum</td>
<td>A. dallachyi F. Muell.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A. queenslandicum R. M. Smith</td>
<td></td>
</tr>
<tr>
<td>Costus</td>
<td>C. potierae F. Muell.</td>
<td>C. dubius (Afzel.) Schumann</td>
</tr>
<tr>
<td>Curcuma</td>
<td>C. australasica J. D. Hook</td>
<td>C. longa L.</td>
</tr>
<tr>
<td>Etinglera</td>
<td>E. australasica (R. M. Smith) R. M. Smith</td>
<td></td>
</tr>
<tr>
<td>Globba</td>
<td>G. marantina L.</td>
<td>H. coronarium Koenig</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H. gardnerianum Sheppard ex Ker Gawler</td>
</tr>
<tr>
<td>Hedychium</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hornstedtia</td>
<td>H. scottiana (F. Muell.) Schumann</td>
<td></td>
</tr>
<tr>
<td>Kaempferia</td>
<td></td>
<td>Kaempferia sp.</td>
</tr>
<tr>
<td>Pleuranthodium</td>
<td>P. racemigerum (F. Muell.) R. M. Smith</td>
<td></td>
</tr>
<tr>
<td>Tapeinochilos</td>
<td>T. ananassae (Hassek.) Schumann</td>
<td>Z. officinale Roscoe</td>
</tr>
<tr>
<td>Zingiber</td>
<td></td>
<td>Z. zerumbet (L.) Smith</td>
</tr>
</tbody>
</table>

2.3 Genus *Zingiber* Boehmer

The genus *Zingiber* comprises approximately 60 species ranging from India through tropical Asia (Mabberley, 1997; Smith, 1987). There are no native Australian representatives of this genus (despite the erroneous statement to the contrary by Mabberley, 1997), but two species, *Z. officinale* Roscoe and *Z. zerumbet* (L.) Smith, have been reported as naturalised in tropical north Queensland (Hnatiuk, 1990; Smith, 1987).
The generic name comes from the Greek *Zingiberi*, which in turn is derived from an Indian word meaning ‘root’ (Smith, 1987).

Several species of *Zingiber* have a history of traditional medicinal, culinary or other ethnobotanical uses. These include Japanese or mioga ginger (*Z. mioga* (Thunb.) Roscoe), used against malaria and as a vermifuge in China (Mabberley, 1997); *Z. montanum* (J. König) Theilade (syn. *Z. cassumunar, Z. purpureum*), used for a wide range of complaints in India and South-East Asia (Johnson, 1999); and *Z. zerumbet* (L.) Smith, which has also been employed for a range of conditions in Asia and the Pacific (Johnson, 1999).

The common ginger (*Z. officinale* Roscoe), which has been a major focus of the present work, is treated in detail below. In addition, other *Zingiber* species included in this work are briefly reviewed.

### 2.3.1 Ginger (*Zingiber officinale* Roscoe)

Ginger (*Zingiber officinale* Roscoe) is a sterile, reed-like plant with a pungent and aromatic rhizome on which it relies for vegetative propagation. The plant is a cultigen, that is, it is only known from cultivation. Its wild origins are not known with certainty but are believed to be India or South-East Asia (Mabberley, 1997; Vaughan & Geissler, 1997). Ginger has a very long history of use, both as a spice and as a medicinal plant, and is mentioned in ancient Sanskrit texts and in classical Buddhist, Arabic, Greek and Roman literature (Govindarajan, 1982a). It was used widely in Europe by the tenth century (Vaughan & Geissler, 1997) and was first exported from Jamaica, where it became a significant agricultural crop, in 1547 (Mabberley, 1997). It is now cultivated in many tropical and subtropical regions including India, Africa, China, the West Indies and Australia, with the annual world production estimated at 100,000 tons in 2000 (Bartley & Jacobs, 2000; Evans, 2002).

Ginger rhizome is valued as a spice for its combination of pungent and aromatic qualities, which arise from its content of phenolic compounds and essential oil, respectively. Ginger is used as flavouring in a vast array of foods, including savoury dishes such as curries, and sweets such as cakes and biscuits, and also in beverages such as ginger ale, ginger beer and ginger wine.
Ginger rhizome (known as *Rhizoma Zingiberis* in pharmacy) is used in several traditional systems of medicine, including Traditional Chinese Medicine, Ayurveda and Western herbal medicine (WHO, 1989; Williamson, 2002). Its traditional uses cover a great variety of complaints including dyspepsia, flatulence and colic, nausea and vomiting, colds and flu, migraine, as well as muscular and rheumatic disorders (WHO, 1999).

### 2.3.1.1 Ginger chemistry

The secondary metabolites found in the rhizome of ginger that are of primary interest can broadly be divided into volatile compounds (extractable by steam distillation) and non-volatile phenolic compounds, the major ones of which have pungent properties. It is generally considered that the pharmacological activity of ginger rhizome resides with compounds from these classes, in particular the non-volatile pungent phenolic compounds.

The term oleoresin, when applied to ginger, refers to the volatile oil, the pungent compounds and other compounds extracted by means of solvents (ethanol or acetone) (Connell, 1969; Govindarajan, 1982a).

#### 2.3.1.1.1 Non-volatile compounds

Ginger owes its pungency to phenolic compounds. In the fresh rhizome the major type comprises a series of homologous phenolic alkanones known as gingerols and derivatives thereof such as gingerdiols. The principal of these compounds is [6]-gingerol with 8- and 10-gingerol occurring in lower concentrations (Connell & Sutherland, 1969; Denniff et al., 1981). When subjected to heat or alkali treatment, however, gingerols are converted to a corresponding series of homologous shogaols by dehydration and/or to the compound zingerone (Connell, 1969; Connell & Sutherland, 1969). The shogaols possess greater pungency than the corresponding gingerols (Denniff et al., 1981).
2.3.1.1.1 Historical background

In 1879, Tresh isolated an oily pungent concentrate from ginger oleoresin and called it gingerol (Tresh, 1879). In 1917, English and Japanese researchers independently isolated two pungent ginger compounds, gingerol (Fig. 2-1) and zingerone (Fig. 2-2) (Lapworth et al., 1917).

![Fig. 2-1. Structure of [6]-gingerol, the most abundant gingerol in ginger rhizome.](image1)

In 1927, the Japanese group published the structural characterisation of another pungent ginger compound, shogaol (Fig. 2-3) (after shoga, the Japanese word for ginger) (Connell, 1969; Govindarajan, 1982a).

![Fig. 2-2. Structure of zingerone.](image2)

![Fig. 2-3. Structure of [6]-shogaol.](image3)
Little progress on the chemistry of pungent ginger compounds was made until the 1960s, when D. W. Connell of the Queensland Department of Primary Industries commenced his work in the area.

### 2.3.1.1.1.2 Gingerols

Comparing the chemical composition of commercially prepared ginger oleoresin and an oleoresin extracted with cold solvent, Connell's group found surprising differences. The major pungent compound identified in the commercial oleoresin was shogaol and despite repeated attempts, gingerol could not be isolated from this sample. The freshly prepared oleoresin extracted with cold solvent, however, had a different major constituent, which was isolated and identified as gingerol (Connell, 1969; Connell & Sutherland, 1969).

Extensive work on ginger oleoresin led Connell and Sutherland to suggest that although both shogaol and zingerone had been isolated from oleoresins, they were in fact both artefacts, or at the most very minor constituents of ginger rhizomes. The presence of shogaol and zingerone in easily detectable quantities, according to Connell and Sutherland, were indicative of the oleoresin having been exposed to excessive heat in the course of extraction (Connell & Sutherland, 1969).

Although gingerol is normally an oily substance, Connell and Sutherland were able to obtain a crystalline solid when storing the gingerol in hexane at –30°C. This solid was shown to consist of a mixture of homologous phenolic ketones, identified as [6]-, [8]- and [10]-gingerol (Fig. 2-4). [4]- and [12]-gingerols were not identified, but their presence in trace amounts could not be excluded.

![Fig. 2-4. Structures of the major gingerols in ginger.](image-url)
He and coworkers also identified [6]-, [8]- and [10]-gingerol in a methanol extract of fresh ginger rhizome analysed by HPLC-electrospray mass spectrometry (He et al., 1998).

### 2.3.1.1.3 Gingerols in fresh ginger

Several studies have reported on the concentration of gingerols in fresh ginger rhizomes. Zhang and colleagues analysed by reversed-phase high-performance liquid chromatography (HPLC) freshly harvested rhizomes from Hawaii extracted with methanol, and found the concentration of [6]-gingerol to be 2100 µg per gram fresh rhizome. The corresponding concentrations of [8]- and [10]-gingerol were 288 and 533 µg/g, respectively (Zhang et al., 1994). Fresh rhizomes purchased locally in the United States, extracted by methylene chloride and analysed by HPLC, yielded 880, 93 and 120 µg per gram fresh rhizome of [6]-, [8]- and [10]-gingerol, respectively (Hiserodt et al., 1998). Fresh rhizome from Taiwan was reported to have a [6]-gingerol concentration of 806 µg/g by HPLC (Young et al., 2002). A supercritical CO₂ extract of ginger grown on the Australian east coast and analysed by negative ion electrospray-MS contained 120, 19 and 24 µg per gram fresh rhizome of [6]-, [8]- and [10]-gingerol, respectively (as well as shogaols that may have formed from gingerols during processing) (Bartley, 1995). The considerable variation in gingerol concentrations across these studies may reflect genetic or environmental differences, as well as the variable methodological approaches, or a combination of these. Because ginger is a sterile cultigen with a very long history of cultivation in different parts of the world, genetic differences between clones are likely to be an important determinant of variation in secondary metabolites.

### 2.3.1.1.4 Gingerol degradation products

Connell (1969) commented on the remarkable extent to which chemical changes occur in ginger. It is interesting to note that these changes are reflected in the different therapeutic applications of fresh and processed ginger in Oriental medicine (Hikino, 1985).
The main pungent compounds in fresh ginger, [6]-, [8]- and [10]-gingerol, are thermally unstable and can undergo at least two reactions (Connell, 1969; Connell & Sutherland, 1969).

Firstly, [6]-, [8]- and [10]-gingerol can undergo dehydration and convert to [6]-, [8]- and [10]-shogaol, respectively, when exposed to high temperature or subjected to prolonged storage (Fig. 2-5) (He et al., 1998; Zhang et al., 1994).

Secondly, a retro-aldol reaction can give rise to zingerone and a series of aliphatic aldehydes, which can cause undesirable flavours in the oleoresin (Fig. 2-6) (Connell, 1969). Experiments showed that aldehyde formation occurred when oleoresin was heated to temperatures above 200°C.
In studies of the fate of pure gingerol at pH 3-5, which is the typical pH-range for oleoresin samples, the conversion to shogaol was found to proceed at a very slow rate at room temperature. At higher temperature, however, the reaction occurred more rapidly. Under alkaline conditions, the dehydration reaction proceeded readily at room temperature, following first order reaction kinetics. Studying this dehydration reaction in the oleoresin at a constant temperature of 120°C, the gingerol content was found to decrease, with the rate of loss increasing with decreasing pH. This reaction also followed first order kinetics. The half-life of gingerol in ginger oleoresin at 120°C was found to range from approximately 3 hours at pH 2.4 to 15 hours at pH 7.2 (Connell, 1969; Connell & Sutherland, 1969).

Connell also extracted an oleoresin from freshly harvested ginger rhizome, using cold acetone, which was subsequently removed from the extract at or below room temperature (Connell, 1969). This extract showed a high gingerol content with little or no shogaol present. From the same batch of ginger rhizome, two other oleoresins were prepared, one from the whole dried rhizome and another from the sliced dried rhizome. Whole rhizomes required longer heating time to dry than did sliced rhizomes. As expected, the oleoresin from the whole dried rhizome had a higher shogaol content.

One of these oleoresins, with a natural pH of 3.5, was stored at ambient temperature for a period of 18 months. During this time, a large proportion of the gingerol was converted to
Whether shogaols occur in fresh ginger rhizome or form as artefacts of various extraction and manufacturing processes has been a matter of some contention. Govindarajan, in his comprehensive review of ginger from 1982, stated that several thin-layer chromatography (TLC) studies had shown that shogaol is ‘definitely’ present in the fresh rhizome as a minor component (Govindarajan, 1982a). This appeared to have been confirmed by liquid chromatography – mass spectrometry (LC-MS) by He and co-workers who reported a small amount of [6]-shogaol (He et al., 1998), but their sample was a methanolic extract prepared by reflux, a process that would likely have provided the necessary heat for [6]-shogaol to form from [6]-gingerol. [6]-Shogaol (and in one case also 8- and 10-shogaol) have also been reported from supercritical carbon dioxide extracts of fresh ginger by GC-MS (Bartley, 1995; Bartley & Jacobs, 2000), but the high temperatures involved in this analytical method (up to 290ºC) readily account for the formation of shogaols from gingerols. Chen and co-workers found only a trace amount of [6]-shogaol in a supercritical CO₂ extract prepared from freeze-dried fresh rhizomes and analysed by HPLC, which does not involve high temperatures (Chen et al., 1986b), and TLC of extracts of fresh Australian ginger did not demonstrate the presence of shogaol (Connell & Sutherland, 1969).

More recent studies have indicated that in aqueous solution [6]-gingerol and [6]-shogaol exist in a pH-dependent equilibrium. One study found that [6]-gingerol was most stable at pH 4, while at pH 1 and 100ºC conversion of [6]-gingerol to [6]-shogaol was relatively fast and reached equilibrium within two hours (Bhattarai et al., 2001). Another study found that in simulated gastric fluid (pH 1 at 37ºC) [6]-gingerol and [6]-shogaol underwent first order reversible dehydration and hydration reactions to form [6]-shogaol and [6]-gingerol, respectively (Bhattarai et al., 2007). The same study found that in simulated intestinal fluid (pH 7.4 at 37ºC) insignificant interconversion of the two compounds took place.

### 2.3.1.1.5 Other non-volatile compounds

A range of other compounds related to gingerols as well as several diarylheptanoids have been isolated from ginger rhizome. These include [4]-, [6]-, [8]- and [10]-gingerdial, [6]-methylgingerdial, [6]-methylgingerdiacetate, [6]-paradol, gerdiones, [6]-gingesulfonic
acid, [6]-hydroxyshogaol, [6]-, [8]- and [10]-dehydroshogaol and hexahydrocurcumin (Kikuzaki, 2000 and references therein).

More recently, workers at the University of Arizona have published numerous compounds from ginger, including many novel compounds (Jolad et al., 2004; Jolad et al., 2005). These include paradols, dihydroparadols, acetyl derivatives of gingerols, 3-dihydroshogaols, gingerdiols, mono- and diacetyl derivatives of gingerdiols, 1-dehydrogingerdiones, diarylheptanoids, methyl [8]-paradol, methyl [6]-isogingerol, methyl [4]-shogaol, [6]-isoshogaol, 6-hydroxy-[8]-shogaol, 6-hydroxy-[10]-shogaol, 6-dehydro-[6]-gingerol, three 5-methoxy-[n]-gingerols (n = 4, 8 and 10), 3-acetoxy-[4]-gingerdione, 5-acetoxy-[6]-gingerdione, diacetoxy-[8]-gingerdione, methyl diacetoxy-[8]-gingerdione, 5-acetoxy-3-deoxy-[6]-gingerol, 1-hydroxy-[6]-paradol and others. Some of these compounds could potentially be artifacts.

Some of the compounds reported from ginger are shown in Fig. 2-7 below.

![Fig. 2-7. Paradols, gingerdiols and gingerdiones from Zingiber officinale.](image)

### 2.3.1.1.2 Volatile oil

Commercial ginger oil is obtained by steam distillation of coarsely ground dried ginger rhizome, and most published compositional analyses refer to oil prepared from dried raw
material. Ginger oil distilled from dry material is characterised by a high proportion of sesquiterpene hydrocarbons and relatively small amounts of monoterpenes hydrocarbons and oxygenated compounds (Govindarajan, 1982a). The major sesquiterpene hydrocarbons are zingiberene, \textit{ar}-curcumene, \textit{\beta}-bisabolene, (-)-\textit{\beta}-sesquiphellandrene and (E,E)-\textit{\alpha}-farnesene (Lawrence, 1995b), although published data indicate the relative abundance of these compounds varies greatly (Fig. 2-8).

\begin{center}
\includegraphics[width=\textwidth]{fig2-8.png}
\end{center}

\textit{Fig. 2-8. Volatile sesquiterpenes from Zingiber officinale.}

Both zingiberene and (-)-\textit{\beta}-sesquiphellandrene can be oxidised to \textit{ar}-curcumene in oil stored under unfavourable conditions (Connell & Jordan, 1971; Govindarajan, 1982a). High levels of \textit{ar}-curcumene may therefore be an indicator of degraded oil, but could also possibly reflect distillation conditions (Govindarajan, 1982a).
Other constituents of ginger essential oil widely reported include α-pinene, camphene, 6-methyl-5-hepten-2-one, myrcene, α- and β-phellandrene, limonene, 1,8-cineole, linalool, borneol, α-terpineol, citronellol, neral, geraniol, geranial, bornyl acetate, 2-undecanone, citronellyl acetate, α-copaene and geranyl acetate (Lawrence, 1995b; Lawrence, 1997; Lawrence, 2000). Some of the monoterpenoids in the oil are shown in Fig. 2-9.

![Monoterpenoid structures](image)

**Fig. 2-9. Volatile monoterpenoids from Zingiber officinale.**

Australian ginger grown in Queensland yields a steam-distilled oil known for its citrus-like aroma. Connell and Jordan compared 35 samples of commercially and laboratory prepared Australian ginger oils with ten samples of commercial oils from other major ginger-producing areas (Jamaica, Nigeria, Sierra Leone, China and India) (Connell & Jordan, 1971). The compounds chiefly responsible for this citrus-like aroma were identified as the monoterpane aldehydes geranial and neral (the mixture of which is known as citral). The 35 Australian oil samples analysed by Connell and Jordan contained 3-20% geranial and 1-10% neral, whereas the oils from other parts of the world contained these compounds from trace amounts to 3 and 1%, respectively (Connell & Jordan, 1971). The same study found that
apart from their high citral content, Australian ginger oils were similar to other oils in terms of their major constituents, these being (-)-zingiberene (20-28%), (-)-β-sesquiphellandrene (7-11%), ar-curcumene (6-10%) and β-bisabolene (5-9%). These sesquiterpenes, however, are only weakly odorous and are believed to make only minor contributions to the aroma and flavour of the oil (Connell & Jordan, 1971).

It is clear from more recent studies, however, that ginger oils from regions other than Australia can also have a high citral content. The highest citral content of ginger oil reported in the literature was 66% in one of five samples (ranging from 28% to 66%) prepared from fresh rhizomes from Fiji (Smith & Robinson, 1981). Rhizomes (probably fresh) from the Central African Republic steam-distilled for five hours yielded an oil containing 34.6% citral (Menut et al., 1994), and a Mauritian ginger oil distilled from freshly harvested rhizomes contained 26.6% citral (Gurib-Fakim et al., 2002). Ekundayo and colleagues compared oils distilled for three hours from fresh and sun-dried Nigerian ginger (Ekundayo et al., 1988). The oil made from fresh and dried plant material contained 23.9% and 14.3% citral, respectively. Some Asian ginger oils have been reported to have low levels of citral (Macleod & Pieris, 1984; Vernin & Parkanyi, 1994), but an Indian oil distilled (4 hours) from fresh rhizomes contained 25% (Sharma et al., 2002), and an oil from Taiwan reportedly had a citral content of 25% (Lawrence, 1995a).

Indian investigators have reported that the citrus-like aroma of ginger oil (which is due to citral) is characteristic of oil distilled from fresh or quickly dried ginger, whereas commercial oils and oils made from sun-dried ginger lack the lemony note (Govindarajan, 1982a). Thus it is presently unclear whether the reported variability in citral content is due to genetic, environmental or ontogenic factors, to factors relating to post-harvest handling or processing, or to a combination of these.

2.3.1.2 Pharmacokinetics of ginger

No information is available regarding the pharmacokinetics of crude ginger preparations. Several in vitro and animal studies and one in vitro study employing human hepatic and intestinal microsomes have investigated the metabolism of the major pungent compound in
ginger, [6]-gingerol. One *in vitro* study has explored the hepatic metabolism of [6]-shogaol. These studies are outlined below.

Experiments in rats with acute renal or hepatic failure showed that the elimination half-life and plasma concentration of intravenously administered [6]-gingerol increased significantly after hepatic failure was induced with carbon tetrachloride. The induction of renal failure, however, did not affect clearance of [6]-gingerol from plasma. These results suggest that the liver but not the kidney is involved in the elimination of [6]-gingerol (Naora *et al.*, 1992). The same study found more than 90% of [6]-gingerol present in plasma was bound to serum protein. [6]-Gingerol incubated with phenobarbital-induced rat liver supernatant containing the NADPH-generating system was converted to two diastereomers of [6]-gingerdiol (Surh & Lee, 1994a).

Another study characterised a number of metabolites following the oral administration of [6]-gingerol to rats (Nakazawa & Ohsawa, 2002). The major metabolite in the bile was *(S)*-[6]-gingerol-4′-O-β-glucuronide, while the β-glucuronidase-treated urine contained vanillic and ferulic acids, 9-hydroxy-[6]-gingerol, *(S)*-(+)-[6]-gingerol and two substituted organic acids. These results demonstrated that orally administered [6]-gingerol undergoes conjugation, and ω-1 oxidation and β-oxidation of a phenolic side chain. About half of the oral dose of [6]-gingerol was excreted in the bile as the glucuronide. Incubation of [6]-gingerol with rat liver produced only *(S)*-[6]-gingerol-4′-O-β-glucuronide and 9-hydroxy-[6]-gingerol (glucuronidation and ω-1 oxidation), suggesting that metabolism must also occur outside the liver. The contribution of gut microflora was demonstrated by the fact that gut sterilisation decreased the amount of urinary metabolites.

In a recent study, [6]-gingerol incubated with human hepatic microsomes fortified with uridine diphosphoglucuronic acid (UDPGA; a source of glucuronic acid) was glucuronidated primarily at the phenolic hydroxyl group with a small amount of glucuronidation also occurring at the aliphatic hydroxyl group, while human intestinal microsomes formed the phenolic glucuronide only (Pfeiffer *et al.*, 2006).

[6]-shogaol incubated with rat liver supernatant containing the NADPH-generating system was converted to saturated ketone and reduced alcohol metabolites as well as an allyl alcohol, 1-(4′-hydroxy-3′-methoxyphenyl)-deca-4-ene-3-ol (Surh & Lee, 1994b).
It is clear from the above that the current state of knowledge of the pharmacokinetics of ginger compounds in humans is embryonic. Expanding this knowledge and including information about oral bioavailability of compounds with known pharmacological activity should be a priority and ought to precede further clinical trials of ginger for inflammatory conditions.

2.3.1.3 Pharmacodynamics of ginger

The pharmacology and therapeutic use of ginger have been the subject of several recent reviews (Afzal et al., 2001; Chrubasik et al., 2005; Grzanna et al., 2005).

Studies conducted in vitro have shown gingerols to act as agonists of the vanilloid receptor (VR1) (Dedov et al., 2002). This receptor is also activated by capsaicin, the major pungent principle in cayenne and chilli pepper, which shares structural features with the gingerols (vanillin-derived compounds with phenolic 3-methoxy-, 4-hydroxy-groups). Earlier work suggested that [6]-shogaol might share its site of action with capsaicin (Onogi et al., 1992); if this were true, [6]-shogaol might also be a VR1 agonist.

Ginger extracts and various ginger constituents have demonstrated a wide range of pharmacological activities in vitro and in vitro. Here the emphasis will be on the activities relevant to inflammation, while other pharmacological activities will be briefly summarised.

2.3.1.3.1 Anti-oxidant activity

Ginger extracts have demonstrated anti-oxidant activity in a number of in vitro assay systems, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, inhibitory effect on oxidation of methyl linoleate under aeration and heating by the Oil Stability Index (OSI) method, inhibitory effect on oxidation of liposome induced by 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) (Masuda et al., 2004), oxygen radical absorbance capacity (ORAC) (Ninfali et al., 2005), Fe$^{2+}$-induced lipid peroxidation, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical quencher assay (Kuo et al., 2005), β-carotene bleaching method (Kaur & Kapoor, 2002) and ferric reducing ability of plasma (FRAP) (Halvorsen et al., 2002).
[6]-Gingerol, [6]-shogaol, [6]-gingerdiol, two other gingerol-related compounds as well as 8 arylheptanoids delayed the oxidation of linoleic acid in the ferric thiocyanate assay (Kikuzaki & Nakatani, 1993). More recently, Masuda and coworkers have identified more than 50 compounds with anti-oxidant activity from ginger rhizome (Masuda et al., 2004). They were either related to gingerols or diarylheptanoids. Structure-activity studies of the gingerol-type compounds suggested that both the substituents on and the length of the alkyl chain might contribute to the anti-oxidant activity. A glucoside of [6]-gingerdiol has also been found to possess strong anti-oxidant activity \textit{in vitro} (Sekiwa et al., 2000).

Several animal studies have demonstrated the anti-oxidant activity of ginger \textit{in vivo}.

Lipid peroxidation was significantly lowered in rats fed ginger (1%); the activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase were maintained, and the blood glutathione content was significantly increased. Similar effects were achieved with ascorbic acid (100 mg/kg) treatment, suggesting that at least in this assay ginger was comparable to ascorbic acid as an antioxidant (Ahmed et al., 2000a).

Rats on a high fat diet supplemented with ginger (35 mg/kg and 70 mg/kg) had lowered levels of tissue thiobarbituric acid reactive substances (TBARS) and hydroperoxides, raised activities of SOD and CAT, and raised levels of reduced glutathione in the aorta, liver, kidney and intestine, compared with unsupplemented controls (Jeyakumar et al., 1999).

In another study, rats fed dried ginger (1%) showed significantly attenuated oxidative stress and lipid peroxidation in response to exposure to the organophosphorous pesticide malathion for 4 weeks (20 ppm) (Ahmed et al., 2000b).

Atherosclerotic, apolipoprotein E-deficient mice fed ginger extract showed a significant reduction in the LDL basal oxidative state, with reduced susceptibility to oxidation and aggregation (Fuhrman et al., 2000).

Pre-treatment with a hydroethanolic extract of ginger reduced the severity of radiation sickness and the mortality in mice exposed to gamma irradiation (Jagetia et al., 2003). Elevated lipid peroxidation and depletion of glutathione following irradiation were both reduced by the ginger treatment. Treatment with ginger after irradiation was not effective (Jagetia et al., 2004).
No studies have as yet directly assessed the anti-oxidant potential of ginger in humans, but based on the substantial amount of evidence from both in vitro and in vivo studies, there are sound reasons to predict that ginger might be a potent anti-oxidant in humans, although any systemic effects could be limited if the compounds responsible have poor oral bioavailability.

2.3.1.3.2 Effects on arachidonic acid metabolism and eicosanoids

Arachidonic acid metabolism is a major inflammatory pathway, and most of the work relevant to the anti-inflammatory activity of ginger has focused on its effects on this pathway. This substantial body of work is reviewed below under the headings (1) Assays using isolated enzymes, (2) Cell-based assays, and (3) Animal studies.

2.3.1.3.2.1 Assays using isolated enzymes

Kiuchi and coworkers were the first group to publish details of the ability of ginger compounds to modulate arachidonic acid metabolism (Kiuchi et al., 1982). They worked on fresh ginger rhizomes obtained from a Japanese market and isolated [6]-gingerol, [6]-gingerdione, [10]-gingerdione, [6]-dehydrogingerdione and [10]-dehydrogingerdione. All of these compounds were shown to be potent inhibitors of prostaglandin-synthetase (cyclooxygenase) in vitro (although details of the assay method were not provided), with IC$_{50}$ values comparable to that of indomethacin. Kiuchi's group subsequently reported the potent inhibitory activity on prostaglandin-synthetase of [6]-gingerol, [10]-gingerol, [6]-shogaol and numerous gingerol derivatives (Kiuchi et al., 1992). Very strong inhibitory activity was found for [10]-gingerol, [6]-acetylgingerol, [6]-shogaol, [6]-dehydrogingerdione, [10]-dehydrogingerdione, [6]-gingerdione and [10]-gingerdione, all of which had IC$_{50}$ values <2.5 μM.

[8]-Paradol has also been shown to be a potent inhibitor of ovine cyclooxygenase-1 (COX-1) with an IC$_{50}$ value of 4±1 μM (Nurjahja-Tjendraputra et al., 2003).

Of particular interest was the finding by Kiuchi’s group that the homologous series of synthetic gingerols (of which [6]-, [8]- and [10]-gingerol occur in ginger) were dual
inhibitors of arachidonic acid metabolism, i.e. inhibitors of both the cyclooxygenase and 5-lipoxygenase pathways (Kiuchi et al., 1992). This finding appears to confirm an earlier finding suggesting that several ginger compounds inhibit both pathways (Flynn et al., 1986), while being at odds with the finding by Srivastava (see below), that fractionated ginger preparations increased the formation of lipoxygenase products (Srivastava, 1984b; Srivastava, 1986). It is possible, however, that these seemingly incongruous results are in fact all accurate and reflect chemical differences between the test preparations.

Dual inhibitors of arachidonic acid metabolism are of significant interest as potential therapeutic substances, because their inhibitory effect on the formation of eicosanoids is likely to some extent to resemble the therapeutic effects of steroidal drugs, which inhibit phospholipase A₂ and therefore inhibit the release from phospholipid membranes of arachidonic acid itself, resulting in potent anti-inflammatory activity.

2.3.1.3.2.2 Cell-based assays

Srivastava published two studies describing the effects of a ginger extract and its fractions on arachidonic acid metabolism and platelet aggregation in washed human platelets (Srivastava, 1984a; Srivastava, 1984b). The preparation used in these studies was described as an aqueous extract, but it is unclear whether it was prepared from fresh or dried plant material. Regardless, thromboxane B₂ (TxB₂) formation was reduced by 73%, while formation of prostaglandin F₂α (PGF₂α), prostaglandin E₂ (PGE₂) and prostaglandin D₂ (PGD₂) and the prostaglandin endoperoxides, prostaglandin G₂ (PGG₂) and prostaglandin H₂ (PGH₂) was also significantly reduced.

Additional data published by Srivastava and colleagues confirmed the previously observed effects on the production of TxB₂ and also found inhibition of lipoxygenase products by ginger components at higher concentrations (Bordia et al., 1997; Srivastava, 1986).

An 80% ethanolic ginger extract (uncertain whether from fresh or dried material) was found to significantly and dose-dependently inhibit prostaglandin release (expressed in terms of PGE₂) by stimulated rat peritoneal leukocytes (Mascolo et al., 1989).

Further evidence of ginger’s inhibitory activity on thromboxane formation was provided by Bordia and coworkers who showed that an n-hexane extract of ginger powder dose-
dependently inhibited thromboxane formation in washed platelets challenged with (1-14C)-labelled arachidonic acid (IC50 =16±8µg/mL, n=3). Using autoradiography, the investigators found that 12-hydroxy-5,8,10-heptadecatrienoic acid (12-HHT) was reduced with a concomitant increase in 12-hydroxyeicosatetraenoic acid (12-HETE) (Bordia et al., 1997).

Inhibition of LPS-induced PGE2 production in human leukaemia HL-60 cells with IC50 values comparable to that of indomethacin was demonstrated for fractions of fresh ginger (Jolad et al., 2004) and commercially processed dry ginger (Jolad et al., 2005; Lantz et al., 2007).

Flynn and colleagues were the first to study the effects of pure synthetic ginger compounds on arachidonic acid metabolism (Flynn et al., 1986). This study used intact human leukocytes and found gingerdione to be a potent inhibitor of both cyclooxygenase and 5-lipoxygenase. [6]-Shogaol and a related compound preferentially inhibited 5-hydroxyeicosatetraenoic acid (5-HETE) formation (i.e. the 5-lipoxygenase pathway), while [6]-gingerol and dehydroparadol appeared to be more potent inhibitors of PGE2 formation (i.e. the cyclooxygenase pathway). All compounds studied possessed a 4-hydroxy-3-methoxyphenyl moiety (which is also a major determinant of flavour). The same study also found curcumin from turmeric (Curcuma longa) to be a dual inhibitor of arachidonic acid metabolism.

Guh et al. investigated the anti-platelet mechanism of ‘gingerol’ isolated from ginger in washed rabbit platelets (Guh et al., 1995). It is not clear from the report whether the plant material was fresh or dried, and the method of isolation is not described. Neither is it clear what is meant by ‘gingerol’. The report includes a diagram of [8]-gingerol that carries the caption ‘Chemical structure of gingerol’. However, since [8]-gingerol is not the major gingerol present in ginger, some uncertainty as to the exact identity of ‘gingerol’ remains. ‘Gingerol’ and indomethacin (a COX inhibitor) were found to significantly inhibit the arachidonic acid-induced formation of TxB2 and PGD2 in a concentration-dependent manner. In contrast, imidazole, which is a thromboxane synthase inhibitor, reduced TxB2 formation while causing a highly significant rise in PGD2 production. These findings are important, because they demonstrate that ‘gingerol’, like indomethacin, inhibits cyclooxygenase rather than thromboxane synthase. It was also demonstrated that the anti-platelet activity of
‘gingerol’ was due to the subsequent reduced production of TxA₂, a potent platelet aggregating agent.

Venkateshwarlu was the first to study the effects of ginger compounds on eicosanoid metabolism in whole blood (Venkateshwarlu, 1997). He showed that [6]- and [10]-gingerol isolated from fresh ginger dose-dependently inhibited the formation of TxB₂ in horse blood. Curcumin and another compound isolated from dried rhizomes of turmeric (Curcuma longa) as well as piperine isolated from dried pepper (Piper nigrum) fruits were also found to be active.

[8]-gingerol and [8]-gingerdiol were potent inhibitors of COX activity in RBL-2H3 cells with IC₅₀ values of 1.54 and 3.3 μM, respectively, which was similar to that of indomethacin (0.76 μM) (Koo et al., 2001). The inhibitory activity of the major pungent phenolic in fresh ginger, [6]-gingerol, was an order of magnitude weaker (50 μM) in this study.

Tjendraputra and co-workers showed that a number of naturally occurring gingerol-type compounds ([8]- and [10]-gingerol, [6]-shogaol, [8]-gingerdiol and [8]-paradol, as well as several synthetic analogues) inhibited COX-2 activity in cultured human airways epithelial A549 cells, with IC₅₀ values in the low micromolar range (Tjendraputra et al., 2001). Interestingly, [6]-gingerol did not inhibit COX-2 in this assay.

### 2.3.1.3.2.3 Animal studies

Sharma and coworkers examined the effects of a steam-distilled ginger oil on chronic adjuvant arthritis in rats (Sharma et al., 1994). The oil, which was obtained from a company in Germany, contained camphene, limonene, 1,8-cineole, linalool, citral and borneol. It is noticeable that three of the major constituents of most ginger oils, zingiberene, ar-curcumene, and β-sesquiphellandrene, were not listed as constituents of this oil. Rats in the verum group (n=8) were given ginger oil (33 mg/kg) orally once daily for 26 days, commencing the day prior to the induction of adjuvant arthritis. Ginger oil treatment produced significant suppression of knee joint swelling on day 2 after induction of oedema (p<0.05) and an even greater suppression of joint swelling on day 26 (p<0.001). In terms of paw swelling, a significant inhibitory effect was evident at 18-26 days (p<0.001). It is noteworthy that this study used a steam-distilled oil, which should lack gingerols and
shogaols. If the ginger preparation indeed was devoid of gingerols and shogaols, these findings indicate that steam-distilled ginger oil contains other compounds with potential anti-inflammatory activity.

In 1997, Sharma et al. re-published the findings of the study first published in 1994 (Sharma et al., 1997). Included were new data on plasma and tissue kallikrein (also known as kallidinogenase) levels in synovial tissue. The kallidinogenases are enzymes responsible for the formation of kinins from their precursors, kininogens and kallidinogens. Kinins have been implicated in many physiological functions, including the regulation of blood flow and blood pressure, allergy and inflammation. Increases in kinin formation or kinin release have been shown to occur in many inflammatory conditions. The presence of high levels of plasma kallidinogenase-like enzyme has previously been demonstrated in blood-free paw tissue from rats with adjuvant inflammation. The study found that raised levels of plasma kallidinogenase fell significantly in rats treated with ginger oil for 26 days (p<0.01). Ginger oil treatment produced no change in synovial tissue kallidinogenase levels. According to the authors, these findings suggest that ginger oil might possess anti-rheumatic and anti-inflammatory properties by inhibiting the kinin-forming kallidinogenase enzyme.

An aqueous preparation made from fresh ginger was given to rats in a study examining the ex vivo effects on TxB2 and PGE2 synthesis (Thomson et al., 2002). Administered orally or intraperitoneally at two doses (50 mg/kg and 500 mg/kg), the low dose significantly reduced serum PGE2 levels only when given orally, whereas the higher dose was effective with either route of administration. Serum TxB2 levels were reduced significantly only with oral administration of the higher dose.

[6]-Gingerol has been tested for anti-inflammatory activity against carrageenan-induced paw oedema in rats (Young et al., 2005). At doses of 50 mg/kg, 100 mg/kg and 250 mg/kg, [6]-gingerol was effective in reducing oedema; an ED50 value of 85 mg/kg suggested a considerable anti-inflammatory effect in this assay.

Penna and co-workers found that rat paw oedema induced by carrageenan, compound 48/80 (a synthetic mast cell-degranulating compound) and serotonin was significantly inhibited by intraperitoneal administration of a 70% aqueous ethanolic extract made from fresh ginger rhizomes (Penna et al., 2003). Local application of the extract also inhibited 48/80-induced (but not Substance P- and bradykinin-induced) skin oedema, and intraperitoneal
administration one hour prior to serotonin injection significantly reduced serotonin-induced skin oedema. It was concluded that the anti-oedematogenic activity of the ginger extract was at least in part due to serotonin receptor antagonism.

A study investigating the anti-tumour promoting effects of [6]-gingerol found that when applied topically to mouse skin stimulated with a tumour promoter, the compound inhibited COX-2 expression in vivo (Kim et al., 2005b). The suggested mechanism of this effect was blocking of the p38 mitogen-activated protein (MAP) kinase–nuclear factor kappa B (NFκB) signalling pathway (NFκB regulates COX-2 induction).

2.3.1.3.2.4 Summary: Effects on arachidonic acid metabolism and eicosanoids

There is a substantial body of evidence from in vitro studies suggesting that ginger extracts and a range of ginger constituents (including [6]-, [8]- and [10]-gingerol, and [6]-shogaol) inhibit cyclooxygenase (COX). This causes a reduction in the formation of prostanoids of the 2-series, including PGE₂, which has potent pro-inflammatory properties in certain circumstances, and TxA₂, which causes platelet aggregation and serotonin release, and contracts smooths vascular muscle. Some phenolic ginger constituents, including [8]- and [10]-gingerol and [6]-shogaol (but not [6]-gingerol), were relatively potent inhibitors of COX-2 in vitro.

Animal studies have confirmed the ability of ginger extracts to inhibit PGE₂, thromboxane and inflammation, and studies using essential oil suggest that compounds other than those of gingerol type possess anti-inflammatory activity. Unlike the finding from the in vitro study of COX-2 inhibition, [6]-gingerol was found to inhibit COX-2 when applied topically to mouse skin. In this instance COX-2 inhibition appeared to be caused by the blocking of a signalling pathway involving NFκB. Serotonin antagonism has also been suggested as a possible mechanism of anti-inflammatory action.

Thus the emerging picture of ginger as an anti-inflammatory agent is consistent with that of a typical phytomedicine: it contains multiple active compounds and as such it appears to have multiple pharmacological targets.
2.3.1.3.3 Effects on cytokines and chemokines

Cytokines are immunoregulatory proteins (such as interleukins, tumour necrosis factor, and interferons) secreted by cells, especially of the immune system. They provide communication between macrophages and various subsets of lymphocytes; all of them act in a non-specific manner to enhance the inflammatory response (McCance & Huether, 2002).

A hot extract of dried ginger in 50% aqueous ethanol was tested for its effects on cytokine secretion by human peripheral blood mononuclear cells (Chang et al., 1995). The secretion of IL-1β, IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) was increased in the presence of the ginger extract at low concentrations (10-30 mg/mL), but only after prolonged reaction time (18 or 24 hours); higher concentrations were less effective. There was no significant effect on tumour necrosis factor-alpha (TNF-α).

Chemokines are chemotactic molecules, at least 50 of which are known to play a role in inflammation. Joint chondrocytes and synoviocytes produce a range of chemokines, which cause migration of leukocytes into the inflamed tissue and mediate cartilage degradation by decreasing proteoglycan synthesis and activating matrix metalloproteinases (MMPs), which catalyse the breakdown of cartilage (Phan et al., 2005).

An extract prepared from dried ginger rhizomes, described as having a high (sic) content of [6]-, [8]- and [10]-gingerol, gingerdione and gingerdiol, was tested in vitro for its effects on the expression of two chemokines, macrophage chemotactic factor (MCP-1) and interferon-γ activated protein (IP-10), in human synoviocytes (Phan et al., 2005). These two chemokines have been shown to be involved in the pathogenesis of both osteoarthritis and rheumatoid arthritis by inducing the migration of leukocytes and monocytes from the blood to areas of inflammation. MCP-1 also has been found to induce MMPs. IP-10 is a chemoattractant for monocytes, natural killer cells and activated T-cell, in particular type 1 helper cells, causing upregulation of interferon-γ. The experiment used two cultivated synoviocyte cell lines obtained from patients with osteoarthritis during knee replacement surgery. Incubation with ginger extract inhibited the secretion of both MCP-1 and IP-10 (by 40% and 54%, respectively), but only in one of the two cell lines tested. Incidentally, the study demonstrated a synergistic effect when extracts of Zingiber officinale and Alpinia galanga (galangal) were combined.
These findings suggest that some aspects of ginger’s anti-inflammatory activity may be mediated by effects on cyto- and chemokines.

2.3.1.3.4 Anti-cancer activity

An ethanolic ginger extract applied topically to mouse skin provided a highly significant protective effect against the development of skin tumours, and this was associated with the inhibition of 12-0-tetradecanoylphorbol-13-acetate (TPA)-caused induction of epidermal ornithine decarboxylase, cyclooxygenase and lipoxygenase activities (Katiyar et al., 1996). A subsequent study showed [6]-gingerol to have similar activity (Park et al., 1998).

A more recent study showed that topical application of [6]-gingerol inhibited COX-2 expression in mouse skin stimulated with the tumour promoter TPA (Kim et al., 2005b). Results from this study suggested that the inhibition of COX-2 expression was the result of the blocking of the p38 MAP kinase- NFκB signalling pathway.

A cytotoxic or cytostatic effect mediated by apoptosis was found for [6]-gingerol and [6]-paradol in human promyelocytic leukaemia HL-60 cells (Lee & Surh, 1998), and also for four diarylheptanoids and two shogaols (Wei et al., 2005).

2.3.1.3.5 Anti-microbial activity

Ginger extracts have demonstrated antimicrobial activity against a wide range of pathogenic micro-organisms; these include both Gram-positive and Gram-negative bacteria and the yeast Candida albicans, but the antimicrobial activity of ginger extracts appears to be moderate in comparison with highly antimicrobial plant extracts.

Of particular interest is an in vitro study showing that a crude methanolic extract (MIC 6-50 µg/mL) and a gingerol-containing fraction (MIC 0.8-12.5 µg/mL) significantly inhibited the growth of 19 strains of Helicobacter pylori, the micro-organism associated with peptic ulcer disease as well as gastric and colon cancer (Mahady et al., 2003).
2.3.1.3.6 Immunomodulatory effects

Few studies have examined the potential immunomodulatory activity of ginger. Non-specific immunity was increased in rainbow trout eating a diet containing 1% of a dried aqueous ginger extract for three weeks (Dugenci et al., 2003). Mice fed a 50% ethanolic ginger extract (25 mg/kg) for seven days had higher haemagglutinating antibody titre and plaque-forming cell counts, consistent with improved humoral immunity (Puri et al., 2000).

One in vitro study found that ginger suppressed lymphocyte proliferation; this was mediated by decreases in IL-2 and IL-10 production (Wilasrusmee et al., 2002). Another study found an aqueous ginger extract significantly increased the production of IL-1β, IL-6 and TNF-α in activated peritoneal mouse macrophages (Ryu & Kim, 2004). The same study found that splenocyte proliferation and cytokine production were stimulated in a dose-dependant manner when mice were given an aqueous ginger extract for 4 weeks (50-500 mg/kg).

2.3.1.3.7 Gastrointestinal effects

An orally administered acetone extract of ginger (75 mg/kg) as well as [6]-shogaol (2.5 mg/kg) and [6]-, [8]- and [10]-gingerol (5 mg/kg) enhanced gastric motility (charcoal meal) in mice (Yamahara et al., 1990). Both acetone and 50% ethanolic extracts (100-500 mg/kg) and ginger juice (2-4 mL/kg) reversed cisplatin-induced delay in gastric emptying in rats when given orally (Sharma & Gupta, 1998). The effect on gastric motility may at least in part explain the anti-emetic properties of ginger.

Both ginger extracts and several ginger compounds including 6-gingerol have demonstrated ulcer-protective activity in rats (Agrawal et al., 2000; Sertie et al., 1992; Yamahara et al., 1988; Yoshikawa et al., 1992). Ginger stimulated bile secretion in rats, with [6]- and [10]-gingerol being chiefly responsible for this activity (Yamahara et al., 1985), and ginger has also been shown to stimulate intestinal lipase, trypsin, chymotrypsin, amylase, sucrase and maltase activity when fed to rats for an 8-week period (Platel & Srinivasan, 1996; Platel & Srinivasan, 2000). These findings support the traditional use of ginger as a digestive stimulant (Mills & Bone, 2000). Ginger may also increase the conversion of cholesterol to
bile acids by increasing the activity of hepatic cholesterol-7-α-hydroxylase, the rate-limiting enzyme of bile acid biosynthesis (Srinivasan & Sambaiah, 1991).

2.3.1.3.8 Metabolic effects

Significant hypoglycaemic activity in rabbits was produced by an ethanolic Soxhlet extract of ginger (Mascolo et al., 1989), and two animal studies have suggested that ginger may improve insulin sensitivity (Goyal & Kadnur, 2006; Kadnur & Goyal, 2005).

2.3.1.3.9 Anti-atherosclerotic effects

A number of animal studies have demonstrated hypocholesterolemic action of ginger and ginger extracts. These studies have shown decreased levels of total cholesterol, low-density lipoprotein (LDL)-, very low-density lipoprotein (VLDL)-cholesterol and triglycerides, and increases in high-density lipoprotein (HDL)-cholesterol (Ahmed & Sharma, 1997; Bhandari et al., 1998; Fuhrman et al., 2000; Giri et al., 1984; Murugaiah et al., 1999).

In a more recent study, air-dried ginger powder (100 mg/kg orally daily) fed to rabbits with experimentally induced atherosclerosis for 75 days inhibited atherosclerotic changes in the aorta and coronary arteries by about 50% (Verma et al., 2004). In this study the ginger treatment did not cause any significant lowering of serum lipids, but lipid peroxidation was decreased and fibrinolytic activity increased.

The effects of an aqueous ginger extract, administered orally or intraperitoneally to rats for 4 weeks, on serum cholesterol and triglyceride levels and on PGE₂ and TxB₂ have been reported (Thomson et al., 2002). At 500 mg/kg both modes of administration led to a significant reduction in serum cholesterol levels, whereas only intraperitoneal administration led to a reduction with a daily dose of 50 mg/kg. Neither dose caused significant changes to serum triglyceride levels. The higher dose administered orally significantly lowered serum PGE₂ and TxB₂ levels, and the lower dose also reduced PGE₂ levels.
It is evident from this that ginger has demonstrated considerable potential as an anti-atherosclerotic agent in animal studies, but as yet this promise has not been confirmed in human trials.

2.3.1.3.10 Other cardiovascular effects

Ginger extracts as well as [6]- and [8]-gingerol have been shown to modulate eicosanoid responses in smooth vascular muscles ex vivo (Hata et al., 1998; Kimura et al., 1989; Pancho et al., 1989).

[6]- and [8]-gingerol and related analogues inhibited arachidonic acid-induced serotonin release by human platelets in a dose range similar to the effective dose aspirin (IC$_{50}$ values between 45 and 83 μM), and the same compounds were also effective inhibitors of arachidonic acid-induced platelet aggregation (Koo et al., 2001). COX inhibition was demonstrated in RBL-2H3 cells, suggesting this might be the underlying mechanism.

An early study found a dose-dependent positive inotropic action of [6]-, [8]- and [10]-gingerol on isolated guinea pig left atria (Shoji et al., 1982), and ‘gingerol’ stimulated the Ca$^{2+}$-pumping ATPase activity of fragmented sarcoplasmic reticulum prepared from mammalian skeletal and cardiac muscle (Kobayashi et al., 1987).

[6]-Gingerol and [6]-shogaol lowered systemic blood pressure in anaesthetised rats at doses of 10-100 μg/kg and caused bradycardia when administered intravenously (Suekawa et al., 1984; Suekawa et al., 1986).

In a recent study a crude extract (70% aqueous methanol) of fresh ginger induced a dose-dependent fall in arterial blood pressure of anaesthetised rats; this effect was shown to be mediated through blockade of voltage-dependent calcium channels (Ghayur & Gilani, 2005).

2.3.1.3.11 Analgesic effects

[6]-gingerol had analgesic effects in mice in both the acid-induced writhing test and the formalin test, suggesting the analgesic activity resulted from peripheral and possible anti-inflammatory action (Young et al., 2005). [6]-shogaol has also been shown to inhibit acetic
acid-induced writhing in mice and to elevate the nociceptive threshold of the yeast-inflamed paw (Suekawa et al., 1984). Experiments carried out by Onogi and co-workers suggested that [6]-shogaol inhibits the release of Substance P by stimulation of the primary afferents from their central terminal and hence shares this site of action with capsaicin (Onogi et al., 1992).

### 2.3.1.3.12 Antipyretic activity

A Soxhlet extract of ginger in 80% ethanol reduced yeast-induced fever in rats by 38% when administered orally (100 mg/kg) (Mascolo et al., 1989). This was comparable to the antipyretic effect of acetylsalicylic acid at the same dose. The ginger extract did not affect the temperature of normothermic rats. This anti-pyretic activity may be mediated by COX inhibition.

### 2.3.1.4 Safety data

#### 2.3.1.4.1 Acute toxicity

A concentrated Soxhlet extract of ginger in 80% ethanol was well tolerated orally in mice at doses up to 2.5 g/kg, but doses of 3.0 and 3.5 g/kg caused 10-30% mortality. Death was caused by involuntary contractions of skeletal muscle; other symptoms included gastrointestinal spasm, hypothermia, diarrhoea and anorexia (Mascolo et al., 1989).

Another study found that a hydroethanolic ginger extract was nontoxic in mice when administered by oral gavage up to a dose of 1.5 g/kg body weight (Jagetia et al., 2004).

#### 2.3.1.4.2 Teratogenicity and embryotoxicity

Pregnant rats administered 20 g/L or 50 g/L ginger tea via their drinking water from gestation day 6 to 15 showed no gross malformation on fetuses (Wilkinson, 2000). However, embryonic loss in the ginger-treated group was twice that of controls. Surviving
fetuses in the ginger group were significantly heavier and showed more advanced skeletal development compared with controls.

A patented ginger extract was tested for teratogenic potential in pregnant rats (Weidner & Sigwart, 2001). The extract caused neither maternal nor developmental toxicity at daily doses of up to 1 g/kg body weight.

2.3.1.4.3 Mutagenicity

[6]-gingerol and to a far lesser extent [6]-shogaol were shown to have mutagenic properties in an assay using Escherichia coli Hs30 as an indicator strain of mutagenesis (Nakamura & Yamamoto, 1983). Despite this finding, ginger is not considered a mutagenic substance, presumably due to its long history of safe use.

2.3.1.5 Clinical studies of ginger

Data from clinical trials support the use of ginger and ginger preparations in motion sickness and seasickness, morning sickness, hyperemesis gravidarum, postoperative nausea and osteoarthritis, although not all clinical trials have produced positive outcomes. The clinical efficacy trials on ginger published prior to 2008 are summarised in Appendix A.

2.3.1.5.1 Clinical studies of ginger in arthritis

Few clinical studies have examined the efficacy of ginger in osteoarthritis. Preceding proper clinical trials, Srivatava and Mustafa published in 1989 a case series of 7 patients suffering from rheumatoid arthritis who had reported improvements in their condition following the consumption of ginger (Srivastava & Mustafa, 1989). The same investigators followed up these observations with a questionnaire-based survey of people who were self-medicating ginger for their condition (Srivastava & Mustafa, 1992). This survey provided data from a total of 56 patients, including the 7 people whose cases had been published in the first report. Of the 56 patients, 28 suffered from rheumatoid arthritis, 18 from osteoarthritis and 10 from
muscular discomfort. The majority of patients reported marked relief in symptoms of pain and swelling as a result of ginger consumption.

The results obtained by Srivastava and Mustafa in their survey were promising. However, the fact that the data came from an uncontrolled study with self-selected subjects meant that these results offered little in terms of proper evidence for the efficacy of ginger in arthritic and rheumatic conditions.

The first proper clinical trial to examine the usefulness of ginger in osteoarthritis was conducted by a Danish group and published in 2000 (Bliddal et al., 2000a). This randomised, placebo-controlled, cross-over study compared the effects of a standardised ginger extract with those of ibuprofen in osteoarthritis. Fifty-six out-patients (41 women, 15 men) completed the study. Of these, 36 had osteoarthritis of the knee and 20 osteoarthritis of the hip. Mean duration of osteoarthritis was 7.7 years (range 1-30 years). One week prior to randomisation, treatment with analgesics and NSAIDs was discontinued, but acetaminophen (paracetamol) was allowed as a rescue drug for pain relief throughout the study, up to a maximum dose of 3 g daily. Subjects were randomised to three treatment periods of three weeks each, during which they received (in different order) capsules containing either 170 mg of a standardised ginger extract t.i.d., ibuprofen 400 mg t.i.d., or placebo t.i.d. The ginger extract had a standardised content of hydroxy-methoxy-phenyl compounds, but no further details were provided, making it impossible to estimate the dose on a fresh or dried equivalent basis. There were no wash-out periods between the three treatments. The primary outcome variable was pain assessed using a visual analogue scale (VAS); secondary outcomes were the Lequesne-index (a questionnaire-based disability score) for either hip or knee, and range of motion.

The results produced a ranking in terms of efficacy on pain level and function with ibuprofen being more effective than the ginger and placebo. The same ranking applied to the consumption of the rescue medication, with ibuprofen treatment being associated with the lowest consumption of the rescue medication. No difference between the ginger extract and placebo was found in a test for multiple comparisons. However, explorative statistical testing of the first period of treatment (before cross-over) found a significantly better effect of both ginger and ibuprofen compared with placebo (p<0.05). This finding is particularly interesting, because the design of this cross-over study was marred by the lack of wash-out periods between treatments. The lack of wash-out periods and the short duration (three
weeks) of each treatment means that the trial carried out by Bliddal’s group failed to convincingly establish whether or not ginger might be useful in the treatment of osteoarthritis.

In 2003, Wigler and co-workers published the results of small clinical trial of ginger in osteoarthritis of the knee (Wigler et al., 2003). This was a randomised, double-blind, placebo-controlled study of 6 months’ duration. Twenty-nine subjects were divided into verum and placebo groups, which were crossed over after 12 weeks. The intervention was a ginger extract produced by supercritical carbon dioxide extraction and formulated into an enteric coated capsule containing 250 mg extract including 10 mg ‘gingerol’. The capsule was designed to release 20% of its content under the acidic conditions of the stomach and the remainder in the intestine. Subjects took one capsule (or identical placebo) four times daily (equivalent to 40 mg ‘gingerol’ daily). Primary outcome measures were pain on movement and handicap as assessed by subjects on a visual analogue scale based on the Western Ontario and McMaster Osteoarthritis Index (WOMAC). Both groups showed a significant decrease in both outcome measures after 12 weeks, but there was no significant difference between the groups. After 24 weeks, however, the difference between the ginger and placebo groups was highly significant for both outcomes in favour of the ginger intervention (p>0.01).

Although the study conducted by Wigler and colleagues also did not include a wash-out phase between treatments, the duration (12 weeks) of each treatment means that this was less likely to invalidate the findings. In fact, the group that started on the active treatment continued to improve for 2 weeks after being switched to placebo, suggesting the effects of the ginger treatment continued after the treatment was withdrawn.

A third larger trial (n=247), rather misleadingly published in 2001 under the title ‘Effects of a ginger extract on knee pain in patients with osteoarthritis’, is often cited as evidence for the efficacy of ginger in osteoarthritis of the knee (Altman & Marcussen, 2001). This assertion is not strictly valid, however, as the intervention employed in this trial was a patented extract containing not only *Zingiber officinale* but also *Alpinia galanga* (galangal). This randomised, double-blind, placebo-controlled, parallel-group, 6-week study found a statistically significant effect of the extract in reducing symptoms of osteoarthritis of the knee.
The reports of the only two randomised controlled trials of ginger extracts as the sole active in the treatment of osteoarthritis (Bliddal et al., 2000b; Wigler et al., 2003) were scored against the CONSORT (Consolidated Standards of Reporting Trials) (Moher et al., 2001) and the elaborated CONSORT for herbal interventions (Gagnier et al., 2006) (see Appendix B). Each item was given a score of 0, 0.5 or 1, depending on whether the information was provided not at all or to an unsatisfactory extent (0), to some extent (0.5), or to a satisfactory or mostly satisfactory extent (1) in the trial report. With total scores of 17.5 (Bliddal) and 17.0 (Wigler) out of a possible 27, the reporting of the two trials was of equal and reasonable quality, although neither provided qualitative chemical information (such as an HPLC chromatogram) about the intervention used, and the study by Bliddal and colleagues did not use the binomial for ginger to unambiguously define the material taxonomically.

The results of the only two randomised controlled trials of ginger in osteoarthritis are encouraging, if not conclusive. The results of the most recent trial suggest that treatment for up to 24 weeks is required for the full benefits to manifest. A large, rigorous trial with a chemically well-defined intervention is now warranted to more conclusively establish the role (if any) of ginger in the treatment of osteoarthritis.

2.3.2 Other Zingiber species

Other Zingiber species included in this work are reviewed below.

2.3.2.1 Thai ginger (Zingiber montanum)

Zingiber montanum (J. König) Theilade is more widely cited in the literature under the synonyms Z. cassumunar Roxb. and Z. purpureum Roscoe.

This species is used in the treatment of asthma in traditional Thai medicine and has been shown to have an anti-histamine effect in asthmatic individuals (500 mg orally) (Piromrat et al., 1986). A methanolic extract inhibited PGE2 production by in human promonocytic U937 cells (IC50 = 7.7 µg/mL) (Jiang et al., 2006b). The rhizome contains a number of phenylbutanoid compounds (Han et al., 2004; Jitoe et al., 1993; Lu et al., 2005; Masuda & Jitoe, 1995), some of which have been shown to have potent anti-inflammatory activity both
in vitro and in vivo through inhibition of COX and lipoxygenase (LOX) pathways (Jeenapongsa et al., 2003; Panthong et al., 1997), including inhibition of COX-2 in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells (Han et al., 2005). One phenylbutenoid dimer had anti-proliferative activity in several human cancer cell lines (Lee et al., 2007). Also present are curcuminooids (cassumunin A and B, cassumunarin A, B and C; Fig. 2-10) with potent anti-oxidant activity (Masuda & Jitoe, 1994; Nagano et al., 1997). The cassumunarins were also shown to have anti-inflammatory activity in vivo (Masuda & Jitoe, 1994). The sesquiterpene zerumbone has antifungal activity (Kishore & Dwivedi, 1992).

Extracts of Z. montanum have demonstrated in vitro antioxidant activity (Chirangini et al., 2004; Habsah et al., 2000a; Jitoe et al., 1992), anti-allergic activity (Tewtrakul & Subhadhirasakul, 2007) and anti-tumour promoter activity (Vimala et al., 1999). Extracts also inhibited P-glycoprotein in human uterine sarcoma cells (Go et al., 2004) and significantly inhibited CYP3A4 in human liver microsomes (Subehan et al., 2006). In animals, extracts have shown anti-inflammatory and analgesic effects (Ozaki et al., 1991; Pongprayoon et al., 1997).

![Chemical structures](https://example.com/structure.png)

**Fig. 2-10. Curcuminooids from Zingiber montanum.**
The essential oil, which primarily consists of monoterpenoids and phenylbutanoids (Taroeno et al., 1991), was active against a wide range of Gram-positive and Gram-negative bacteria, dermatophytes and yeasts (Pithayanukul et al., 2007).

2.3.2.2 *Zingiber ottensii*

*Zingiber ottensii* Valeton is another species native to South-east Asia. The rhizome is reportedly used to treat lumbago and is an ingredient in a sedative lotion used in the treatment of convulsions (Sirirugsa, 1999).

The rhizome contains a number of terpenoids and diarylheptanoids (Akiyama et al., 2006; Sirat, 1994). Zerumbone is the major constituent of the essential oil (Sirat & Nordin, 1994), which had moderate cytotoxicity in the brine shrimp assay (Thubthimthed et al., 2005).

Extracts of the rhizome have been found to possess antimicrobial (Azmi Muda et al., 2002; Mohtar et al., 1998) and anti-oxidant (Habsah et al., 2000b) activities. No other information about the pharmacological activity of this species was located.

2.3.2.3 Beehive ginger (*Zingiber spectabile*)

*Zingiber spectabile* Griff. is native to Thailand and the Malayan peninsula and is widely used as an ornamental in tropical and subtropical areas. An infusion of the leaves has been reported to have been used to treat infected eyelids (Sirirugsa, 1999).

The rhizome contains an essential oil reported to contain terpinen-4-ol (23.7%), labda-8 (17),12-diene-15,16-dial (24.3%), \(\alpha\)-terpineol (13.1%), and \(\beta\)-pinene (10.3%) as major constituents (Sirat & Leh, 2001). A methanolic extract inhibited PGE\(_2\) production in human promonocytic U937 cells (IC\(_{50}\) = 1.2 \(\mu\)g/mL) (Jiang et al., 2006b), and a solvent extract demonstrated antimicrobial activity (Ghosh et al., 2000). No other information pertaining to the chemistry and pharmacology of this species was located.
2.4 Genus *Curcuma* L.

The genus *Curcuma* contains approximately 40 mostly tropical Asian species. Best known is turmeric (*C. longa* L.), a cultigen of likely Indian origin, which is widely used as a spice and as an orange and yellow dye (Mabberley, 1997). Several other species are also used for culinary purposes including mango ginger (*C. amada* Roxb.), Bombay or Indian arrowroot (*C. angustifolia* Roxb.) and zedoary (*C. zedoaria* (Christm.) Roscoe, syn. *C. zerumbet*) (Mabberley, 1997).

In Australia, the genus is represented by a single native species, *C. australasica* J. D. Hook, which is sometimes, rather misleadingly, called Cape York lily, and finds use as an ornamental plant. Its natural habitat is shady rainforest margins in tropical parts of Queensland and the Northern Territory, and it also occurs in New Guinea (Smith, 1987).

More than a dozen species of *Curcuma* have been used in traditional systems of medicine (Johnson, 1999).

2.4.1 *Curcuma longa*

Turmeric (*C. longa* L., syn. *C. domestica* Val.) and its major active constituent curcumin have been the subject of hundreds of scientific studies, the majority of which have been published since 2000. The interest has focused on curcumin and its anti-oxidant, anti-carcinogenic and anti-inflammatory actions.

2.4.1.1 Chemistry

The major constituents of interest in turmeric are the coloured diarylheptanoids known as curcuminoids, which constitute around 5% of the dried rhizome. The major of these is the unsaturated β-diketone curcumin (diferuloylmethane), which together with desmethoxycurcumin and bisdesmethoxycurcumin make up 50-60% of the curcuminoids present in the rhizome (Fig. 2-11). Dihydrocurcumin has also been reported (Evans, 2002; WHO, 1999).
Turmeric also contains 5-6% volatile oil made up of mono- and sesquiterpenes including zingiberene, curcumene, α- and β-turmerone (Evans, 2002; WHO, 1999).

![Structural diagrams of the major curcuminoids in turmeric rhizome: curcumin, demethoxycurcumin and bisdemethoxycurcumin.](Fig. 2-11)

Curcumin exists in a pH-dependent equilibrium between its bis-keto and enolate forms. (After Higdon, 2007; Sharma et al., 2005).

### 2.4.1.2 Pharmacology

The biological and pharmacological activities of curcumin have been the subject of many reviews. These have included general reviews (Bengmark, 2006; Maheshwari et al., 2006; Sharma et al., 2005) and reviews focussing on anti-carcinogenic and chemopreventive
activities (Duvoix et al., 2005; Karunagaran et al., 2005; Leu & Maa, 2002; Lin, 2004; Narayan, 2004; Thangapazham et al., 2006), mechanism of action (Joe et al., 2004), and the potential role of curcumin in the treatment of Alzheimer’s disease (Ringman et al., 2005).

2.4.1.2.1 Pharmacokinetics of curcumin

In rodents, curcumin has low oral bioavailability and may undergo partial intestinal metabolism. Following absorption, curcumin undergoes extensive first-pass metabolism and excretion with the bile (Sharma et al., 2005). The major curcumin metabolites in suspensions of both human and rat hepatocytes were hexahydrocurcumin and hexahydrocurcuminol and these were less potent inhibitors of PGE2 production in human colonic epithelial cells than curcumin itself (Ireson et al., 2001).

In humans, curcumin also has low oral bioavailability. In one study, a dose of up to 200 mg failed to produce serum levels detectable at the level of detection (0.63 ng/mL) (Ruffin et al., 2003). Administration of 2 g turmeric powder to fasting volunteers resulted in curcumin plasma concentrations below 10 ng/mL, but this was increased 20-fold when piperine (the pungent alkaloid from Piper species) was co-administered (Shoba et al., 1998). Curcumin sulfate and curcumin glucuronide have been identified as metabolites in human urine (Sharma et al., 2004) and in intestinal tissues of patients with colorectal cancer (Garcea et al., 2005).

2.4.1.2.2 Pharmacodynamics of curcumin

2.4.1.2.2.1 Anti-oxidant activity

Numerous studies have shown curcumin to possess potent anti-oxidant activity both in vitro and in vivo. Mechanistic studies have found that curcumin is a phenolic chain-breaking anti-oxidant, which donates hydrogen atoms from its phenolic groups (Barclay et al., 2000; Priyadarsini et al., 2003; Sun et al., 2002). The resulting phenoxy radical can be repaired by water-soluble anti-oxidants such as vitamin C (Jovanovic et al., 2001).
Curcumin can act directly as a scavenger of free radicals such as singlet oxygen (Das & Das, 2002), superoxide (Biswas et al., 2005; Mishra et al., 2004) and hydroxyl radical (Biswas et al., 2005), but also exerts its anti-oxidant activity by enhancing endogenous defenses against oxidative damage. Cultured alveolar epithelial cells (A549) showed increased glutathione expression compared with controls (Biswas et al., 2005), and mice receiving dietary curcumin (2%) had significantly increased activities of glutathione peroxidase, glutathione reductase, glucose 6-phosphate dehydrogenase and catalase in the kidneys compared with controls (Iqbal et al., 2003). This induction of detoxification enzymes may play a role in the chemopreventive effects of curcumin.

The anti-oxidant activity of turmeric and curcumin has been demonstrated in a variety of in vitro assay systems, including oxygen radical absorbance capacity (ORAC), radical scavenging assays using 1,1-diphenyl-2-picryl hydrazyl (DPPH) and 2,2'-azobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), the ferric reducing antioxidant power (FRAP) assay (Tilak et al., 2004), and the Trolox equivalent antioxidant capacity (TEAC) assay (Betancor-Fernandez et al., 2003).

Radical scavenging and anti-oxidant activities have been confirmed in vivo in a variety of animal models. Some of these studies are briefly summarised here.

Curcumin was shown to protect rat brain homogenate against lipid peroxidation caused by lead and cadmium (Daniel et al., 2004), and pre-treatment with curcumin significantly reduced or abolished cadmium-induced hepatic oxidative damage in rodents (Eybl et al., 2004; Eybl et al., 2006). Curcumin protected the rat forebrain against cerebral ischaemia/reperfusion oxidative injury (Ghoneim et al., 2002).

A hydroethanolic turmeric extract fed to rabbits (1.66 mg/kg) receiving a high-fat, atherogenic diet for 30 days significantly reduced the oxidative damage to erythrocyte and liver microsome membranes (Mesa et al., 2003), and dietary curcumin (0.2%) reduced lipid peroxidation and the depletion of intracellular anti-oxidants (thiols and glutathione) in erythrocytes of rats fed a high-fat diet for 8 weeks (Kempaiah & Srinivasan, 2004). Mice fed a turmeric extract (1% of diet) for just one week had reduced levels of erythrocyte phospholipid hydroperoxides and liver triacylglycerol levels about half those of controls (Asai et al., 1999).
Curcumin (200 mg/kg) significantly reduced lipid peroxidation in chronic streptozotocin-diabetic rats, but had no significant effect on superoxide dismutase, catalase, or reduced glutathione levels (Majithiya & Balaraman, 2005). The same curcumin dose administered for 8 weeks to diabetic rats had a preventive effects on the cross-linking of collagen and the formation of advanced glycation end products, a process linked to oxidative stress (Sajithlal et al., 1998). Low dietary levels of curcumin (0.002% and 0.01%) delayed the progression of diabetic cataract in the lens of diabetic rats (Kumar et al., 2005).

Rats subjected to oxidative stress caused by dietary ethanol or oxidised polyunsaturated fatty acids had elevated liver marker enzymes and lipid peroxidative indices, but curcumin abrogated this effect, suggesting it potentially could provide some protection against alcoholic liver disease (Rukkanmani et al., 2004). Lipid peroxidation was significantly modulated and anti-oxidant status improved in rats suffering lung toxicity caused by subcutaneous injection of nicotine (Kalpana & Menon, 2004).

Curcumin has also been shown to protect rodents against drug-induced oxidative organ injury. Gentamycin-induced renal oxidative damage in rats (Farombi & Ekor, 2006), nephro- and cardiotoxicity in rats caused by adriamycin (Venkatesan, 1998; Venkatesan et al., 2000), and inflammatory and oxidative lung injury caused by bleomycin in rats (Venkatesan et al., 1997) were all reduced by curcumin. Curcumin also reduced cisplatin-induced clastogenesis in rats through it radical scavenging activity (Antunes et al., 2000), but did not protect against cisplatin-induced lipid peroxidation and nephrotoxicity (Antunes et al., 2001).

Several studies have found that curcumin can also promote the generation of reactive oxygen species (ROS) under some circumstances (Ahsan et al., 1999; Atsumi et al., 2005a; Atsumi et al., 2005b). One such study found that in human myeloid leukemia (HL-60) cells, low curcumin concentration reduced ROS generation, while high concentrations promoted it (Chen et al., 2005). A recent study using myelomonocytic U937 cells found that the anti-oxidative action of curcumin was preceded by a time and dose dependent oxidative stimulus and suggested that excessive curcumin concentrations may be harmful to cells (Strasser et al., 2005).
In a placebo-controlled study involving 20 subjects with tropical pancreatitis, 500 mg of curcumin (with 5 mg piperine to enhance the bioavailability of curcumin) reduced markers of erythrocyte lipid peroxidation, but did not improve pain (Durgaprasad et al., 2005).

2.4.1.2.2 Anti-inflammatory activity

Numerous studies have indicated that the key to the anti-inflammatory activity of curcumin is its ability to inhibit the activation of the transcription factor NFκB (Brennan & O'Neiill, 1998; Funk et al., 2006; Jobin et al., 1999; Jung et al., 2006; Kang et al., 2004; Kumar et al., 1998; Moon et al., 2005; Singh & Aggarwal, 1995; Strasser et al., 2005). NFκB plays a critical role in the transcriptional regulation of pro-inflammatory gene expression in various cells. Jobin and colleagues demonstrated that curcumin blocks a signal upstream of NFκB-inducing kinase and IκB kinase, both of which are involved in the activation of NFκB itself (Jobin et al., 1999).

Curcumin has also been found to inhibit the production of inflammatory cytokines involved in NFκB activation. In human peripheral blood monocytes and alveolar macrophages stimulated with phorbol ester or lipopolysaccharide (LPS), curcumin inhibited IL-8, monocyte inflammatory protein-1 (MIP-1α), monocyte chemotactic protein-1 (MCP-1), IL-1β, and TNF-α (Abe et al., 1999). At a concentration of 5 μM, curcumin inhibited LPS-induced production of TNF and IL-1 by a human monocytic macrophage cell line (Chan, 1995), and in rats with adjuvant inflammation curcumin reduced levels of IL-1β and C-reactive protein (Banerjee et al., 2003). Curcumin also inhibited IL-1β-mediated intracellular adhesion molecule-1 (ICAM-1) and IL-8 gene expression in several epithelial intestinal cell lines, resulting in the inhibition of NFκB activation (Jobin et al., 1999).

Crude organic turmeric extracts inhibited LPS-induced TNF-α and PGE₂ production in HL-60 cells with IC₅₀ values of 15.2 μg/mL and 0.92 μg/mL, respectively (Lantz et al., 2005).

An important aspect of the inflammatory response is the migration of endothelial cells from the vascular system into the tissues, following their recruitment of leukocytes. Treatment of endothelial cells with TNF caused monocytes to adhere as a result of NFκB-dependent expression of ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (ELAM-1); pre-treatment with curcumin completely blocked
adhesion (Kumar et al., 1998). These findings suggest that inhibition of leukocyte recruitment by endothelial cells (as a consequence of NFκB inhibition) may contribute to the anti-inflammatory activity of curcumin.

The inhibition of NFκB activation also leads to subsequent reduction in the expression of COX-2 and other inflammatory mediators whose genes are regulated by NFκB, and several studies have shown curcumin and turmeric to reduce PGE₂ levels (Funk et al., 2006; Hong et al., 2004; Huang et al., 1991; Ireson et al., 2001; Lantz et al., 2005). Although one study reported the direct inhibition of prostaglandin E synthase-1 and COX-2 (Moon et al., 2005), other studies have suggested that PGE₂ levels are reduced not through inhibition of COX catalytic activity, but as a result of upstream inhibition of COX-2 expression (Funk et al., 2006; Hong et al., 2004). Curcumin strongly inhibited COX-2 mRNA and protein expression in UVB-irradiated keratinocytes; the mechanism appeared to involve the suppression of p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) activities (Cho et al., 2005).

In addition to inhibiting COX-2 expression, curcumin also affects arachidonic acid metabolism by blocking the phosphorylation and thus the activation of PLA₂, the enzyme controlling the release of arachidonic acid from membranes, and by inhibiting the activity of 5-LOX (Hong et al., 2004).

Curcumin inhibits DNA binding of another transcription factor, activator protein-1 (AP-1), which together with NFκB controls a key mediator of coagulation, endothelial tissue factor (Bierhaus et al., 1997). Curcumin also inhibited DNA binding of AP-1 in microglial cells (Kang et al., 2004), but did not reduce activation of AP-1 in mice suffering non-alcoholic steatohepatitis, although NFκB activation was prevented (Leclercq et al., 2004).

Curcumin also inhibits the expression of nitric oxide (NO), which along with its derivatives acts as an inflammatory mediator. The derivatives nitrite and peroxynitrite are capable of damaging DNA and are therefore carcinogenic. The inhibition of nitric oxide production by stimulated RAW 264.7 macrophages when cells were treated with curcumin (10 μM) was shown to result from reduced expression of inducible nitric oxide synthase (iNOS) (Brouet & Ohshima, 1995). Similar inhibition of nitric oxide production has been observed in mouse peritoneal cells (Chan et al., 1995), while inhibition of both NO production and expression of iNOS was demonstrated in rat primary microglial cells (Jung et al., 2006). In the latter
study, several signaling pathways (incl. NFκB, p38 MAPK and JNK) were found to be involved in curcumin’s inhibition of NO, suggesting that the compound has multiple molecular targets.

In an *in vivo* study with two oral treatments of curcumin (92 ng/g body weight), the expression of hepatic iNOS RNA was reduced by 50-70% in mice injected with LPS (Chan *et al.*, 1998). Interestingly, inhibition was seen only when the curcumin was administered after fasting, suggesting that concurrent food intake interfered with the bioavailability of curcumin. This study is also important because it showed that curcumin can exert important pharmacological activity when administered orally in very low doses, at least in mice.

In rodent brain microglial cells, curcumin reduced the inflammatory response (induction of COX-2 and iNOS in response to stimuli) by inhibiting Janus kinase-STAT signalling (Kim *et al.*, 2003).

### 2.4.1.2.2.3 Anti-carcinogenic and anti-tumour activity

The chemopreventive and anti-tumour properties of curcumin (and turmeric) have been the subject of numerous studies and, as previously mentioned, a number of reviews (Duvoix *et al.*, 2005; Karunagaran *et al.*, 2005; Leu & Maa, 2002; Lin, 2004; Narayan, 2004; Thangapazham *et al.*, 2006).

Anti-tumour activity of curcumin has been demonstrated in many human cancer cell lines, including colorectal (Goel *et al.*, 2001; Lev-Ari *et al.*, 2006), renal (Jung *et al.*, 2005), lung (Lee *et al.*, 2005a; Shishodia *et al.*, 2003), oral (Sharma *et al.*, 2006), breast epithelial (Lee *et al.*, 2005b), pancreatic (Li *et al.*, 2005) and bladder cells (Park *et al.*, 2006). This anti-tumour activity has been confirmed in several animal studies (Pal *et al.*, 2005; Rao *et al.*, 1995; Volate *et al.*, 2005).

Few human studies have been conducted in this area; those that have been carried out have mostly been small and explorative in nature. Colorectal cancer patients who ingested 3.6 g curcumin daily for 7 days had significantly fewer nucleotide adducts in malignant tissue (p<0.05) but unchanged levels of COX-2 (Garcea *et al.*, 2005). Both an ethanolic turmeric extract and a curcumin-containing ointment was reported to have provided remarkable symptomatic relief (smell, itch) in 62 patients with external cancers (Kuttan *et al.*, 1987).
Numerous studies on curcumin suggest that it exerts its anti-cancer activities through a variety of mechanisms at the initiation, promotion and progression stages of carcinogenesis (Chauhan, 2002; Kawamori et al., 1999).

A major mechanism appears to be the induction of apoptosis (Anto et al., 2002; Atsumi et al., 2005b; Divya & Pillai, 2006; Li et al., 2005). This occurs via the mitochondrial pathway, where curcumin reduces mitochondrial membrane potential (Banjerdpongchai & Wilairat, 2005; Karunagaran et al., 2005; Volate et al., 2005). Curcumin-induced apoptosis in human renal cancer cells was found to be associated with upregulation of death receptor 5 expression (Jung et al., 2005), and in human colon cancer cell lines the induction of apoptosis by curcumin was accompanied by down-regulation of COX-2 and reduced PGE$_2$ synthesis (Lev-Ari et al., 2006).

Elevated levels of COX-2 are commonly seen in various types of malignant cells, and COX-2 is therefore recognized as a molecular target in chemoprevention (Lee et al., 2005b). COX-2 has also been implicated in tumour growth and invasiveness (Li et al., 2005). In particular, COX-2 is selectively over-expressed in colon tumours and believed to play an important role in colon carcinogenesis (Plummer et al., 1999). Many studies have shown that curcumin down-regulates COX-2 (and subsequent PGE$_2$ production) in cancer cells (Atsumi et al., 2005b; Divya & Pillai, 2006; Goel et al., 2001; Lee et al., 2005a; Lee et al., 2005b; Lev-Ari et al., 2006; Plummer et al., 1999; Sharma et al., 2006; Tunstall et al., 2006; Yoysungnoen et al., 2006; Zhang et al., 1999). As outlined in the review of curcumin’s anti-inflammatory activity above, it appears that the inhibition of the transcription factor NFkB is the central mechanism by which curcumin inhibits the expression of COX-2 in cancer cells (Divya & Pillai, 2006; Lee et al., 2005a; Lee et al., 2005b; Li et al., 2005; Plummer et al., 1999; Sharma et al., 2006; Shishodia et al., 2003).

Curcumin has also demonstrated other anti-cancer mechanisms. These include protection of DNA against genotoxic agents (Polasa et al., 2004), cytotoxicity (Chen et al., 1999; Divya & Pillai, 2006; Su et al., 2006), causing cell cycle arrest (at G2/M) (Chauhan, 2002; Chen et al., 1999; Park et al., 2006), inhibition of viral oncogenes (Divya & Pillai, 2006), inhibition of tumour angiogenesis (Li et al., 2005; Yoysungnoen et al., 2006), production of reactive oxygen species (Atsumi et al., 2005a; Banjerdpongchai & Wilairat, 2005), and the induction of detoxification enzymes (glutathione S-transferases) (Piper et al., 1998; Singhal et al., 1999).
2.4.1.2.2.4 Other pharmacological activities

Curcumin has demonstrated a range of other pharmacological activities. These include immunological effects (Kang et al., 1999; Kobayashi et al., 1997; Ranjan et al., 1998; Youn et al., 2006), anti-diabetic effects (Mahesh et al., 2005; Sharma et al., 2006), and hepatoprotective action (Kang et al., 2002; Nanji et al., 2003; Park et al., 2000; Sreepriya & Bali, 2006; Sugiyama et al., 2006; Xu et al., 2003).

Histamine release by leukemia cells was markedly decreased by both curcumin and tetrahydrocurcumin (Suzuki et al., 2005). Curcumin is a potent inhibitor of Kv1.4 K⁺ channels (Liu et al., 2006) and inhibits arachidonic acid- and PAF-induced platelet aggregation and platelet TxA₂ synthesis (Shah et al., 1999). Curcumin reversed endothelial dysfunction caused by homocysteine (Ramaswami et al., 2004) and had anti-atherogenic effects in animals (Olszanecki et al., 2005; Ramirez-Tortosa et al., 1999). Curcumin had anti-fibrotic action in rats with glomerular disease (Gaedeke et al., 2005) and inhibited or reduced a range of inflammatory molecules and signals (incl. NFκB, AP-1, IL-6, TNF-α and iNOS) in rat models of both ethanol and non-ethanol pancreatitis (Gukovsky et al., 2003).

Dietary curcumin (0.002%) protected rats against galactose-induced cataract formation (Suryanarayana et al., 2003), and curcumin had potent anti-ulcer effects in a rat model by preventing glutathione depletion, lipid peroxidation, and protein oxidation (Swarnakar et al., 2005).

Curcumin improved wound healing following gamma-irradiation (Jagetia & Rajanikant, 2004) and also facilitated wound healing in diabetic rodents (Sidhu et al., 1999).

Curcumin has produced positive results in animal models of inflammatory bowel disease; this effect is associated with the inhibition of NFκB (Jian et al., 2005; Jiang et al., 2006a; Salh et al., 2003; Ukil et al., 2003; Zhang et al., 2006). A small pilot study with patients with ulcerative proctitis or Crohn’s disease has yielded promising results (Holt et al., 2005).

Several *in vitro* and *in vivo* studies have identified curcumin as a promising agent for the prevention and treatment of Alzheimer’s disease (Baum & Ng, 2004; Lim et al., 2001; Ono et al., 2004; Yang et al., 2005). Curcumin has also been proposed as an agent worth investigating for the treatment of cystic fibrosis (Davis & Drumm, 2004), but the interest in pursuing this appears to have waned following further studies (Mall & Kunzelmann, 2005; Song et al., 2004).
2.4.2 Curcuma parviflora

*Curcuma parviflora* Wallich is native to Thailand, where it has been used in the topical treatment of cuts (Sirirugsa, 1999) and for the detoxification of scorpion bites (Takahashi *et al.*, 2003). It has been shown to contain a series of dimeric sesquiterpenes, parviflorenes A-H with cytotoxic properties (Takahashi *et al.*, 2003; Toume *et al.*, 2004; Toume *et al.*, 2005). Parviflorene F (Fig. 2-12) induced apoptosis in HeLa cells (Ohtsuki *et al.*, 2008).

![Parviflorene A and F from Curcuma parviflora.](image)

2.4.3 Other Curcuma species

No information regarding the chemistry or pharmacology was identified for the other two *Curcuma* species included in this work, *C. australasica* J. D. Hook and *C. cordata* Wallich.

Several other species of *Curcuma* are used for medicinal purposes, particularly in Asia. These include Javanese turmeric *C. xanthorrhiza* Roxb. (included in the *British Pharmacopoeia* (Anonymous, 2007), *C. kwangsiensis* S. G. Lee & C. F. Liang, *C.*
phaeocaulis Valeton, *C. wenyujin* Y. H. Chen & C. Ling (all three are included in the *Pharmacopoeia of the People’s Republic of China* (Anonymous, 2005), and *C. zedoaria* Roscoe (included in the *Japanese Pharmacopoeia* (Anonymous, 2001)).

However, since these species were included in the present work, they are not reviewed here.

2.5 Genus *Boesenbergia* Kuntze

The genus *Boesenbergia* comprises about 30 species and is distributed from India to New Guinea (Mabberley, 1997). There are no native or naturalised members of this genus in Australia.

Fingerroot (*Boesenbergia rotunda* (L.) Mansf. (syn. *Boesenbergia pandurata* Schltr., *Kaempferia pandurata* Roxb., *Gastrochilus panduratus* (Roxb.) Ridl.) is used as a culinary and medicinal herb in Southeast Asia. Fresh rhizomes are slightly pungent and have a characteristic aroma. They are used for the treatment of colic, fungal infections, dry cough, rheumatism and muscular pains, and for their reputed aphrodisiac properties (bin Jantan et al., 2001; Murakami et al., 1993; Trakoontivakorn et al., 2001).

*Boesenbergia rotunda* was included in the present work.

2.5.1 Chemistry

A range of flavonoids have been identified in the rhizome of *B. rotunda*, notably chalcones (including boesenbergin A and B [Fig. 2-13], panduratin A, cardamomin and dihydromethoxychalcone) and flavanones (including pinostrobin, pinocembrin, alpinetin and 5-hydroxy-7-methoxyflavanone) (Murakami et al., 1993; Trakoontivakorn et al., 2001). A chalcone derivative, 4-hydroxypanduratin A, has also been identified (Trakoontivakorn et al., 2001).

Steam distilled rhizome oils from *B. rotunda* rhizomes from Malaysia, Indonesia and Thailand showed considerable qualitative and quantitative differences in composition (bin
The major volatile oil constituents identified were camphor (16.1-32.1%), geraniol (16.2-26.0%), (E)-\( \beta \)-ocimene (19.0-23.7%) and 1,8-cineole (7.5-13.9%).

![Boesenbergin A and B](image)

**Fig. 2-13. Boesenbergin A and B from Boesenbergia rotunda.**

### 2.5.2 Pharmacology

Fingerroot has attracted attention as a potential chemopreventive agent. Both chalcones and flavanones from this species have demonstrated anti-mutagenic activity against mutagenic heterocyclic amines in *Salmonella typhimurium* TA98 assays (Trakoontivakorn *et al.*, 2001).

An ethanolic extract of rhizomes had potent inhibitory activity (similar to that of turmeric, *Curcuma longa*) against human HT-29 colon cancer and MCF-7 breast cancer cell lines (Kirana *et al.*, 2003).

A methanolic extract of fresh *B. rotunda* rhizomes showed strong inhibition of tumour promoter-induced Epstein-Barr virus activation (Murakami *et al.*, 1993). This *in vitro* assay is used to screen for agents with possible anti-tumour promoting properties. The chalcone cardamonin was isolated and identified as having potent inhibitory activity in this assay (IC\(_{50}\)
= 3.1 μM). Panduratin A has also been shown to induce apoptosis in human colon cancer HT-29 cells (Yun et al., 2005).

These in vitro findings are at odds with a medium-term bioassay of hepatocarcinogenesis in rats (Tiwawech et al., 2000). This study found that the administration of B. rotunda dried rhizome, combined with a hepatocarcinogenic heterocyclic amine found in cooked meats, resulted in an increase in the genesis of preneoplastic liver cell foci, suggestive of a possible cancer promoting effect.

Panduratin A isolated from B. rotunda potently and dose-dependently inhibited nitric oxide (IC$_{50}$ = 0.175 μM) and PGE$_2$ (IC$_{50}$ = 0.0195 μM) production in RAW264.7 cells, suppressed the expression of iNOS and COX-2 and reduced NFκB transcriptional activity without significant cytotoxicity (Yun et al., 2003).

### 2.6 Genus *Hedychium* Koenig

The genus *Hedychium* comprises approximately 50 species distributed from the Himalayas to New Guinea. The genus is also possibly native to Madagascar. Many species, including *H. coronarium* Koenig, are cultivated as ornamental plants and often called ginger lilies (Mabberley, 1997). There are no *Hedychium* species native to Australia, but two species, *H. coronarium* and *H. gardnerianum* Sheppard ex Ker-Gawler, have become naturalised (Smith, 1987).

Several species of *Hedychium* have been reported to have ethnobotanical uses including *H. coronarium*, which has been employed for rhinitis, tonsillitis, halitosis, swelling and tumours (Johnson, 1999). In Mauritius, the rhizomes of *H. coronarium* and *H. flavescens* are both used in traditional medicine as topical applications for rheumatism (Gurib-Fakim et al., 1997). *Hedychium* species are not used for culinary purposes (Omata et al., 1991).

The ground rhizome of *H. spicatum* Koenig is the major component of *abir*, a scented powder used in Hindu ceremonies (Mabberley, 1997). This species has also been used medicinally. The rhizome has been employed as a stomachic, carminative and stimulant, whereas the root and stalk are reported to have analgesic and anti-inflammatory actions, having been used in liver, urinary and respiratory disorders (Tandan et al., 1997).
Only *Hedychium coronarium* was included in the present work.

### 2.6.1 Chemistry

The steam-distilled volatile oil from the rhizome of *H. gardnerianum* was found to contain 67% monoterpenes and 31% sesquiterpenes, including 18% cadinane derivatives (Weyerstahl *et al.*, 1998). The major constituents of the oil were $\beta$-pinene (24.5%), $\alpha$-pinene (21.5%), $p$-cymene (6.2%), $\alpha$-cadinol (4.9%), 10-epi-$\alpha$-cadinol (2.4%), ar-curcumene (2.3%) and linalool (2.1%).

Rhizome oil of *H. coronarium* from India was found to contain $\alpha$- and $\beta$-pinene, limonene, $\delta$-3-carene, $\beta$-phellandrene, $p$-cymene, 1,8-cineole, $\beta$-caryophyllene, $\beta$-caryophyllene oxide, linalool and elemol in varying quantities (Dixit *et al.*, 1990). In addition to these constituents, the following compounds also occur in the oil: myrcene, $\alpha$-phellandrene, borneol, methyl salicylate and eugenol (Omata *et al.*, 1991).

Steam-distilled essential oil of fresh *H. coronarium* rhizome from Tahiti, to where the species has been introduced from Indo-China, contained 1,8-cineole (40.2%), $\beta$-pinene (24.8%), $\alpha$-pinene (7.8%) and $\alpha$-terpineol (5.4%) as major constituents (Lechat-Vahirua *et al.*, 1993).

In a study related to the present work steam-distilled oil from rhizomes of *H. coronarium* grown in Mauritius was characterised by $\alpha$-muurolol (17%), $\alpha$-terpineol (16%) and 1,8-cineole (11%) (Gurib-Fakim *et al.*, 2002). Seven labdane diterpenes have also been isolated from the rhizome of *H. coronarium* (Nakatani *et al.*, 1994).

### 2.6.2 Pharmacology

Anthelmintic properties *in vitro* (Dixit & Varma, 1975) and fungitoxicity against *Aspergillus flavus* (Singh *et al.*, 1984) have been reported for the essential oil of *H. coronarium*. The ethanolic extract of the rhizome of this species does not appear to have been studied
previously, but in vivo analgesic and anti-inflammatory effects have been reported for an ethanolic extract of *H. spicatum* (Tandan et al., 1997).

### 2.7 Genus *Kaempferia* L.

The genus *Kaempferia* contains approximately 50 species ranging from India to Southern China and Malaysia. Among these, *K. rotunda* L. and in particular *K. galanga* L. are used for medicinal and culinary purposes (Johnson, 1999; Mabberley, 1997).

The rhizome of lesser galangal (*K. galanga*) (also known as East Indian galangal, kacholam, kentjur, chekur, and sometimes, misleadingly, simply ‘galangal’) is used medicinally and cultivated in several countries, including India, China, Vietnam, Indonesia and the Sudan (Luger et al., 1996; Vimala et al., 1999). It is considered to be warming, promoting vital energy circulation, and is used in the treatment of complaints caused by cold, including headache, dyspepsia, vomiting, diarrhoea, toothache, and abdominal and pectoral pain, while a liniment applied topically through massage is used in rheumatism (Luger et al., 1996).

It should be noted that the binomial *Kaempferia pandurata* is a synonym for *Boesenbergia rotunda* (refer to Section 2.4 above).

*Kaempferia galanga* and *K. rotunda* were included in the present work.

#### 2.7.1 Chemistry

The rhizome of *K. galanga* contains a volatile oil with ethyl-*p*-methoxy-*E*-cinnamate as a constituent (Luger et al., 1996). Other constituents reported from the rhizome of this species are ethyl cinnamate, cinnamaldehyde, camphene, *l*-Δ⁴-carene, borneol, *p*-methoxystyrene and pentadecane (Noro et al., 1983).

#### 2.7.2 Pharmacology

Several studies have investigated the biological activity of *K. galanga*. An ethanolic extract exhibited moderate activity in a cell assay of anti-tumour promoter activity (inhibition of 12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced Epstein-Barr virus early antigen (EBV-
EA) in Raji cells) (Vimala et al., 1999). Another study showed moderate inhibition of tumour promoter-induced Epstein-Barr virus activation (Murakami et al., 1993). This is a similar in vitro assay, which like the first is used to screen for agents with possible anti-tumour promoting properties.

A methanolic extract of K. galanga rhizomes showed in vitro cytotoxic activity against HeLa cells, with an IC$_{50}$ value of 80 $\mu$g/mL (Kosuge et al., 1985). Ethyl-p-methoxy-E-cinnamate was isolated from this extract and identified as a cytotoxic compound, with an IC$_{50}$ value of 35 $\mu$g/mL.

Ethyl-p-methoxy-trans-cinnamate isolated from K. galanga rhizomes has also been shown to inhibit monoamine oxidase in vitro, with an IC$_{50}$ value of $6.8 \times 10^{-5}$ M (Noro et al., 1983).

### 2.8 Genus Scaphochlamys Baker

The genus Scaphochlamys includes 30 species, which occur in India, Southeast Asia and New Guinea (Mabberley, 1997).

Scaphochlamys biloba and S. kunstleri were included in the present work.

No information about the ethnobotany, chemistry or pharmacology of this genus was located.

### 2.9 Genus Alpinia Roxburgh

The genus Alpinia comprises some 200 species distributed mainly in Asia and the Pacific (Mabberley, 1997). Five species of Alpinia are indigenous to Australia: A. arctiflora (F. Muell.) Benth., A. arundelliana (F. M. Bailey) Schumann, A. caerulea (R. Br.) Benth. (syn. A. coerulea Benth.), A. hylandii R. M. Smith and A. modesta Schumann (Hnatiuk, 1990). The species formerly known as A. racemigera F. Muell. is now named Pleuranthodium racemigerum (F. Muell.) R. M. Smith (refer to Section 2.10). Both A. caerulea (commonly known as ‘native ginger’ in eastern Australia) and A. arundelliana grow in rainforest and wet sclerophyll forest in coastal New South Wales, north from the Central Coast, and in
Queensland, while the other three species are restricted to north-eastern Queensland (Hnatiuk, 1990; Wilson & Hastings, 1993).

Many members of the genus are used as spices and/or as medicinal plants in indigenous systems of traditional medicine around the world. This is particularly the case in China, India and South-East Asia, but medicinal use of Alpinia species has also been reported from Saudi Arabia and Brazil (Tewari et al., 1999). Galangal (greater galangal), A. galanga (L.) Swartz is widely cultivated in Southeast Asia, where it is used as a spice and a traditional medicine (Kubota et al., 1998; Someya et al., 2001).

The following Alpinia taxa were included in the present work: A. arctiflora (F. Muell.) Benth., A. caerulea (R. Br.) Benth., A. calcarata Rosc., A. galanga (L.) Swartz, A. luteocarpa Elmer, A. malaccensis Rosc., A. modesta Schumann, A. mutica Roxb., A. purpurea ‘Eileen McDonald’, A. spectabile ‘Giant Orange’ and A. zerumbet (Pers.) B.L. Burtt & R.M. Sm.

2.9.1 Chemistry

The chemistry of a range of Alpinia species has been reviewed by Tewari and co-workers (Tewari et al., 1999), although it is evident that this review was not comprehensive. Essential oil from Alpinia species, mostly extracted from rhizomes but also in some cases from leaves, stems and seeds, contain terpenoids and phenylpropanoids typical of essential oils. This includes monoterpenoids such as α- and β-pinene, geraniol, borneol, citronellol, linalool, 1,8-cineole and camphor, sesquiterpenoids including eudesmol, β-sesquiphellandrene and curcumene, and phenylpropanoids such as methyl eugenol (Tewari et al., 1999). Four acetoxy-cineole isomers, viz. the (trans and cis)-2- and 3-acetoxy-1,8-cineoles, have been identified as the major aroma constituents in the rhizome of A. galanga (Kubota et al., 1998; Kubota et al., 1999). The major pungent compound in A. galanga is 1’-acetoxychavicol acetate (Fig. 2-14), which is at least ten times less pungent and produces a more short-lived pungent sensation than capsaicin (Gautschi et al., 1999). Several pungent diarylheptanoids have also been isolated from Alpinia species (Tewari et al., 1999).
Two kava-pyrones, 5,6-dehydrokawain and dihydro-5,6-dehydrokawain, have been isolated from the leaves of *A. zerumbet* (Pers) B. L. Burtt & R. M. Smith (syn. *A. speciosa* K. Schum.), a species used as a diuretic and hypotensive agent in Brazil (Kuster *et al.*, 1999).

Computerised searches of the databases Chemical Abstracts, MEDLINE, CAB Abstracts, Current Contents Connect and Web of Science using the search terms ‘Alpinia caerulea’ and ‘Alpinia coerulea’ yielded no results relating the chemistry or pharmacology of this Australian species.

### 2.9.2 Pharmacology

Several species of *Alpinia* are used in traditional medicine. Greater galangal (*A. galanga*) is used for the treatment of stomach ache in China and Thailand (Yang & Eilerman, 1999), and a number of species are used in traditional Ayurvedic medicine in India for inflammation, cancer, urinary calculi, and as a digestive, liver and spleen tonic for the treatment of various digestive complaints (Tewari *et al.*, 1999). *A. zerumbet* is used medicinally in Brazil (Kuster *et al.*, 1999).

*Alpinia galanga* has demonstrated gastric anti-secretory and anti-ulcer effects in experimental animals (Tewari *et al.*, 1999) and is reported to possess anti-tumour (Itokawa *et al.*, 1987), anti-fungal (Haraguchi *et al.*, 1996; Scheffer *et al.*, 1981) and anti-oxidant activity (Cheah & Abu, 2000). An extract of *A. galanga* in combination with a ginger (*Zingiber officinale*) rhizome extract is marketed as an anti-inflammatory agent for use in arthritis under the name Zinaxin™ (http://www.zinaxinrapid.com/). This product was the study...
medication in one clinical trial in patients with osteoarthritis (Altman & Marcussen, 2001) (refer to Section 2.2.1.5.1) (Altman & Marcussen, 2001).

A methanolic extract of *A. galanga* rhizome showed strong inhibitory effect in an *in vitro* assay using inhibition of Epstein-Barr virus activation as a means of identifying potential anti-tumour promoters (Murakami *et al.*, 1994). This study identified 1’-acetoxychavicol acetate, the major pungent principle in *A. galanga*, as the active principle with an IC₅₀ value of 1.5 µM.

*Alpinia malaccensis* and *A. mutica* had *in vitro* anti-bacterial and potent anti-oxidant activity (Habsah *et al.*, 2000b).

In Brazil, *A. zerumbet* is used as a diuretic and anti-hypertensive agent (Kuster *et al.*, 1999). The anti-hypertensive effect is likely due to the presence of several flavonoids with calcium channel blocking activity. The kava pyrones present (see above) also have anti-platelet, anti-convulsant, analgesic, sedative and anxiolytic actions. An aqueous extract of the leaves was found to promote central nervous system excitation, followed by depression and hypokinesia.

The major pungent principle of *A. galanga*, 1’-acetoxychavicol acetate, possesses considerable biological activity, with anti-ulcer, anti-tumour and anti-microbial properties, as well as inhibition of xanthine oxidase having been demonstrated (Kubota *et al.*, 1998). Most recently, the compound has been shown to inhibit the replication of human immunodeficiency virus type 1 (Ye & Li, 2006).

Acute (24 h) and chronic (90 days) oral toxicity studies on the ethanolic extract of *A. galanga* rhizome were carried out in mice (Qureshi *et al.*, 1992). Acute extract dosages were 0.5, 1.0, and 3 g per kg body weight, while the chronic dosage was 100 mg/kg/day. No significant mortality was observed compared with controls, but galangal treated mice experienced a significant weight gain and an increased level of red blood cells. In male mice, a highly significant increase in sperm count, sperm motility and weight of sexual organs was recorded.

Diarylheptanoids possess potent anti-inflammatory properties. An early study found that diarylheptanoids including yakuchinone A isolated from *A. officinarum* were dual inhibitors of arachidonic acid metabolism, i.e. they inhibited both prostaglandin synthetase (COX) and
5-lipoxygenase (Kiuchi et al., 1992). The anti-inflammatory activity of diarylheptanoids may in part result from their suppressive effect on the surface expression of inducible adhesion molecules in endothelial cells and subsequent leukocyte adhesion (Yamazaki et al., 2000), but other mechanisms may also be involved. Two diarylheptanoids isolated from *A. oxyphylla* Miquel, yakuchinone A and B, were found to down-regulate the expression of COX-2 and iNOS through suppression of NFκB in mouse skin treated with a tumour promoter (Chun et al., 2002).

### 2.10 Genus *Pleuranthodium* (K. Schum.) R. M. Smith

The genus *Pleuranthodium* comprises some 23 species distributed in New Guinea, Australia and the Pacific (Mabberley, 1997). In Australia, *Pleuranthodium racemigerum* (F. Muell.) R. M. Smith (syn. *Alpinia racemigera* F. Muell., *Psychanthus racemiger* (F. Muell.) R. M. Smith) occurs in the Wet Tropics region of Far North Queensland and was included in the present work. No information about the chemistry or pharmacology of this species was located in the literature.

### 2.11 Genus *Etlingera* Giseke

The genus *Etlingera* is made up of around 60 species distributed from India to New Guinea. The genus is closely allied to, and sometimes included in, the genus *Amomum* (Mabberley, 1997).

One species is native to Australia, *E. australasica* R. M. Smith, which is confined to rainforest in the north-eastern Cape York Peninsula in North Queensland (Smith, 1987).

Torch ginger (*E. elatior* (Jack) R. M. Smith) from Malesia is cultivated as an ornamental, and its flowers are used as a condiment in cooking (Mabberley, 1997). The leaves of a Tahitian species, *E. cevuga* Smith (syn. *Amomum cevuga*, *Geanthus cevuga*), are used as a yellow dyeing material for dying vegetable fibres for traditional dresses (Lechat-Vahirua *et al.*, 1993).
The present work included *Etlingera australasica* R.M. Sm. and *E. elatior* ‘Burma Torch’. No information about the chemistry and pharmacology of these taxa was identified, but the essential oil of *E. cevuga* has been reported to contain methyl eugenol (47%) and (E)-methyl isoeugenol (18%), as principal constituents (Lechat-Vahirua *et al.*, 1993). No pharmacological data pertaining to the genus *Etlingera* was located.

### 2.12 Genus *Elettaria* Maton

The genus *Elettaria* comprises seven species and is distributed from India to western Malesia. Cardamom (*E. cardamomum* Maton), a native of India, produces aromatic seeds, which are used in cooking and medicine (Mabberley, 1997). Today cardamom seeds are obtained from the cultivated variety *E. cardamomum* var. *minuscula*, and the main producing countries are Sri Lanka, India and Guatemala (Evans, 2002).

The medicinal use of cardamom seeds dates back several thousand years in the Indian system of Ayurveda, and they were known to the Greeks in the 4th century BCE. In India, cardamom is used for a variety of complaints including digestive problems, asthma, bronchitis, kidney stones, anorexia and debility (Chevallier, 2001).

Cardamom fruit is included in the *British Pharmacopoeia* (Anonymous, 2007), and the seed was approved by the German Commission E for the treatment of dyspepsia (Blumenthal, 1998).

#### 2.12.1 Chemistry

Cardamom seeds yield 2.8-6.2% volatile oil with a high content of terpinyl acetate and 1,8-cineole (Fig. 2-15) and smaller amounts of other monoterpenes including alcohols and esters (Evans, 2002; Lawrence, 1998).
2.12.2 Pharmacology

Cardamom seed oil had anti-inflammatory activity against acute carrageenan-induced paw oedema in the rat and analgesic activity in mice, producing 50% protection against writhing induced by intraperitoneal administration of 0.02% \( \beta \)-benzoquinone (Al-Zuhair et al., 1996). The same authors also reported anti-spasmodic activity in rabbit intestine \textit{in vitro} resulting from muscarinic receptor blockage. The seed oil administered intravenously (5-20 \( \mu \)L per kg) caused dose-dependent decrease in arterial blood pressure in the rat, and large doses relaxed spontaneously contracting rabbit jejunum \textit{in vitro} (El Tahir et al., 1997).

The volatile oil of cardamom has been shown to enhance transdermal absorption of indomethacin, as a result of its monoterpene content (Huang et al., 1993; YawBin et al., 1999).

An aqueous cardamom seed extract (10% w/v) was shown to increase gastric acid secretion almost three-fold in anaesthetised rats (Vasudevan et al., 2000).

Ethanolic extracts of combinations of cardamom leaves and root and also of roots and seeds inhibited the growth of \textit{Staphylococcus aureus} (Daswani & Bohra, 2000). No other pharmacological information pertaining to root or rhizome preparations was located.
2.13 Genus *Hornstedtia* Retz.

The 24 species in the genus *Hornstedtia* occur in South-east Asia, Papua New Guinea and Australia (Mabberley, 1997).

The species included in the present work, *Hornstedtia scottiana* (F. Muell.) Schum., is found in New Guinea and Far North Queensland (Anonymous, 2008). It is reportedly edible (Dick, 1992), but no information about its chemical composition or biological activity was located.

A labdane diterpene, labda-8(17),12-diene-15,16-dial, has been reported from *H. scyphifera* (Koenig) Steud. (Sirat *et al.*, 1994), but no other information about the chemistry or pharmacology of the genus was located.

2.14 Genus *Renealmia* L.f.

The genus *Renealmia* consists of some 60 species in tropical parts of the Americas and Africa (Mabberley, 1997). Several species are reported to be used locally as medicinal plants (Dos Santos & Sant'Ana, 2000; Lans *et al.*, 2001; Otero *et al.*, 2000; Zhou, 1997). Diterpenoids (Boukouvalas *et al.*, 2006; Sekiguchi, 2001; Yang, 1999), dihydrochalcones (Gu, 2002), diarylheptanoids (Sekiguchi, 2002) and sesquiterpenes (Kaplan *et al.*, 2000) have been reported from the genus.

The Central American species *Renealmia cernua* (Sw. ex Roem. & Schult.) J.F. Macbr. was included in the present work. No information about the chemistry or pharmacology of this species was identified.

2.15 Genus *Costus* L.

The genus *Costus* is a tropical genus with 42 species and is placed in the family Costaceae by some authorities (Mabberley, 1997). There is one endemic species in Australia, *C. potierae* (Smith, 1987). Various species have been used in traditional medicine in Europe and the Americas for a wide range of ailments (Johnson, 1999). *C. speciosus* (Koenig) Sm. from Indomalesia is a commercial source of diosgenin, which occurs in the rhizome in a
concentration up to 2.6% on a dry weight basis (Gupta et al., 1981; Kaphai et al., 1977; Mabberley, 1997).

It should be noted that the Asian drug ‘costus root’ is not derived from the genus Costus but from Saussurea lappa (Asteraceae) (de Kraker et al., 2001; Moeslinger et al., 2000).

The following species of Costus were included in the present work: C. barbatus Suess., C. leucanthis Maas, C. malortieanus H. Wendland, C. productus Gleason ex Maas, C. pulverulentus C. Presl and C. tappenbeckianus J. Braun et K. Schum.

2.15.1 Chemistry

Diosgenin (Fig. 2-16) has been reported from C. malortieanus (Prasad & Ammal, 1983), and other steroidal glycosides (including furostanol and spirostanol glycosides) have been identified in the rhizomes of several other species (Agrawal et al., 1984; Inoue et al., 1995; Pereira et al., 1999; Rui et al., 1997; Silva et al., 1998; Willuhn & Pretzsch, 1985).

![Diosgenin molecule](image)

**Fig. 2-16. Diosgenin from Costus malortieanus.**

No further information was located pertaining to the chemistry of the species included in the present work.
2.15.2 Pharmacology

No information about the pharmacology of any of the species included in the present work was located, but *C. spiralis*, which is used in Brazilian folk medicine for urinary tract complaints and calculi, reduced the growth of foreign body-induced calculi in the bladder of rats (Viel *et al.*, 1999), and *C. lucanusianus*, a traditional anti-abortion drug in the Ivory Coast, produced complete inhibition of oxytocin-induced contractions in isolated rat uterus (Foungbe *et al.*, 1987).

2.16 Genus *Tapeinochilos* Miquel

The genus *Tapeinochilos* comprises approximately twelve species distributed in Indonesia, New Guinea and Australia (Mabberley, 1997; Smith, 1987). The genus is closely related to *Costus*, and is placed in the family Costaceae by some taxonomists (Mabberley, 1997). One species, *T. ananassae* (Hassk.) K. Schum. (syn. *T. queenslandiae* K. Schum) occurring in Queensland and New Guinea (Smith, 1987), was included in the present work.

No information about the chemistry or pharmacological activity of *T. ananassae* or any other members of this genus was located.

2.17 Summary

The Zingiberaceae contains many species that have been used as medicinal plants or spices, in particular in Southeast Asia. Extensive information about the chemistry and pharmacology of these plants is available only for a few species, in particular ginger and turmeric. For the majority of species, including many species traditionally used as medicines, limited or no information is available, and this is also true for whole genera in some cases.

Since the family clearly produces compounds with interesting pharmacological action, the investigation of little known species should prove rewarding.
2.18 Aims and research questions

The overall aim of this work has been to contribute to the existing body of knowledge about members of the Zingiberaceae as medicinal plants, in particular in terms of their potential use as anti-inflammatory agents. The work comprised two distinct parts. The first part focused on the phytochemistry of a number of ginger (*Zingiber officinale*) clones with the aim of identifying one or more with unique chemistry and consequent particular therapeutic prospects. The second part involved the screening of species from various genera in a number of bioassays relevant to potential anti-inflammatory activity, with the aim of increasing the understanding of established medicinal species and the potential identification of species with novel therapeutic prospects.

2.18.1 Part 1: Phytochemical investigations of 17 ginger clones

That pharmacologically active constituents in medicinal plants can vary both qualitatively and quantitatively is well established, and a range of genetic, environmental, ontogenic and biologic factors can affect the production of secondary metabolites in plants (Evans, 2002; Wijesekera, 1991).

Ginger is particularly interesting in this context, because the species is sterile and can only propagate by vegetative means. This, combined with the fact that the plant has been widely cultivated in tropical regions around the world for several hundreds of years, makes it probable that genetically stable and potentially chemically distinct clones exist.

Access to 17 ginger clones (including 12 of tetraploid genotype) through the Queensland Department of Primary Industries represented an excellent opportunity to investigate phytochemical variability resulting from genotypic differences.

2.18.1.1 Research questions

A number of research questions arose from the literature review in relation to the phytochemical variability of ginger clones:
1. Are there significant qualitative and/or quantitative phytochemical differences between different clones of ginger that are genetically determined and independent of environmental, ontogenic or biologic factors?

2. If such genetically determined phytochemical differences exist, would it be possible to select one or more clones with characteristics that would make them particularly suitable for pharmaceutical or other specific uses?

3. Does fresh ginger contain shogaols, or do they only form from gingerols post harvest?

4. Does the essential oil composition vary significantly between clones and if so, how?

2.18.1.2 Specific aims

Based on the research questions above, a number of specific aims were formulated for this part of the project. They were:

1. To assess ginger extracts, in a pilot study, for in vitro COX-1 inhibitory activity, determine their content of gingerols and shogaols, and examine whether a correlation between activity and levels of gingerols/shogaols exist.

2. To compare the 17 ginger clones in terms of their yield of pungent gingerols and shogaols, both of which have demonstrated anti-inflammatory activity in vitro and which are responsible for the pungency of ginger.

3. To identify one or more clones that yield particularly high amounts of these compounds and therefore would be suitable for pharmaceutical use.

4. To determine whether fresh ginger contains shogaols.

5. To compare the 17 clones in terms of their essential oil composition.

6. To determine if all clones have high citral content (Australian ginger is said to possess a particular ‘lemony’ aroma).
2.18.1.3 Research methods

The following methods were employed in order to meet the specific aims of this part of the project:

1. Cultivation of ginger clones under uniform conditions.
2. Standardised extraction of ginger rhizomes.
4. Quantification of gingerols and shogaols by HPLC.
5. Extraction of essential oil from ginger rhizomes by steam distillation.
6. Analysis of essential oils by GC-MS.

2.18.2 Part 2: Screening Zingiberaceae for pharmacological activity

The second part of this work involved the screening of a number of Zingiberaceae species in a range of bioassays. This work adds to the existing knowledge base regarding the pharmacology of several traditional medicinal plants and provides novel information about species that have not previously been investigated in this way.

Also, the work was designed to test the hypothesis that the combination of ethnobotanical and taxonomic information is a productive strategy to identify previously unrecognised plant species with therapeutic potential, either in the form of phytomedicines or by providing lead compounds for subsequent drug development.

As evidenced by the literature review above, the Zingiberaceae contains many species that have been used as traditional medicines. Among the most well known medicinal species from the family are ginger (Zingiber officinale), turmeric (Curcuma longa), galangal (Alpinia galanga) and fingerroot (Boesenbergia rotunda). Thus ethnobotanical information has been used to identify a plant family and specific genera with significant pharmacological and therapeutic activity. Subsequently, taxonomic information (based on phylogenetic relationships) has been applied to identify species related to established medicinal species at
the generic or family level. This approach is based on the well established concept that phylogenetically related plant species usually display a significant degree of similarity in the kinds of secondary metabolites they produce (Evans, 2002).

2.18.2.1 Research questions

A number of research questions arose in relation to the second part of this work:

1. Can the mode of action of well known medicinal species from the Zingiberaceae be further elucidated through bioassays?

2. Can Zingiberaceae species with previously unrecognised pharmacological activity be identified through screening in bioassays?

3. Can species with previously unrecognised pharmacological activity be identified from genera with recognised medicinal species (such as *Zingiber*, *Curcuma* and *Alpinia*)?

4. If species with previously unrecognised pharmacological activity are identified, do they contain active compounds that are similar to active compounds in related medicinal species?

2.18.2.2 Specific aims

In accordance with the research questions, the specific aims of this part of the work were:

1. To screen a number of Zingiberaceae species in a range of bioassays (inhibition of PGE\(_2\), antioxidant activity, inhibition of nitric oxide, stimulation of natural killer cell activity, cytotoxicity).

2. Through bioassay work, to contribute to the understanding of the mode of action of established medicinal plants.

3. To identify previously unrecognised species with pharmacological activity that makes them candidates for novel phytomedicines or drug leads.
4. To isolate and determine the structure of major active compounds in previously unrecognised medicinal species.

2.18.2.3 Research methods

To address the specific aims of this part of the project, the following methods were employed:

1. Inhibition of PGE$_2$ in 3T3 murine fibroblasts.

2. Oxygen radical absorption capacity (ORAC) assay.


5. Cytotoxicity in murine and human cell lines.


8. Structural elucidation by nuclear magnetic resonance (NMR) spectroscopy.
3. INHIBITION OF CYCLOOXYGENASE-1 BY GINGER RHIZOME EXTRACTS

3.1 Introduction

The aims of this study were: (i) to determine the most suitable method for extraction of ginger rhizome; (ii) to determine the most suitable extraction medium for ginger rhizome; (iii) to establish a protocol for the quantitative determination of major pungent ginger compounds by high-performance liquid chromatography (HPLC); and (iv) to establish a protocol for the determination of the biological activity of ginger rhizome extracts in a cyclooxygenase-1 (COX-1) bioassay. The determination of the most suitable extraction method and medium was guided by the biological activity observed in the bioassay.

3.2 Materials and Methods

3.2.1 Plant material

A single rhizome of *Zingiber officinale* 'Queensland', grown outdoors under sprinklers at Southern Cross University over the previous 7 months, provided the raw material for all ginger extracts used in this study. The plant was grown in a black 300 mm plastic pot in a sand-peat 1:1 growth medium containing the following nutrients (g per m$^3$): dolomite 3600, ammonium sulfate 544, superphosphate 184, potassium sulfate 248, magnesium sulfate 472, copper sulfate 7.2, zinc sulfate 9.6, iron sulfate 7.2 (Smith & Hamill, 1996).

The freshly harvested rhizome was peeled and chopped finely with a knife. For hot extractions, 10 g of chopped rhizome was added to 150 ml of extraction medium (water or ethanol), ground with a tissue tearer (Heidolph Diax 600) and extracted in a Soxhlet apparatus for 5 hours. Extracts were reduced to near dryness under vacuum on a rotary evaporator, then reconstituted in 100 ml fresh extraction medium. In Experiment 1, residues from ethanolic extractions were dissolved in 75% ethanol and 25% water, whereas residues from aqueous extractions were dissolved in 75% water and 25% ethanol. Laboratory grade potable alcohol (96%) was used in the preparation of ginger extracts.
For extractions at ambient temperature (approximately 23°C), 1 g of chopped rhizome was added to 10 ml of extraction medium, ground with a tissue tearer (Heidolph Diax 600), then placed in a sonicator and macerated at ambient temperature for 20 minutes. The macerate was then centrifuged (1800 RCF for 5 minutes), the supernatant removed, and the pellet extracted again with 10 ml fresh extraction medium following the same procedure. The supernatant fluids from both extractions were combined, reduced to near dryness under vacuum on a rotary evaporator, then reconstituted in 10 ml fresh extraction medium. In Experiment 1, residues from ethanolic extractions were dissolved in 75% ethanol and 25% water, whereas residues from aqueous extractions were dissolved in 75% water and 25% ethanol.

A preliminary experiment showed that two subsequent extractions with ethanol yielded 96% of the substances extracted by 4 subsequent extractions (data not shown).

3.2.2 HPLC analysis

Reversed-phase HPLC analysis of the plant extracts was performed on a Hewlett Packard 1100 HPLC fitted with a HP LiChrospher® 100 RP-18e (5µm) column. Mobile phase was pumped at 1mL/min (90% H2O/10% acetonitrile to 10% H2O/90% acetonitrile over 20 min). Injection volume was 10 µL. Data were collected using a UV/visible diode array detector scanning from 195 to 330 nm.

Identification of [6]-gingerol, [8]-gingerol, [10]-gingerol, [6]-shogaol and [8]-shogaol was based on comparisons of retention time and UV-spectra with pure synthesised standards obtained from the Department of Pharmacy at the University of Sydney. Absolute ethanol (99%) analytical grade was used in the preparation of standards. Quantification of the compounds mentioned above was based on standard curves prepared with pure standards and eugenol as an external standard. Duplicate injections were analysed of all samples.

3.2.3 Cyclooxygenase-1 assay

The COX-1 assay was carried out using 14C-labelled arachidonic acid as a substrate. Ginger extracts were incubated with labeled arachidonic acid and cyclooxygenase-1 for 15 min at
37°C, after which the reaction was stopped. Arachidonic acid metabolites were extracted and separated by thin layer chromatography and quantified using a liquid scintillation counter. Samples were assayed in triplicates. Details of the assay are given below.

3.2.3.1 Enzyme reaction

40 µl Cox-buffer (0.1 M TRIS at pH 8.0 containing 0.5 mM sodium EDTA and 0.5 mM phenol), 10 µl co-factor-1 (aqueous solution of 10 µM hematin [Sigma] and 0.02 M NaOH) and 10 µl co-factor-2 (0.1 mM TRIS buffer containing 10 mM reduced glutathione [Sigma] and 10 mM (-)-epinephrine-(+)-bitartrate [Sigma] adjusted to pH 8.0) was added to Eppendorf tubes and incubated for 5 minutes at 37°C. 10 µl dilute ovine cyclooxygenase-1 solution (Cayman Chemicals; 20 µl in 190 µl Cox-buffer) was added to each tube followed by 10 µl sample or control solvent. 40 µl 14C-arachidonic acid (Amersham, Australia) was diluted with 360 µl Cox-buffer and 20 µl of this solution added to each tube. Tubes were vortexed gently after each addition of material. Tubes were incubated for 15 minutes at 37°C with gentle shaking after which the reaction was stopped by the addition of 40 µl formic acid solution to each tube.

3.2.3.2 Extraction of arachidonic acid and metabolites

After the enzymatic reaction was stopped, 200 µl chloroform (CHCl₃) was added to each tube, which was then vortexed at high speed for approximately 10 seconds. The chloroform (bottom) layer was then transferred to a clean Eppendorf tube using a micropipette, and the chloroform evaporated under a nitrogen gas stream.

3.2.3.3 Separation of arachidonic acid and metabolites

This separation was carried out by means of thin-layer chromatography (TLC). Two mobile phases were prepared as follows: Mobile Phase-1 (hexane 70 ml, diethyl ether 30 ml, glacial acetic acid 1 ml) and Mobile Phase-2 (ethyl acetate 40 ml, methanol 10 ml, distilled water 25
ml, combined in a separation funnel). Extracted substances were reconstituted in 20 µl CHCl₃ and applied to a 200 x 200 mm TLC plate (0.2mm Silicon F₂₅₄; Merck, Germany). Two plates were used at a time. Standards (arachidonic acid 2 µl and prostaglandin E₂/D₂ 10 µl [Sigma]) were applied to one lane on each plate. After the application of samples and standards, the plates were allowed to dry, then run in a TLC tank (CAMAG) containing Mobile Phase-1 until the solvent front reached a groove cut in the gel at 10 cm. Plates were removed from the tank, allowed to dry for 15 minutes, then inserted into another TLC tank containing Mobile Phase-2 and run until the solvent front reached a pencil mark at 3 cm. Plates were again removed from the tank, allowed to dry for 15 minutes and then placed in an iodine chamber for approximately 45 minutes. After this development, the arachidonic acid standard (Rf 65-70) and the prostaglandin standard (just below the 3 cm line) were marked with a pencil on both plates. Two parallel lines, 10-13 mm apart and equidistant from the marked prostaglandin standard, were drawn horizontally across the plate and the number of each lane written between the lines. The plate was then cut up along the parallel lines and the grooves separating the lanes, resulting in 9 pieces of silica gel containing the prostaglandin metabolites of arachidonic acid from each plate. The silica gel from each numbered piece was then scraped off the aluminium plate and transferred to a scintillation tube to which 200 µl distilled water and 200 µl Soluene-350 (Packard, Australia) was added. After thorough vortexing, tubes were left to stand for 30 minutes, after which 2 ml Insta-Gel (Packard, Australia) and 2 ml Hionic-Fluor (Packard, Australia) was added to each tube. Tubes were vortexed for 10 seconds, then placed in a liquid scintillation counter (Minaxiβ Tri-carb® 4000; United Technologies Packard) running Program 4. Results were recorded as counts per minute (CPMA/K).

### 3.2.4 Statistical analysis

Data were analysed using Excel 97-SR-1 (Microsoft Corporation, Redmond, WA). Statistics calculated were Student's 2-tailed t-test and the correlation coefficient.
3.3 Results and Discussion

3.3.1 Experiment 1

This experiment aimed to determine the most efficient extraction method for gingerols. Hot Soxhlet extraction was compared with maceration/sonication at room temperature (23°C) using two different solvents, water and ethanol. The biological activity in the cyclooxygenase-1 assay and the content of assayed pungent compounds of these extracts are shown in Table 3-1.

Table 3-1. Effect of extraction method: cyclooxygenase-1 inhibitory activity and content of major pungent compounds of ginger extracts.

All extracts were prepared from fresh rhizome; the rhizome:solvent ratio was 1:4 (w/v). a mean±s.d of 6 injections; b mean±s.d. of 2 injections, c mean±s.d of 4 samples; d mean±s.d of 3 samples; e mean±s.d. of 2 samples; f 75% ethanol-25% water; g 75% water-25% ethanol. CPMA/K: counts per minute. * Two-tailed t-test for COX-inhibitory effect, sample v control.

<table>
<thead>
<tr>
<th></th>
<th>Cold EtOH</th>
<th>Hot EtOH</th>
<th>EtOH Control</th>
<th>Cold Water</th>
<th>Hot Water</th>
<th>Water Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-gingerol (mg/mL)</td>
<td>0.2922 ±0.0005a</td>
<td>0.2841 ±0.0005b</td>
<td>0.1913 ±0.012b</td>
<td>0.2398 ±0.0001b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-gingerol (mg/mL)</td>
<td>0.0842 ±0.0005a</td>
<td>0.0829 ±0.0006b</td>
<td>0.0491 ±0.0013b</td>
<td>0.0356 ±0.0001b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-gingerol (mg/mL)</td>
<td>0.0868 ±0.0036a</td>
<td>0.0810 ±0.0003b</td>
<td>0.0402 ±0.0016b</td>
<td>0b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-shogaol (mg/mL)</td>
<td>0a</td>
<td>0.0282 ±0.0000b</td>
<td>0b</td>
<td>0.0476 ±0.0001b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPMA/K</td>
<td>12791 ±2199c</td>
<td>12225 ±942c</td>
<td>58496 ±9886c</td>
<td>51878 ±2595d</td>
<td>41476 ±1832d</td>
<td>78417 ±4502d</td>
</tr>
<tr>
<td>Inhibition of Cox-1 (%)</td>
<td>78.13±17.19</td>
<td>79.10±7.71</td>
<td>0±16.90</td>
<td>33.84±5.00</td>
<td>47.11±4.42</td>
<td>0±5.74</td>
</tr>
<tr>
<td>p-value*</td>
<td>0.002</td>
<td>0.002</td>
<td>0.038</td>
<td>0.036</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All extracts demonstrated statistically significant inhibition of cyclooxygenase-1 in the bioassay (two-tailed t-test; Table 3-1). The inhibitory effect ranged from approximately 34% for the cold aqueous extract to almost 80% for both ethanolic extracts, compared with controls. The data show that the choice of solvent was a stronger determinant of biological
activity than the extraction method. The cold ethanolic extract showed significantly more potent inhibitory activity than did the cold aqueous extract (p = 0.00003), while the hot Soxhlet ethanolic extract was also significantly more potent than the hot Soxhlet aqueous extract (p = 0.00022). Overall, the ethanolic extracts were significantly more potent in terms of cyclooxygenase-1 inhibition than were the aqueous extracts (p = 0.00002).

It is interesting to note that while the extraction method appeared to have little effect on the inhibitory activity when ethanol was used as the solvent, the aqueous hot Soxhlet extract was significantly more active than the cold aqueous extract (47% vs 34% inhibition, p = 0.00653).

The [6]-gingerol content of the extracts showed a very high correlation to the inhibitory activity in the cyclooxygenase-1 assay (r = 0.976). The [8]-gingerol content also showed considerable correlation to the inhibitory activity (r = 0.891).

The amount of [10]-gingerol in the ethanolic extracts was approximately twice the amount measured in the cold aqueous extract, while no [10]-gingerol was identified in the hot Soxhlet extract. Being less polar than [6]- and [8]-gingerol, smaller amounts of [10]-gingerol would be expected to be extracted in an aqueous solvent. It is possible that what small amount of [10]-gingerol may have been extracted was degraded by the heat in the hot Soxhlet extraction process.

It is noteworthy that [6]-shogaol was identified only in the hot Soxhlet extracts. This is in agreement with the notion that [6]-shogaol is a degradation product of [6]-gingerol that forms when [6]-gingerol is exposed to excessive heat (Connell & Sutherland, 1969; He et al., 1998; Zhang et al., 1994). The present data also suggest that 6-shogaol is in fact absent from fresh ginger rhizome.

Based on the findings of this experiment, it was decided to employ maceration with sonication at ambient temperature in subsequent experiments, since hot Soxhlet extraction is considerably more time consuming and was not found to provide additional extraction efficiency.
3.3.2 Experiment 2

This experiment aimed to determine what extraction medium would yield the most active extract in terms of inhibitory activity in the cyclooxygenase-1 bioassay. Water and ethanol were identified as potential solvents because of their widespread use and acceptance as solvents for herbal medicines. Five different extraction media were compared: ethanol (96%), 75% ethanol-25% water, 50% ethanol-50% water, 25% ethanol-75% water, and water (100%). All extractions were carried out as macerations with sonication at room temperature (23°C) as described earlier.

The biological activity in the cyclooxygenase-1 assay and the content of assayed pungent compounds of these extracts is shown in Table 3-2.

Table 3-2. Effect of extraction solvent: cyclooxygenase-1 inhibitory activity and content of major pungent compounds of ginger extracts.

All extracts were prepared from fresh rhizome; the rhizome:solvent ratio was 1:4 (w/v). a mean±s.d of 2 injections; b mean±s.d of 3 samples; c mean±s.d of 2 samples; d control values calculated on the basis of values for ethanol and water; CPMA/K: counts per minute. * Two-tailed t-test for COX-inhibitory effect, sample v control.

<table>
<thead>
<tr>
<th></th>
<th>EtOH (96%)</th>
<th>75%EtOH+25%Water</th>
<th>50%EtOH+50%Water</th>
<th>25%EtOH+75%Water</th>
<th>100% Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-gingerol (mg/mL)</td>
<td>0.3701±0.0005</td>
<td>0.3211±0.0028</td>
<td>0.3003±0.0008</td>
<td>0.2744±0.0016</td>
<td>0.1772±0.0007</td>
</tr>
<tr>
<td>8-gingerol (mg/mL)</td>
<td>0.0648±0.0008</td>
<td>0.0577±0.0006</td>
<td>0.0482±0.0008</td>
<td>0.0440±0.0010</td>
<td>0.0155±0.0002</td>
</tr>
<tr>
<td>10-gingerol (mg/mL)</td>
<td>0.1076±0.0009</td>
<td>0.0941±0.0003</td>
<td>0.0754±0.0005</td>
<td>0.0570±0.0001</td>
<td>0.0136±0.0002</td>
</tr>
<tr>
<td>CPMA/K</td>
<td>3396±390</td>
<td>6609±993</td>
<td>12507±2270</td>
<td>21353±5213</td>
<td>23030±5348</td>
</tr>
<tr>
<td>Control value</td>
<td>41197±6165</td>
<td>40303d</td>
<td>39410d</td>
<td>38516d</td>
<td>37622±1951c</td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>91.76±11.49</td>
<td>83.60±15.02</td>
<td>68.26±18.16</td>
<td>44.56±24.41</td>
<td>38.78±23.22</td>
</tr>
<tr>
<td>p-value*</td>
<td>0.009</td>
<td>0.009</td>
<td>0.008</td>
<td>0.014</td>
<td>0.029</td>
</tr>
</tbody>
</table>

All extracts demonstrated statistically significant inhibition of COX-1 in the bioassay (two-tailed t-test; Table 3-2). The ethanol content of the solvent was highly correlated to inhibitory activity (r = 0.983) as well as [6]-, [8]- and [10]-gingerol content (r= 0.956, 0.939...
and 0.971, respectively). This clearly shows that pure ethanol is a superior solvent to hydroethanolic mixtures with respect to COX-1 inhibitory activity of the resulting extracts, as well as in terms of gingerol content of these extracts.

Both [6]-, [8]- and [10]-gingerol content of the extracts showed high correlation to inhibitory activity in the bioassay ($r = 0.904, 0.889$ and 0.941, respectively). It is not possible from this experiment to determine to what extent each of these compounds contributes to the inhibitory activity. Kiuchi and colleagues found the IC$_{50}$ values of [6]-, [8]- and [10]-gingerol to be 4.6, 5.0 and 2.5µM, respectively, against prostaglandin synthase (cyclooxygenase), suggesting that [10]-gingerol may be the most potent of the three gingerols (Kiuchi et al., 1992). If this is the case, [10]-gingerol may contribute significantly to the inhibitory activity of the extracts, despite its relative low concentration compared with the major gingerol, [6]-gingerol.

The inhibitory activity of more than 90% recorded for the ethanolic extract was very promising and encouraging for future studies.

The gingerol content of the extracts is shown in Fig. 3-1. [6]-Shogaol was not detected in any of the extracts, a further confirmation that this compound does not occur in fresh ginger (see above).

![Fig. 3-1. Gingerol content (mg/mL extract) of ginger extracts.](image-url)
In Table 3-3, the ratios of [6]-gingerol : [8]-gingerol : [10]-gingerol in the extracts are given and compared to values from the literature.

**Table 3-3. Gingerol ratios in ginger extracts compared with values from the literature.**

<table>
<thead>
<tr>
<th>Extraction Medium</th>
<th>[6]-gingerol: [8]-gingerol: [10]-gingerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (96%)</td>
<td>5.7 : 1 : 1.7</td>
</tr>
<tr>
<td>75% Ethanol + 25% Water</td>
<td>5.6 : 1 : 1.6</td>
</tr>
<tr>
<td>50% Ethanol + 50% Water</td>
<td>6.2 : 1 : 1.6</td>
</tr>
<tr>
<td>25% Ethanol + 75% Water</td>
<td>6.2 : 1 : 1.3</td>
</tr>
<tr>
<td>100% Water</td>
<td>11.4 : 1 : 0.9</td>
</tr>
<tr>
<td>Fresh ginger, Hawaii</td>
<td>7 : 1 : 2</td>
</tr>
<tr>
<td>Freeze-dried ginger, Taiwan</td>
<td>7 : 1 : 1.4</td>
</tr>
<tr>
<td>Ginger oleoresin</td>
<td>4.3 : 1 : 2.4</td>
</tr>
</tbody>
</table>

From Table 3-3 it can be seen that the ratio between the three gingerols is relatively stable when the extraction medium contains at least 25% ethanol. In pure water, however, the ratio is shifted significantly in favor of the most polar of the compounds, [6]-gingerol.

The results obtained in this experiment clearly show that of the extraction media tested, ethanol (96%) was the most effective. The ethanol extract showed the greatest inhibitory activity in the COX-1 assay and had the highest concentration of the putative active compounds.

In conclusion, this study found that fresh ginger rhizome extracted by maceration with sonication at room temperature, using ethanol (96%) as the extraction medium, resulted in extracts with a high concentration of pungent gingerols and potent inhibitory activity in the cyclooxygenase-1 bioassay.
4. GINGEROL CONTENT OF SEVENTEEN GINGER (ZINGIBER OFFICINALE) CLONES

4.1 Introduction

As outlined in Chapter 2, the main pungent compounds in fresh ginger are a series of homologous phenolic ketones known as gingerols. The major gingerol is [6]-gingerol, while [8]- and [10]-gingerol occur in smaller quantities. The gingerols are thermally unstable and are converted under high temperature to [6]-, [8]- and [10]-shogaol (after shoga, the Japanese word for ginger) (He et al., 1998). Shogaols, which are more pungent than gingerols, are the major pungent compounds in dried ginger rhizome. Both gingerols and shogaols have pharmacological activity including inhibiting COX in vitro (refer to Chapter 2).

In Australia, the most widely grown ginger cultivar is ‘Queensland’, and it is estimated that 40% of the world’s confectionary ginger products are prepared from this cultivar (Smith et al., 2004b). The origin of this cultivar remains uncertain. Various authors have suggested that it arrived in Australia from the Cochin coast of India (Connell, 1986), from Fiji (Leverington, 1975), or from China in the early 1900s (Miles, 1980).

This chapter reports on the analysis of 17 ginger clones grown in Eastern Australia, including commercial cultivars and 12 experimental tetraploid clones derived from ‘Queensland’, and the quantification by HPLC of the major pungent phenolic compounds, viz. gingerols and shogaols. The objectives of this study were to explore the variability of Australian ginger clones in terms of their content of pungent phenolic compounds with a view to identify one or more high-yielding clones as candidates for commercial cultivation for flavour or pharmaceutical use.
4.2 Materials and Methods

4.2.1 Plant materials

Seventeen clones of ginger (Table 4-1) were obtained from the Queensland Department of Primary Industries & Fisheries, Maroochy Research Station at Nambour, Queensland. They included two selections of the cultivar ‘Queensland’, which is grown commercially in Queensland, the cultivars ‘Jamaican’, ‘Brazilian’ and ‘Canton’, which were introduced to Queensland between 1970 and 1972 for cultivar evaluation studies, and twelve experimental clones developed at the Maroochy Research Station at Nambour, including the newly released cultivar ‘Buderim Gold’ (Smith & Hamill, 2002). The experimental clones were obtained after in vitro colchicine treatment of shoots of diploid (2n=22) ‘Queensland’ parent material provided by J. Roscoe. They were confirmed as solid tetraploids except for one (Z30), which proved to be a periclinal chimera with both diploid and tetraploid tissues (Smith et al., 2004b).
Table 4-1. Ginger clones studied, their genotype and origin.

Ploidy: *: confirmed as solid tetraploids by flow cytometry; $: chimera with both diploid and tetraploid tissue sectors; ^: presumed to be tetraploid from stomatal measurements; #: unknown but presumed to be diploid. BGL = Buderim Ginger Ltd.

<table>
<thead>
<tr>
<th>ID</th>
<th>Genotype</th>
<th>Cultivar name</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z22</td>
<td>Tetraploid^</td>
<td>(Unnamed)</td>
<td>Derived from ‘Queensland’ (Selection 1) by colchicine treatment</td>
</tr>
<tr>
<td>Z23</td>
<td>Tetraploid^</td>
<td>(Unnamed)</td>
<td></td>
</tr>
<tr>
<td>Z24</td>
<td>Tetraploid*</td>
<td>(Unnamed)</td>
<td></td>
</tr>
<tr>
<td>Z25</td>
<td>Tetraploid^</td>
<td>(Unnamed)</td>
<td></td>
</tr>
<tr>
<td>Z26</td>
<td>Tetraploid*</td>
<td>‘Buderim Gold’</td>
<td></td>
</tr>
<tr>
<td>Z27</td>
<td>Tetraploid*</td>
<td>(Unnamed)</td>
<td></td>
</tr>
<tr>
<td>Z28</td>
<td>Tetraploid^</td>
<td>(Unnamed)</td>
<td></td>
</tr>
<tr>
<td>Z29</td>
<td>Tetraploid^</td>
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<td>Z30</td>
<td>Tetraploid$</td>
<td>(Unnamed)</td>
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</tr>
<tr>
<td>Z31</td>
<td>Tetraploid*</td>
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</tr>
<tr>
<td>Z32</td>
<td>Tetraploid^</td>
<td>(Unnamed)</td>
<td></td>
</tr>
<tr>
<td>Z33</td>
<td>Tetraploid*</td>
<td>(Unnamed)</td>
<td></td>
</tr>
<tr>
<td>Z44</td>
<td>Diploid</td>
<td>‘Queensland’ (Selection 1)</td>
<td>Selected by J. Roscoe, BGL</td>
</tr>
<tr>
<td>Z45</td>
<td>Diploid</td>
<td>‘Queensland’ (Selection 2)</td>
<td>Selected by L. Palmer, BGL</td>
</tr>
<tr>
<td>Z46</td>
<td>Diploid</td>
<td>‘Jamaican’</td>
<td>Imported from Jamaica</td>
</tr>
<tr>
<td>Z47</td>
<td>Diploid#</td>
<td>‘Brazilian’</td>
<td>Imported from Brazil</td>
</tr>
<tr>
<td>Z58</td>
<td>Diploid</td>
<td>‘Canton’</td>
<td>Imported from China</td>
</tr>
</tbody>
</table>

The clones were grown from rhizome stock in raised, outdoor beds under uniform, irrigated conditions at Southern Cross University, Lismore, New South Wales (28° 49’ S, 153° 18’ E) for approximately eight months.
4.2.2 Sample preparation

Fresh rhizomes were washed and a cylindrical sample was taken from the thickest part of the rhizome using an apple corer. The epidermis was removed and the sample cut into cubes approximately $1.5 \times 1.5 \times 1.5 \text{ mm}$. A five-gram sample was placed in a large centrifuge tube to which twice the sample mass of 99% ethanol was added. The preparation was sonicated for 20 minutes (Ultrasonic Cleaner 50 Hz, Unisonics Pty Ltd, Manly Vale, Australia) and subsequently centrifuged for 5 minutes at 4000 rotations per minute (Hettich Universal 16A Centrifuge, Tuttingen, Germany). The supernatant was transferred to a brown glass vial using a transfer pipette, stored at 4°C and filtered through a Zymark/Millipore Automation Certified Filter (Glassfiber APFB 1.0 μm prefilter, hydrophilic PTFE membrane 0.45 μm) before being injected onto the HPLC column.

In order to take account of variation between individual plants, three samples from different rhizomes were prepared for each clone.

4.2.3 HPLC methods

The same extracts were analyzed by HPLC on two occasions, approximately 5 months apart, during which period they were stored at 4°C. Measurement reproducibility was determined by repeated analyses of a mixed standard solution (see below).

**Method 1.** The first (baseline) reversed-phase HPLC analysis of the extracts was performed on an Agilent (Palo Alto, California) 1100 HPLC fitted with a HP LiChrospher® 100 RP-18e (5μm) column. Mobile phase A consisted of HPLC-grade water obtained from an in-house Milli-Q system (Waters, Milford, MA), mobile phase B consisted of HPLC-grade acetonitrile (EM Science, Gibbstown, NJ); both contained 0.05% trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO). The gradient eluting mobile phase was A:B (70:30, v/v) to A:B (10:90, v/v) over 20 min followed by A:B (10:90, v/v) for 10 min. Mobile phase was pumped at 1.00 mL/min, the column temperature was 40 °C, and the injection volume was 10.0 μL. Data were collected using a UV/visible diode array detector collecting absorption spectra from 200 to 400 nm with quantification performed at 228 nm.
Method 2. The second analysis (at 5 months) was carried out on an Agilent 1100 Series LC/MSD system fitted with a Phenomenex (Torrance, California) Luna® 5µ (150 x 4.6mm) C-18 column. Mobile phase A and mobile phase B were as described above. The gradient eluting mobile phase was A:B (70:30, v/v) to A:B (10:90, v/v) over 20 min followed by A:B (10:90, v/v) for 5 min. Mobile phase was pumped at 0.40 mL/min, the column temperature was 40 ºC, and the injection volume was 10.0 µL. Data were collected using a UV/visible diode array detector collecting absorption spectra from 200 to 400 nm with quantification performed at 228 nm.

Standards. Synthetic standards of [6]-gingerol (99%), [8]-gingerol (99%), [10]-gingerol (98%), [6]-shogaol (99%) and [8]-shogaol (99%) were obtained from the Department of Pharmacy, University of Sydney (Sydney, NSW). A known quantity of each standard was dissolved in 99% ethanol and made up to 10 mL in a volumetric flask. A mixed standard solution was prepared by combining 100 µL of each of the individual standard solutions of [6]-, [8]-, and [10]-gingerol, and [6]- and [8]-shogaol. A ten-fold dilution of this mixed standard was employed in the analyses. It contained the standard compounds in concentrations ranging from 15 to 25 µg/mL. Identification of gingerols and shogaols in samples was based on comparisons of retention time and UV-spectra with the standards. Quantification was based on standard curves prepared with pure standards.

4.2.4 Statistical analysis

Statistical analyses were performed using SPSS (Chicago, Illinois) for Windows Release 11.0.0. The mean, standard deviation and coefficient of variance were calculated for repeated measurement of the standard solution. The mean and standard error were calculated for the analyses of the samples. Two-factor repeated measure analysis of variance was used to compare the content of the three gingerols (‘gingerol’ = the within factor) from the 17 clones (‘clone’ = the between factor). The analysis was based on three replicates for each clone. The assumption of compound symmetry was not met, so tests of effects were adjusted using the Greenhouse-Geisser method. Pairwise comparisons were also employed to compare the gingerol content across the clones. The correlation between the different gingerols was examined by way of scatter plots and Pearson Product-Moment correlations. The stability over time of the gingerols was explored by repeated measure t-tests. The mean,
standard error and range were calculated for the gingerol content of diploid and tetraploid clones, respectively.

4.3 Results

4.3.1 Measurement reproducibility

In order to determine the reproducibility of the HPLC measurements, repeated analyses of the mixed standard solution were carried out by both methods. The analysis by Method 1 showed a high degree of reproducibility when the same sample was injected 9 times over a 5-day period. The coefficient of variance ranged from 2.2% to 6.4% for the five standard compounds. The reproducibility of the measurements by Method 2 was assessed by comparing eleven injections of the mixed standard over a 37-hour period. This analysis showed a very high degree of reproducibility with the coefficient of variance being less than 2% for all compounds.

4.3.2 Quantification of gingerols in test samples

Rhizome extracts of seventeen clones of ginger were analyzed twice, approximately five months apart. For each clone, three extracts were prepared from different rhizomes. The mean concentrations of [6]-gingerol, [8]-gingerol and [10]-gingerol in three rhizomes of each clone were calculated from the peak areas obtained at 228 nm. A typical HPLC trace is shown in Fig. 4-1.
The concentrations of gingerols in freshly extracted ginger are shown in Fig. 4-2. The most abundant gingerol in all clones was [6]-gingerol, which occurred at concentrations ranging from 132 to 339 µg per gram fresh rhizome (mean 215±54 µg/g). [8]-Gingerol and [10]-gingerol occurred in lower concentrations; [8]-gingerol ranged from 40 to 177 µg/g (mean 75±31), while [10]-gingerol ranged from 37 to 148 µg/g (mean 73±26). The cultivar ‘Jamaican’ (Z46) had the highest total gingerol content of any clone (664 µg/g). Neither [6]-nor [8]-shogaol was identified in any of the samples (freshly prepared or stored 5 months).
Two-factor repeated measure analysis of variance (Greenhouse-Geisser test) of the clone by gingerol interaction effect showed that the mean values of [6]-, [8]- and [10]-gingerol varied significantly across the 17 clones (\(F=2.335, p=0.01\)). When the mean total gingerol content of each clone was compared with the mean value for all clones, only two clones showed a statistically significant difference from the overall mean. These were the cultivar ‘Jamaican’ (Z46), which contained a significantly higher concentration of gingerols (\(p=0.002\)), and one of the experimental tetraploid clones (Z25), which had a gingerol content that was significantly lower than the overall mean (\(p=0.028\)).

The difference in gingerol content between the clones was also explored by way of pairwise comparisons on each of the three gingerols. This analysis is summarised in Table 4-2, which shows the number of significantly (\(p<0.05\)) different cases between each clone and the other 16 clones for each of the three gingerol compounds. The pairwise analysis confirms that the cultivar ‘Jamaican’ (Z46) is the most outstanding clone in terms of gingerol content. This is
particularly true in terms of [10]-gingerol concentration, which is significantly higher in ‘Jamaican’ than in all but one other clone (Z31).

Table 4-2. Results of pairwise analyses of 17 ginger clones in terms of [6]-, [8]- and [10]-gingerol content.

Each clone is compared with all other clones and the number of significantly (p<0.05) different cases (max. 16) is shown for each gingerol. Note that Clone Z46 (‘Jamaican’) showed 32 significantly different comparisons, distinguishing it from all other clones.

| Clone (Z no.) | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 44 | 45 | 46 | 47 | 58 |
|--------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| [6]-gingerol | 2  | 2  | -  | 7  | 2  | -  | 4  | 2  | 1  | 1  | 2  | 2  | 1  | 8  | 8  | 2  | 2  |
| [8]-gingerol | 1  | 5  | 1  | 3  | 2  | -  | 1  | -  | 1  | 6  | -  | 2  | -  | 2  | 9  | 2  | 2  |
| [10]-gingerol| 1  | 4  | 1  | 2  | 1  | 2  | 1  | 1  | 1  | 2  | 1  | 1  | 1  | 1  | 15 | 1  | 1  |
| Total number | 4  | 11 | 2  | 12 | 5  | 2  | 6  | 3  | 3  | 9  | 1  | 5  | 2  | 11 | 32 | 5  | 5  |

4.3.3 Correlation between gingerols

The correlations between the mean concentrations of [6]-, [8]- and [10]-gingerol in the freshly prepared extracts of 17 clones are illustrated by way of scatter plots in Fig. 4-3. A linear relationship between the concentrations of [6]-, [8]- and [10]-gingerol is apparent. The cultivar ‘Jamaican’ (Z46) stands out from the others by containing higher concentrations of [8]- and [10]-gingerol relative to [6]-gingerol (Fig. 4-3A-B).
Fig. 4-3. Scatter plots illustrating the correlation between concentrations (µg/g) of [6]- and [8]-gingerol (A), [6]- and [10]-gingerol (B), and [8]- and [10]-gingerol (C) in fresh ginger rhizomes. Based on HPLC data from 17 different clones.
The Pearson Product-Moment correlations between the concentrations of [6]-, [8]- and [10]-gingerol in the rhizomes at zero and five months were calculated (Table 4-3). Since the scatter plots clearly show a positive correlation between the different gingerols a one-tailed test for significance was chosen. The highly significant correlations confirm the strong positive correlation between the three gingerols.

Table 4-3. Pearson Product-Moment correlations between the concentration of gingerols in fresh rhizomes of seventeen ginger clones assayed by HPLC at zero and five months.

*: p<0.0005 (one-tailed)

<table>
<thead>
<tr>
<th></th>
<th>[6]-gingerol</th>
<th>[8]-gingerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 months</td>
<td>0.882*</td>
<td>0.850*</td>
</tr>
<tr>
<td>5 months</td>
<td>0.803*</td>
<td>0.914*</td>
</tr>
</tbody>
</table>

4.3.4 Gingerol ratios

The ratio of [6]-gingerol:[8]-gingerol:[10]-gingerol was calculated for the 17 clones based on the HPLC data. The mean ratio of [6]-gingerol:[8]-gingerol:[10]-gingerol was 3:1:1 across the clones, but some clones deviated considerably from this ratio, for example Z29 (4.4:1:1.2) and Z46 (‘Jamaican’) (1.9:1:0.8). Since both [8]- and [10]-gingerol possess considerable pungency (albeit less than [6]-gingerol) (Govindarajan, 1982b), the relatively high levels of [8]- and [10]-gingerol present in ‘Jamaican’ in addition to the high concentration of [6]-gingerol make this clone by far the most pungent of the 17 clones assayed. This was confirmed organoleptically.
4.3.5 Stability of gingerols

Ethanolic extracts were assayed twice approximately 5 months apart. Between analyses the extracts were refrigerated at 4º C. The concentrations of [6]-, [8]- and [10]-gingerol at zero and five months were compared by way of repeated measure (paired samples) t-tests. The mean concentrations of [6]- and [8]-gingerol did not change significantly in the 17 clones over the 5-month period, and these compounds therefore appear to be stable in ethanolic solution at 4º C for this period of time. The concentration of [10]-gingerol, however, showed a small (5%) but statistically significant (p=0.01) decrease over the same period.

4.3.6 Gingerol content of tetraploid clones

The mean, standard error and ranges for gingerol concentrations in two commercial selections of the diploid ‘Queensland’ cultivar and twelve experimental tetraploid clones are shown in Table 4-4. ‘Queensland’ (Selection 1) is the parent clone from which the tetraploids were generated by colchicine treatment. Both diploid and tetraploid clones displayed considerable variation in gingerol concentrations. On average, the diploid ‘Queensland’ clones contained higher levels of all three gingerols but the differences were not statistically significant.

<table>
<thead>
<tr>
<th></th>
<th>[6]-gingerol</th>
<th>[8]-gingerol</th>
<th>[10]-gingerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Queensland’ clones (n=2)</td>
<td>245.2±77.7</td>
<td>92.2±49.9</td>
<td>83.2±39.9</td>
</tr>
<tr>
<td></td>
<td>(139.3-339.4)</td>
<td>(50.9-176.6)</td>
<td>(45.2-147.9)</td>
</tr>
<tr>
<td>Tetraploid clones (n=12)</td>
<td>202.8±37.9</td>
<td>68.1±18.2</td>
<td>67.7±18.4</td>
</tr>
<tr>
<td></td>
<td>(131.6-252.5)</td>
<td>(40.2-92.0)</td>
<td>(36.6-98.5)</td>
</tr>
<tr>
<td>p-value (t-test)</td>
<td>0.058</td>
<td>0.279</td>
<td>0.471</td>
</tr>
</tbody>
</table>
4.4 Discussion

An extensive survey of fresh rhizomes of five diploid ginger cultivars, eleven experimental tetraploid clones, and one recently released tetraploid clone grown in Australia was conducted with respect to their content of pungent gingerols and shogaols. Ethanol was chosen as the extraction solvent, as it is the solvent of choice for herbal medicine preparations.

4.4.1 Gingerols

The three pungent compounds, [6]-, [8]- and [10]-gingerol, were identified and quantified in all samples. [6]-Gingerol was the most abundant gingerol in all clones, which is in accordance with the literature (Bartley, 1995; Chen et al., 1986a; Govindarajan, 1982a). The mean ratio of [6]-gingerol:[8]-gingerol:[10]-gingerol was 3:1:1 across all 17 clones. A strong, positive, linear correlation between levels of the three gingerols was found in all clones, reflecting the close biosynthetic relationship between these compounds.

The mean content of gingerols obtained in the present study are considerably higher than those found by Bartley in a supercritical CO$_2$ extract of Australian ginger (Bartley, 1995), but lower than levels reported from other parts of world (Table 4-5). This variability may reflect genetic differences between clones in different regions or physiological responses to environmental factors such as climate, soil characteristics or predation, but they may also be due to differences in extraction and analytical methodologies.
Table 4-5. Literature data on gingerol content of fresh ginger rhizomes.

Values are µg per gram fresh rhizome. n.d. = not determined.

<table>
<thead>
<tr>
<th>Country (reference)</th>
<th>Solvent/ extraction</th>
<th>Analytical method</th>
<th>[6]-gingerol</th>
<th>[8]-gingerol</th>
<th>[10]-gingerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia (present study)</td>
<td>Ethanol</td>
<td>HPLC</td>
<td>215</td>
<td>75</td>
<td>72</td>
</tr>
<tr>
<td>Hawaii (Zhang et al., 1994)</td>
<td>Methanol</td>
<td>HPLC</td>
<td>2100</td>
<td>288</td>
<td>533</td>
</tr>
<tr>
<td>United States (Hiserodt et al., 1998)</td>
<td>Methylene chloride</td>
<td>HPLC</td>
<td>880</td>
<td>93</td>
<td>120</td>
</tr>
<tr>
<td>Taiwan (Young et al., 2002)</td>
<td>Ground; acetate buffer solution (pH 4.0) added</td>
<td>HPLC</td>
<td>806</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Australia (Bartley, 1995)</td>
<td>Supercritical CO₂</td>
<td>NIES-MS</td>
<td>120</td>
<td>19</td>
<td>24</td>
</tr>
</tbody>
</table>

The cultivar ‘Jamaican’ contained the highest concentration of all three gingerols on a fresh weight basis and was therefore the most pungent of the clones assayed. It also contained higher levels of [8]- and [10]-gingerol relative to [6]-gingerol than any other clone. ‘Jamaican’ may thus be suitable for commercial production of highly pungent ginger rhizomes with potential application in both the pharmaceutical and flavour industries, even though, eventually, the viability of commercial production of this clone will depend on biomass yield.

Repeated analyses of ethanolic extracts five months apart showed that [6]- and [8]-gingerol did not degrade during this period when stored at 4º C. Concentrations of [10]-gingerol showed a small but statistically significant decrease (5%). It is not known whether [10]-gingerol is in fact less stable than [6]- and [8]-gingerol under these conditions or this finding represents a type 1 error resulting from the small sample size.
4.4.2 Shogaols

Neither [6]- nor [8]-shogaol were identified in these samples, which were prepared at ambient temperature from fresh rhizomes. In contrast, [6]-shogaol was identified in fresh rhizome extracts prepared by hot Soxhlet extraction (data not shown, refer to Chapter 3). These findings support the hypothesis that shogaols are not native constituents of fresh ginger rhizomes but form from gingerols by dehydration as a result of heat treatment or alkaline or acidic conditions (Connell & Sutherland, 1969; Vesper et al., 1995; Zhang et al., 1994). Earlier reports of shogaols in fresh ginger extracts analyzed by GC-MS (Bartley, 1995; Bartley & Jacobs, 2000) can probably be explained by the high temperatures samples are exposed to during this form of analysis, resulting in the formation of shogaols as artifacts of analysis.

4.4.3 Ploidy

The mean concentrations of all three gingerols were lower for the tetraploid clones than for the parent diploid ‘Queensland’ cultivar, although the differences were not statistically significant. This observation is in marked contrast to the findings of Nakasone and colleagues who reported that tetraploid clones of three Japanese ginger cultivars contained higher concentrations of total gingerols ([6]-, [8]- and [10]-gingerol) and in particular of [10]-gingerol than did their parent diploid genotypes (Nakasone et al., 1999).

Comparative quantitative studies of secondary metabolites in diploid versus polyploid genotypes have been conducted on numerous medicinal plants. Although polyploidy often appears to result in increased expression of secondary metabolites, this is not always the case, and the effects of polyploidy are not predictable (Evans, 2002).

The findings of this study do not therefore preclude the possibility of identifying a tetraploid clone with elevated gingerol biosynthesis. In this context it would be of particular interest to monitor experimental tetraploid clones derived from the cultivar ‘Jamaican’, which are currently under development at the Maroochy Research Station.

This study has described the variability of gingerol compounds in Australian commercial and experimental ginger clones. When combined with agronomic data, the present information
should allow for selection of clones with specific levels of pungency, a characteristic which, along with the aroma produced by the essential oil, determines the flavour characteristic of ginger.
5. ESSENTIAL OIL COMPOSITION OF SEVENTEEN GINGER (ZINGIBER OFFICINALE) CLONES

5.1 Introduction

Ginger owes its unique flavour properties to the combination of pungency and aroma. The pungency is provided by non-volatile phenolic compounds, whereas the essential oil gives ginger its characteristic aroma. Ginger rhizome yields two primary extracts: oleoresin and essential (or volatile) oil. The oleoresin is a solvent extract (usually in acetone or ethanol) containing both essential oil and the phenolic compounds responsible for the pungency of ginger, chiefly [6]-gingerol and to a lesser extent [8]- and [10]-gingerol. The corresponding shogaols, which are dehydration products of gingerols formed in heat-treated ginger, are also found in the oleoresin. Ginger oleoresin is used extensively as a flavouring agent in the food and beverage industries.

Commercial ginger oil is normally extracted by steam distillation from dried rhizomes. Typical ginger oil is characterized by a high content of sesquiterpene hydrocarbons, in particular zingiberene, ar-curcumene, β-bisabolene, and β-sesquiphellandrene, while important monoterpenoids normally include geranial, neral, and camphene (Lawrence, 1997; Lawrence, 2000; Martins et al., 2001; Vernin & Parkanyi, 1994). Although these compounds are characteristic of ‘typical’ ginger oils, the literature clearly shows that ginger oil composition is highly variable (Asfaw & Abegaz, 1995; Connell & Jordan, 1971; Ekundayo et al., 1988; Gurib-Fakim et al., 2002; Lawrence, 1995a; Lawrence, 1997; Lawrence, 2000; Macleod & Pieris, 1984; Martins et al., 2001; Menut et al., 1994; Vernin & Parkanyi, 1994). Factors such as geographical origin, whether the oil is distilled from fresh or dried rhizome material, drying process and temperature, and analytical methodology may all contribute to the disparity of published ginger oil analyses.

Ginger ‘oil’ obtained by supercritical fluid extraction using carbon dioxide is also commercially available, but this product differs radically from steam-distilled oil due to the presence of the pungent gingerols and shogaols (Bartley & Jacobs, 2000).
Ginger oil is used in the beverage and fragrance industries, and the world production of ginger oil was estimated at 100-200 t in 2000 (Weiss, 2002). In the classic work, Perfume and Flavor Materials of Natural Origin, Arctander described the odour of ginger oil as “warm, but fresh-woody, spicy and with a peculiar resemblance to orange, lemon, lemon-grass, coriander weed oil, etc. in the initial, fresh topnotes […] the] sweet and heavy undertone is tenacious, sweet and rich, almost balsamic-floral” (Arctander, 1994).

This chapter reports the essential oil composition (analysed by GC-MS) of the clones of ginger previously analysed for their gingerol content (refer to Chapter 4). This is the first comprehensive survey of steam-distilled Australian ginger oils since the early work by Connell and Jordan (Connell & Jordan, 1971).

5.2 Materials and Methods

5.2.1 Plant materials and distillation

Details of the seventeen commercial and experimental clones of ginger included in this study were provided in Section 4.2.1 of the previous chapter. Rhizomes were harvested in late July and stored at ambient temperature for approximately seven weeks before being distilled. Prior to distillation, unpeeled rhizomes were washed and had any diseased tissue removed before being chopped into pieces approximately 1 × 3 × 7 mm. Equal parts of rhizome from three different plants were pooled, and approximately 200 g of this material was hydrodistilled in a Clevenger distillation apparatus for three hours.

5.2.2 Chemical analysis

Oils were analyzed on an Agilent Technologies (Palo Alto, California) 6890/5973 GC-MSD system using helium as the carrier gas at a constant linear flow velocity of 29 cm/s. Oil samples (150 µL) were diluted with pentane (1500 µL) and 1 µL of this solution was injected. The column was a 50 m × 0.22 mm capillary column, 1.00 µm film thickness (BPX-5, SGE Ltd., Melbourne). The split ratio was 25:1. The column oven was programmed from 70°C to 280°C at a ramp rate of 4°C/min (final hold time 4 minutes), and
the injector temperature was 250°C. Composition values were recorded as percentage area based on the total ion current chromatogram. Retention indices were determined using a C-8 to C-22 n-alkane mixture. Compound identification was based on comparisons with mass spectra and retention indices of authentic reference compounds where possible and by reference to WILEY275, NBS75K and Adams terpene library (Adams, 1995) and published data. Myrcene, 1,8-cineole, linalool, α-terpineol, β-citronellol, citral, geraniol, (–)-bornyl acetate, citronellol acetate, geranyl acetate and (E)/(Z)-nerolidol were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI); (–)-borneol and (Z)-nerolidol were obtained from Fluka Chemie (Buchs, Switzerland); 6-methyl-5-hepten-2-one was obtained from ants (Formicidae) (Tomalski et al., 1987) and (E,E)-α-farnesene from the peel of ‘Granny Smith’ apples (Pechous & Whitaker, 2004). Germacrene-D and elemol were identified by comparison with these compounds in authentic clary sage (Salvia sclarea L.) oil (Anonymous, 2004) and elemi (Canarium luzonicum (Miq.) Asa Gray; Berjé Inc., Bloomfield NJ) oil (Villanueva et al., 1993), respectively.

5.2.3 Statistical analysis

Statistical analyses were performed using SPSS (Chicago, Illinois) for Windows Release 11.5. Mean values, standard deviations and ranges were calculated for major oil constituents. The oil composition of the 17 clones was the subject of principal components analysis and cluster analysis based on the ten most abundant constituents. The relationship between neral and geranial was examined by way of a scatter plot and Pearson’s correlation coefficient.

5.3 Results

5.3.1 Oil composition

The essential oil composition for the 17 clones is shown in Table 5-1. The mean, standard deviation and range for the 14 most abundant constituents in the 16 homogenous or ‘typical’ clones are shown in Table 5-2, which also gives the percentage content for the atypical oil from the cultivar ‘Jamaican’ (Z46). The ‘typical’ oils had a mean citral (neral + geranial)
content of 58%, whilst the five major sesquiterpene hydrocarbons typically found in ginger oil (ar-curcumene, \((E,E)\)-\(\alpha\)-farnesene, zingiberene, \(\beta\)-bisabolene and \(\beta\)-sesquiphellandrene (Lawrence, 1995b)) made up only 17%. In contrast, the oil from ‘Jamaican’ had a comparatively low citral content (28%) and contained 35% of the main sesquiterpene hydrocarbons. Some of the major oil constituents are shown in Fig. 5-1.

Fig. 5-1. Volatile constituents from *Zingiber officinale* essential oil.
### Table 5.1: Composition of essential oils of 17 clones of ginger analyzed by GC-MS on a BPX-5 column

| Compound | RI  | 0.00 | 0.03 | 0.05 | 0.07 | 0.09 | 0.12 | 0.15 | 0.18 | 0.20 | 0.22 | 0.24 | 0.26 | 0.28 | 0.30 | 0.32 | 0.34 | 0.36 | 0.38 | 0.40 | 0.42 | 0.44 | 0.46 | 0.48 | 0.50 |
|----------|-----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| general  |     | 112  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| eugenol  |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| p-eugenol |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| p-eugenol dimuente |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| p-epigallocatechin |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-1 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-2 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-3 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-4 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-5 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-6 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-7 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-8 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-9 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-10 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-11 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-12 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-13 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-14 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-15 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-16 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| epigallocatechin-17 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |

**Note:** Mass spectral data and retention index (degradation resistance). Values are percentage content. RI: Retention index. Mass spectral data and retention index compared with those of reference compound b, based on GC-MS on a BPX-5 column.
Table 5-2. Content of 14 constituents in essential oils of 16 ‘typical’ clones of ginger and one ‘atypical’ clone, ‘Jamaican’ (Z46).

Mean, standard deviation and range are shown for the 16 clones. All values are percentage content.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>16 ‘typical’ clones mean±SD (range)</th>
<th>‘Jamaican’ (Z46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,8-cineole</td>
<td>1.52±0.52 (0.39-2.63)</td>
<td>0.79</td>
</tr>
<tr>
<td>linalool</td>
<td>1.21±0.20 (0.97-1.55)</td>
<td>1.02</td>
</tr>
<tr>
<td>borneol</td>
<td>2.41±0.39 (1.84-2.94)</td>
<td>3.91</td>
</tr>
<tr>
<td>α-terpineol</td>
<td>1.81±0.21 (1.49-2.18)</td>
<td>1.13</td>
</tr>
<tr>
<td>citronelol</td>
<td>1.93±0.28 (1.48-2.49)</td>
<td>1.09</td>
</tr>
<tr>
<td>neral</td>
<td>21.44±1.63 (19.39-26.49)</td>
<td>10.60</td>
</tr>
<tr>
<td>geraniol</td>
<td>4.91±1.28 (2.73-7.30)</td>
<td>1.54</td>
</tr>
<tr>
<td>geranial</td>
<td>36.50±3.26 (31.29-44.31)</td>
<td>17.51</td>
</tr>
<tr>
<td>geranyl acetate</td>
<td>2.02±0.92 (0.52-3.45)</td>
<td>0.26</td>
</tr>
<tr>
<td>ar-curcumene</td>
<td>3.24±0.73 (2.43-5.31)</td>
<td>5.72</td>
</tr>
<tr>
<td>(E,E)-α-farnesene</td>
<td>3.02±0.68 (2.10-4.30)</td>
<td>4.35</td>
</tr>
<tr>
<td>zingiberene</td>
<td>4.82±2.03 (1.86-9.00)</td>
<td>11.24</td>
</tr>
<tr>
<td>β-bisabolene</td>
<td>1.51±0.31 (0.97-2.16)</td>
<td>4.05</td>
</tr>
<tr>
<td>β-sesquiphellandrene</td>
<td>4.36±0.85 (2.93-5.62)</td>
<td>9.40</td>
</tr>
</tbody>
</table>

5.3.2 Principal components analysis

The percentage composition of the 17 oil samples was subjected to principal components analysis based on the ten most abundant oil constituents (borneol, neral, geraniol, geranial, geranyl acetate, ar-curcumene, (E,E)-α-farnesene, zingiberene, β-bisabolene and β-sesquiphellandrene). This analysis revealed the presence of three components with eigenvalues exceeding one. These components explained 61.6%, 18.8% and 10.7% of the variability, respectively. Two components (together explaining 80% of the variability) were retained for further investigation, and Varimax rotation was performed to assist in the interpretation (Table 5-3).
Table 5-3. Varimax rotated component matrix for two component solution for the ten most abundant ginger essential oil constituents.

<table>
<thead>
<tr>
<th>Component</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>neral</td>
<td>-.96</td>
<td>-.25</td>
</tr>
<tr>
<td>geranial</td>
<td>-.96</td>
<td>-.15</td>
</tr>
<tr>
<td>β-bisabolene</td>
<td>.89</td>
<td>.39</td>
</tr>
<tr>
<td>borneol</td>
<td>.87</td>
<td>-.21</td>
</tr>
<tr>
<td>β-sesquiphellandrene</td>
<td>.78</td>
<td>.61</td>
</tr>
<tr>
<td>ar-curcumene</td>
<td>.61</td>
<td>.30</td>
</tr>
<tr>
<td>zingiberene</td>
<td>.55</td>
<td>.54</td>
</tr>
<tr>
<td>geraniol</td>
<td>-.23</td>
<td>-.89</td>
</tr>
<tr>
<td>geranyl acetate</td>
<td>.12</td>
<td>-.87</td>
</tr>
<tr>
<td>(E,E)-α-farnesene</td>
<td>.45</td>
<td>.74</td>
</tr>
<tr>
<td>Percentage of variance explained</td>
<td>49.3%</td>
<td>31.2%</td>
</tr>
</tbody>
</table>

The rotated solution revealed a complex structure in which both components had several strong loadings (coefficients relating the variables to the components), but some compounds loaded substantially on both components. Neral, geranial, β-bisabolene, borneol and β-sesquiphellandrene were strongly associated with component 1, indicating a high degree of interrelationship (positive or negative) between the concentrations of these compounds. Similarly, geraniol, geranyl acetate and (E,E)-α-farnesene were strongly associated with component 2. More broadly, an inverse relationship between levels of citral (geranial + neral) and the sesquiterpene hydrocarbons (zingiberene, ar-curcumene, β-sesquiphellandrene, β-bisabolene and (E,E)-α-farnesene) was evident by inspection of the component plot in Fig. 5-2.
5.3.3 Cluster analysis

In order to examine the degree of similarity displayed by the 17 clones in terms of oil composition, a hierarchical, between-groups linkage, cluster analysis based on the ten most abundant constituents was performed. This is a multivariate procedure that allows for the classification of cases (or variables) into groups based on Euclidian distances between cases. For each constituent, percentage values were rescaled to have a mean value of one, so that all constituents were equally weighted for the purpose of the analysis, and Euclidian distances were calculated between pairs of clones.

Fig. 5-3 shows a dendrogram of the 17 essential oils. This diagram confirms the unique nature of the essential oil from the cultivar ‘Jamaican’ (Z46) when compared with oils from the other 16 clones. The dendrogram also shows that the oil from the clone Z22 stands out from the others. This oil had an extremely high citral content (71%). The remaining 15
clones fall into two clusters. The similarity between these two clusters was examined using a multivariate general linear model, comparing the set of clones in Cluster 1 (Z25, Z32, Z33, Z27, Z26, Z31, Z30, Z58, Z28, Z29, Z45) and Cluster 2 (Z24, Z44, Z47, Z23). Cluster 1 and Cluster 2 showed a near-significant difference (Wilks’ Lambda = 0.064; F = 5.838; df = 10 and 4; p = 0.052). In terms of individual constituents, six (borneol, geraniol, geranyl acetate, (E,E)-α-farnesene, β-bisabolene, β-sesquiphellandrene) were significantly different between clusters at p ≤ 0.05 and four (neral, geranial, ar-curcumene, zingiberene) were not.

Fig. 5-3. Dendrogram of hierarchical cluster analysis of 17 essential oils of ginger.

The diagram shows average linkage (between groups), and values shown along the horizontal axis are Euclidian distances rescaled to an arbitrary scale showing the levels of relative similarity where clusters join.

5.3.4 Citral content

The ginger oils analyzed in the present study had citral contents ranging from 28% in the ‘Jamaican’ cultivar (Z46) to 71% in the tetraploid clone Z22. The mean citral content of the 16 ‘typical’ ginger oils (‘Jamaican’ excluded) was 57.9±4.9% (range: 50.7-70.8%), which was more than double the corresponding value for ‘Jamaican’ (28%). A similar trend was
evident for geraniol, which is a precursor to citral, with a mean concentration of 4.9%±1.3% in the ‘typical’ oils compared with 1.5% in ‘Jamaican’.

**5.3.5 Neral to geranial ratio**

The 17 ginger oils contained neral and geranial in a ratio that was remarkably constant. The neral to geranial ratio ranged from 0.55 to 0.64, with a mean value of 0.61±0.01. This fixed, linear relationship between the two isomers, which persisted regardless of the percentage content of citral, is illustrated in Fig. 5-4. The very strong correlation between the two compounds is quantified by the Pearson’s correlation coefficient of 0.987 (p<0.001).

It is particularly interesting to note that the neral to geranial ratio also was 0.6 in the cultivar ‘Jamaican’ (Z46), which otherwise yielded an oil that was distinctly different from those of the other 16 clones.

![Fig. 5-4. Scatter plot showing the relationship between the percentage content of the stereoisomers neral and geranial in essential oils from 17 clones of ginger.](image-url)
5.4 Discussion

Essential oils of 17 clones of Australian ginger were prepared by hydrodistillation of rhizomes and analyzed by GC-MS. Compared with values in the literature (Asfaw & Abegaz, 1995; Connell & Jordan, 1971; Ekundayo et al., 1988; Gurib-Fakim et al., 2002; Lawrence, 1995b; Lawrence, 1997; Lawrence, 2000; Macleod & Pieris, 1984; Martins et al., 2001; Menut et al., 1994; Vernin & Parkanyi, 1994), all the samples were characterized by a very high citral content (28% or greater) and a relatively low content of sesquiterpene hydrocarbons.

5.4.1 Oil composition

There was no distinct difference in the composition of the oils of the tetraploid clones compared with the diploid parent cultivar ‘Queensland’ or the cultivars ‘Canton’ and ‘Brazilian’. These oils were characterized by very high levels of citral (geranial + neral) (51-71%) and relatively low levels of the sesquiterpene hydrocarbons characteristic of ginger oil (Lawrence, 1995b), namely zingiberene (4.8±2.0%), ar-curcumene (3.2±0.7%), β-sesquiphellandrene (4.4±0.9%), β-bisabolene (1.5±0.3%) and (E,E)-α-farnesene (3.0±0.7%). These values contrast with those reported in 1971 by Connell and Jordan, whose analysis of 35 Australian ginger oils found citral levels ranging from 4 to 30% and much higher concentrations of sesquiterpene hydrocarbons (zingiberene 20-28%, ar-curcumene 6-10%, β-sesquiphellandrene 7-11% and β-bisabolene 5-9% (but no (E,E)-α-farnesene) (Connell & Jordan, 1971). The results obtained in the present study are better aligned with more recent analyses of ginger oils from Mauritius (Gurib-Fakim et al., 2002), Sao Tomé e Príncipe (Martins et al., 2001) and Nigeria (Ekundayo et al., 1988) by being high in citral, relatively low in the major sesquiterpene hydrocarbons and by containing (E,E)-α-farnesene. In general, the published values for these compounds vary greatly; whether this is due to true natural variability or differences in raw material (fresh or dried, time of harvest), distillation conditions or analytical methodologies is uncertain.

An inverse relationship between levels of citral on the one hand and β-bisabolene, β-sesquiphellandrene and borneol on the other was demonstrated by principal components analysis. Likewise, an inverse relationship was shown to exist between geraniol/geranyl
acetate and \((E,E)\)-\(\alpha\)-farnesene. Terpenoids are derived from \(C_5\) isoprene units in the form of the diphosphate (pyrophosphate) esters dimethylallyl diphosphate and isopentenyl diphosphate and are products of the mevalonate pathway (Dewick, 1997). The common biosynthetic origin of monoterpenoids (such as geranial, neral, geraniol and geranyl acetate) and sesquiterpenoids (including \(\beta\)-bisabolene, \(\beta\)-sesquiphellandrene and \((E,E)\)-\(\alpha\)-farnesene) provides a possible explanation for the inverse relationship seen between mono- and sesquiterpenoids, as the pathways to mono- and sesquiterpenes would compete for common precursors. Similarly, the fact that citral and borneol share the monoterpene precursor geranyl pyrophosphate could explain the inverse relationship observed between these compounds.

The oils analysed in this study were prepared from fresh rhizomes seven weeks post-harvest by a short distillation process and analysed within a short period of time. They may differ significantly from oils prepared from dried rhizome material by longer distillation processes followed by prolonged storage under conditions that favour oxidation. It has been reported that both zingiberene and \(\beta\)-sesquiphellandrene can undergo oxidation to \(\alpha\)-curcumene (Connell & Jordan, 1971; Govindarajan, 1982a), and high levels of this compound may therefore be indicative of a degraded oil.

The essential oil from the cultivar ‘Jamaican’ (Z46) differed distinctly in composition from all the others, primarily by having lower citral content and higher sesquiterpene hydrocarbon content. This oil contained 28\% citral while the total amount of the five main sesquiterpene hydrocarbons was 35\%, which is more than double the mean concentration found in the 16 other clones. The distinctiveness of the oil of ‘Jamaican’ was confirmed by hierarchical cluster analysis. As reported in Chapter 4, this cultivar was also the most pungent of the 17 clones tested due to its high content of gingerols. The dissimilar essential oil composition coupled with its high pungency gives ‘Jamaican’ flavour and aroma qualities that are distinct from the other clones examined.

Cluster analysis identified one other distinct clone (Z22), characterized by extremely high (71\%) citral content, while the remaining clones fell into two clusters. These two clusters did not appear to reflect breeding history, as the different tetraploid clones produced from the same parent cultivar (‘Queensland’) were split between the two clusters, as were the other two cultivars, ‘Brazilian’ and ‘Canton’.
5.4.2 Citral content

The ginger oils analysed in this study contained remarkably high levels of citral (above 50% in all samples except ‘Jamaican’ and up to 71% in one sample). The highest citral content described previously in ginger oil was 56% in a sample from Fiji (Smith & Robinson, 1981). Although Australian ginger has a reputation for its ‘lemony’ aroma, a 1971 study of 35 Australian ginger oils by Connell and Jordan found considerably lower citral levels, ranging from 4% to 30% (Connell & Jordan, 1971). The same study found only very low citral levels in oils from other parts of the world, and many other studies have reported very low citral levels in non-Australian ginger oils (Asfaw & Abegaz, 1995; Humphrey et al., 1993; Lawrence, 1995b; Lawrence, 2000; Macleod & Pieris, 1984). Conversely, several more recent investigations have found substantial citral levels in ginger oils from other parts of the world, for example from the Central African Republic (35%) (Menut et al., 1994), Mauritius (27%) (Gurib-Fakim et al., 2002), Sao Tomé e Príncipe (22-25%) (Martins et al., 2001), Nigeria (24%) (Ekundayo et al., 1988) and Tahiti (14-25%) (Lawrence, 1997; Lechat-Vahirua et al., 1996).

There are several possible, not mutually exclusive, explanations for these somewhat incongruent findings regarding citral content. One relates to the changes in gas chromatography technology (especially detection systems) since the early 1970s, which might explain some of the differences between the results of the current study and those of Connell and Jordan (Connell & Jordan, 1971). It is also likely that ginger oils distilled from fresh rhizomes generally have a higher citral content than oils obtained from dried rhizomes. The notion that sun drying and storage of ginger could cause a loss of citral was put forward by Govindarajan more than two decades ago (Govindarajan, 1982a), and studies from Sri Lanka (Macleod & Pieris, 1984) and Nigeria (Ekundayo et al., 1988) have documented citral losses in ginger oils ranging from 40% to 74%, when rhizomes were dried before distillation. A significant difference in citral content has also been observed in supercritical fluid extracts of fresh and dried Australian ginger, which contained 19.9% and 6.2% citral, respectively (Bartley & Jacobs, 2000). These data would seem to suggest that many ginger oils contain considerable amounts of citral, provided they are distilled from fresh rhizomes or rhizomes dried at low temperature. On the other hand, the existence of genuine low-citral genotypes of ginger is supported by a recent study from India, where the essential oil distilled from fresh rhizomes contained less than 4% citral (Singh et al., 2005).
A number of other factors could also have contributed to the unexpectedly high citral values obtained in this study. They include the particular growth period (8 months), the harvest time (mid-winter), the 7-week delay between harvest and distillation, and the conditions of cultivation (including climate) at Southern Cross University, which is located some 250 km south of the commercial ginger-growing area in Australia.

### 5.4.3 Neral to geranial ratio

Neral (Z-citral) and geranial (E-citral) are stereoisomers occurring in many plants in mixtures often referred to simply as citral. The neral to geranial ratios found in ginger oils in this study were remarkably constant and close to 2:3 (mean 0.61±0.01, range 0.55-0.64). The ratios reported in the literature for ginger oil vary greatly, from 0.3 in a Taiwanese oil (Lawrence, 1995b) to 4.7 in a Chinese oil (Lawrence, 2000). However, a ratio of 0.6 was also reported for a Mauritian oil made from fresh rhizomes (Gurib-Fakim et al., 2002), a Nigerian oil also prepared from fresh rhizomes (Ekundayo et al., 1988) and an oil made from dried rhizomes from Sao Tomé e Principe (Martins et al., 2001), while oils from Madagascar (Möllenbeck et al., 1997) and the Central African Republic (Menut et al., 1994) were reported to contain neral to geranial ratios of 0.4 and 0.9, respectively. The data obtained in the current study support the hypothesis that neral and geranial occur in ginger in a relatively fixed ratio of approximately 2:3 and calls into question the validity of analyses suggestive of very different ratios.

Neral and geranial also occur in other plants in a similar ratio. Species of lemongrass produce essential oil with a citral content typically exceeding 70%. Nine samples of West Indian lemongrass (*Cymbopogon citratus*, family Poaceae) oil from Africa, China, New Zealand and Cuba had neral to geranial ratios ranging from 0.61 (New Zealand) to 0.96 (China) with a mean of 0.76, while four Indian cultivars of East Indian lemongrass (*C. flexuosus*) produced oils with a neral to geranial ratio ranging from 0.61 to 0.67 (Lawrence, 2002).

This study has shown that essential oil from the three ginger cultivars ‘Queensland’, ‘Canton’ and ‘Brazilian’ and twelve tetraploid clones derived from ‘Queensland’ had a high degree of compositional similarity and were characterized by a very high citral content. Tetraploid clones were shown to have retained the characteristics of the parent cultivar in
terms of essential oil composition. In contrast, the cultivar ‘Jamaican’ produced an oil that was distinctly different, characterized by a lower citral content and higher levels of sesquiterpene hydrocarbons. The distinctive aroma profile of this cultivar, which also contains significantly higher levels of pungent gingerols, should make it of commercial interest to the flavour and fragrance industries.
6. **PHARMACOLOGICAL ACTIVITY OF ZINGIBERACEAE SPECIES**

6.1 Introduction

Forty-one taxa were screened for inhibition of prostaglandin E\(_2\) (PGE\(_2\)) production. Some of the samples were also screened for antioxidant activity in the oxygen radical absorbance (ORAC) assay, for inhibition of nitric oxide production, and for modulation of natural killer cell activity. This work should be regarded as a preliminary survey, and it was constrained by the cost of some of the assays.

Prostaglandin E\(_2\) is a largely pro-inflammatory eicosanoid produced from arachidonic acid by the action of cyclooxygenase (COX). COX, in particular the isoform COX-2, is an important pharmacological target for the mitigation of inflammation, which is an underlying pathological mechanism in many chronic diseases including arthritic disorders, cardiovascular disease, diabetes, some types of cancer, and osteoporosis (Libby, 2007). Salicylates and other non-steroidal anti-inflammatory drugs target COX, and many plant extracts and compounds have been shown to inhibit COX and the generation of PGE\(_2\) (Calixto *et al*., 2003; Calixto *et al*., 2004). Prostaglandins and COX were described in more detail in Chapter 1.

Reactive oxygen species and oxidative stress have been linked to a large number of pathologies including inflammation, cancer, cardiovascular disease, Parkinson’s disease and Alzheimer’s disease, and the importance of antioxidant defenses (both endogenous and exogenous) is well recognised (Gilbert, 1999). Higher plants are rich sources of antioxidant compounds (well known examples are carotenoids and polyphenols), and plant extracts with potent antioxidant activity are of interest as sources of potential therapeutic substances.

Nitric oxide (NO) is a free radical with numerous functions in both normal and pathological processes. Nitric oxide is produced by the oxidation of L-arginine by inducible nitric oxide synthase (iNOS) and is involved in pathological processes associated with an overproduction of nitric oxide including inflammatory disorders of the joint, gut and lungs. Nitric oxide functions as a messenger molecule in the immune system, and many immune cells produce
and respond to nitric oxide (Morikawa et al., 2005; Sharma et al., 2007; Tripathi et al., 2007). Nitric oxide and iNOS play key roles in the up-regulation of the inflammatory response, and nitric oxide mediates many of the destructive effects of interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-α) on cartilage in osteoarthritis. The inhibition of nitric oxide and iNOS therefore represent an attractive therapeutic target in inflammatory conditions (Cuzzocrea, 2006; Sharma et al., 2007; Vuolteenaho et al., 2007).

Inhibition of nitric oxide production and/or iNOS expression has been demonstrated for a number of medicinal plants and plant compounds in recent years, for example *Andrographis paniculata* (Batkhuu et al., 2002), *Salvia miltiorrhiza* (Jang et al., 2003), *Hypericum perforatum* (Di Paola et al., 2007), *Citrus reticulata* (Jung et al., 2007) as well as the reishi mushroom (*Ganoderma lucidum*) (Woo et al., 2005). It is widely considered that this property likely contributes to the anti-inflammatory activity of these plants.

Natural killer (NK) cells are specialised bone marrow-derived lymphocytes that form part of the innate immune system. NK cells have the ability to lyse virally infected and tumour cells. They comprise approximately 5-20% of the peripheral lymphocyte population in the peripheral blood, liver and spleen and are present at lower frequencies in the lymph nodes, bone marrow, and thymus. The anticancer activity of NK cells in the form of tumour rejection has been well established in a variety of animal models of cancer. In addition to their anti-tumour and anti-viral activity, NK cells are also involved in other pathophysiological processes such as inflammation and autoimmunity, and stimulated NK cells can produce cytokines including interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), and granulocyte-macrophage colony stimulating factor (GM-CSF) (Wallace & Smyth, 2005; Yokoyama et al., 2004).

Little has been published on the effects of medicinal plant extracts on NK cell activity. Medicinal plants for which an ability to increase NK cell activity has been demonstrated include garlic (*Allium sativum*) (Abuharfeil et al., 2001), *Viscum album* (Schink, 1997), *Echinacea* spp. (Currier & Miller, 2000; Zhai et al., 2007) and *Nigella sativa* (Abuharfeil et al., 2001).
6.2 Materials and Methods

6.2.1 Plant materials

A total of 53 samples representing rhizome extracts of 43 Zingiberaceae taxa were included in this study, although not all taxa were screened in all assays. Plant material was obtained from a variety of sources. Nineteen samples were originally obtained from the live collection in the George Brown Darwin Botanic Garden, Darwin, Northern Territory (latitude S 12° 26'; longitude E 130° 50'), and a number of these were cultivated in pots at Southern Cross University, Lismore, New South Wales (latitude S 28° 49'; longitude E 153° 18') before harvest of the rhizome material. Twelve samples were obtained from the living collection in the Flecker Botanic Gardens, Cairns, Queensland (latitude S 16° 54'; longitude E 145° 45'). Z129 Alpinia arctiflora, Z127 Alpinia modesta, Z126 Hornstedtia scottiana, Z128 Pleuranthodium racemigerum, and Z125 Tapeinochilos ananassae were collected in the wild in the Wet Tropics region of North Queensland under a permit issued by the Queensland Environmental Protection Agency (voucher specimens were deposited in the Queensland Herbarium; voucher number AQ736264, AQ736265, AQ736255, AQ736263, AQ736256, respectively). Z107 Etlingera australasica was obtained from a tub specimen grown in Brisbane, Queensland from wild stock collected at Bog Hole Creek, Silver Plains, Queensland (latitude S 13° 42'; longitude E 143° 29') by Paul Forster of the Queensland Herbarium (PIF30425 (BRI)). Z45 Zingiber officinale ‘Queensland’ rhizome stock was obtained from the Queensland Department of Primary Industries & Fisheries Maroochy Research Station at Nambour, Queensland (latitude S 26° 37'; longitude E 152° 57') and grown in raised beds at Southern Cross University. J-2 and J-4-4 Zingiber officinale ‘Jamaican’ were grown at the Maroochy Research Station. Z108/9 Zingiber officinale was obtained from a produce store in Lismore, NSW. Twelve samples were obtained from the living collections of the Medicinal Plant Garden and the Lismore campus grounds of Southern Cross University.

Table 6-1 provides a list of the samples, their origin and methods of extraction.
Table 6-1. Zingiberaceae samples screened for biological activity.

<table>
<thead>
<tr>
<th>ID</th>
<th>Taxon</th>
<th>Source</th>
<th>Extraction method³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z129$</td>
<td><em>Alpinia arctiflora</em> (F. Muell.) Benth.</td>
<td>Danbulla State Forest, c. 18 km WSW of Gordonvale, North Qld. (Qld Herbarium AQ736264)</td>
<td>3</td>
</tr>
<tr>
<td>Z13^</td>
<td><em>Alpinia caerulea</em> (R. Br.) Benth.</td>
<td>Darwin Botanic Garden (D94418)</td>
<td>1</td>
</tr>
<tr>
<td>Z102#</td>
<td><em>Alpinia caerulea</em> (R. Br.) Benth.</td>
<td>Southern Cross University (NCM03-029)</td>
<td>2</td>
</tr>
<tr>
<td>Z49^</td>
<td><em>Alpinia calcata</em> Roscoe</td>
<td>Southern Cross University</td>
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<td>Z07^</td>
<td><em>Alpinia galanga</em> (L.) Swartz</td>
<td>Darwin Botanic Garden (D94418)</td>
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<tr>
<td>Z103#</td>
<td><em>Alpinia galanga</em> (L.) Swartz</td>
<td>Southern Cross University (NCM04-002)</td>
<td>2, 5</td>
</tr>
<tr>
<td>Z111</td>
<td><em>Alpinia luteocarpa</em> Elmer</td>
<td>Flecker Botanic Gardens, Cairns</td>
<td>3</td>
</tr>
<tr>
<td>Z48^</td>
<td><em>Alpinia malaccensis</em> Roscoe</td>
<td>Southern Cross University</td>
<td>1</td>
</tr>
<tr>
<td>Z127$</td>
<td><em>Alpinia modesta</em> Schumann</td>
<td>Nyleta Creek, 16 km NNE of Tully, North Qld. (Qld Herbarium AQ736265)</td>
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<tr>
<td>Z08*</td>
<td><em>Alpinia mutica</em> Roxb.</td>
<td>Darwin Botanic Garden (D950346)</td>
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<tr>
<td>Z11*</td>
<td><em>Alpinia purpurea</em> ‘Eileen McDonald’</td>
<td>Darwin Botanic Garden (D93105)</td>
<td>1</td>
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<tr>
<td>Z53^</td>
<td><em>Alpinia spectabile</em> Griff. ‘Giant Orange’</td>
<td>Southern Cross University</td>
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<tr>
<td>Z52^</td>
<td><em>Alpinia zerumbet</em> (Pers.) B.L. Burtt &amp; R. M. Sm.</td>
<td>Southern Cross University</td>
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<tr>
<td>Z104++</td>
<td><em>Boesenbergia rotunda</em> (L.) Mansf.</td>
<td>Southern Cross University (NCM04-121)</td>
<td>2, 6</td>
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<tr>
<td>Z03^</td>
<td><em>Costus barbatus</em> Suess.</td>
<td>Darwin Botanic Garden (D971488)</td>
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<tr>
<td>Z112</td>
<td><em>Costus barbatus</em> Suess.</td>
<td>Flecker Botanic Gardens, Cairns</td>
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<tr>
<td>Z113</td>
<td><em>Costus leucanthus</em> Maas</td>
<td>Flecker Botanic Gardens, Cairns</td>
<td>3</td>
</tr>
<tr>
<td>Z114</td>
<td><em>Costus malortieanus</em> H. Wendl.</td>
<td>Flecker Botanic Gardens, Cairns</td>
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<tr>
<td>Z14^</td>
<td><em>Costus productus</em> Gleason ex Maas</td>
<td>Darwin Botanic Garden (D971499)</td>
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<tr>
<td>Z116</td>
<td><em>Costus productus</em> Gleason ex Maas</td>
<td>Flecker Botanic Gardens, Cairns</td>
<td>3</td>
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<tr>
<td>Z115</td>
<td><em>Costus pulverulentus</em> C. Presl</td>
<td>Flecker Botanic Gardens, Cairns</td>
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<tr>
<td>Z15*</td>
<td><em>Costus tappenbeckianus</em> J. Braun &amp; K. Schum.</td>
<td>Darwin Botanical Garden (D971491)</td>
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<tr>
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<td><em>Curcuma australasica</em> J.D. Hook</td>
<td>Southern Cross University (NCM03-036)</td>
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<tr>
<td>Z101#</td>
<td><em>Curcuma australasica</em> J.D. Hook</td>
<td>Southern Cross University (NCM03-036)</td>
<td>2, 5</td>
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<tr>
<td>Z117</td>
<td><em>Curcuma cordata</em> Wallich</td>
<td>Flecker Botanic Gardens, Cairns</td>
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<tr>
<td>Z18^</td>
<td><em>Curcuma longa</em> L.</td>
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<td>Z106#</td>
<td><em>Curcuma longa</em> L.</td>
<td>Southern Cross University (NCM04-013)</td>
<td>2, 5</td>
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<tr>
<td>Z118</td>
<td><em>Curcuma parviflora</em> Wallich</td>
<td>Flecker Botanic Gardens, Cairns (NCM05-040)</td>
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<tr>
<td>Z12^</td>
<td><em>Elettaria cardamomum</em> (L.) Maton</td>
<td>Darwin Botanic Garden (D95162)</td>
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<tr>
<td>Z107</td>
<td><em>Ettlingera australasica</em> (R.M. Smith) R.M. Smith</td>
<td>Brisbane (PIF30425 (BRI))</td>
<td>2, 5</td>
</tr>
<tr>
<td>Z04*</td>
<td>Etlingera elatior (Jack) R.M. Smith</td>
<td>Darwin Botanic Garden (D95335)</td>
<td>1</td>
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<tr>
<td>Z10^</td>
<td>Hedychium coronarium Koenig</td>
<td>Darwin Botanic Garden (D960188)</td>
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<tr>
<td>Z126$</td>
<td>Horntedtia scottiana (F.Muell.) Schumann</td>
<td>Nyleta Creek, 16 km NNE of Tully, North Qld. (Qld Herbarium AQ736255)</td>
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<tr>
<td>Z05*</td>
<td>Kaempferia galanga L.</td>
<td>Darwin Botanic Garden (D970234)</td>
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<tr>
<td>Z120</td>
<td>Kaempferia rotunda L.</td>
<td>Flecker Botanic Gardens, Cairns</td>
<td>3</td>
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<tr>
<td>Z128$</td>
<td>Pleuranthodium racemigerum (F. Muell.) R.M. Smith</td>
<td>Gillies Range, c. 13 km NE of Yungaburra, North Qld. (Qld Herbarium AQ736263)</td>
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<tr>
<td>Z20*</td>
<td>Pleuranthodium racemigerum (F. Muell.) R.M. Smith</td>
<td>Darwin Botanic Garden (D971598)</td>
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<tr>
<td>Z121</td>
<td>Renealmia cernua (Sw. ex Roem. &amp; Schult.) J.F. Macbr.</td>
<td>Flecker Botanic Gardens, Cairns</td>
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<tr>
<td>Z01*</td>
<td>Scaphochlamys biloba (Ridley) Holttum</td>
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<td>Z16*</td>
<td>Scaphochlamys kunstleri (Baker) Holttum</td>
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<td>Z122</td>
<td>Scaphochlamys kunstleri (Baker) Holttum</td>
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<td>Z09*</td>
<td>Tapeinochilos ananassae (Hassk.) K. Schum.</td>
<td>Darwin Botanic Garden (D971993)</td>
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<td>Z125$</td>
<td>Tapeinochilos ananassae (Hassk.) K. Schum.</td>
<td>Mt. Bellenden Ker, North Qld. (Qld Herbarium AQ736256)</td>
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<td>Z06*</td>
<td>Zingiber longipedunculatum Ridley</td>
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<tr>
<td>Z45^</td>
<td>Zingiber officinale Roscoe 'Queensland'</td>
<td>Maroochy Research Station, Nambour, Qld.</td>
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</tr>
<tr>
<td>Z108/9</td>
<td>Zingiber officinale Roscoe</td>
<td>Purchased fresh in produce store, Lismore (NCM04-207)</td>
<td>2, 5</td>
</tr>
<tr>
<td>J-2</td>
<td>Zingiber officinale Roscoe ‘Jamaican’, diploid</td>
<td>Maroochy Research Station, Nambour, Qld.</td>
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</tr>
<tr>
<td>J-4-4</td>
<td>Zingiber officinale Roscoe ‘Jamaican’, tetraploid</td>
<td>Maroochy Research Station, Nambour, Qld.</td>
<td>4</td>
</tr>
<tr>
<td>Z124</td>
<td>Zingiber ottensii Valeton</td>
<td>Flecker Botanic Gardens, Cairns</td>
<td>3</td>
</tr>
<tr>
<td>Z02*</td>
<td>Zingiber montanum (J. König) Link ex. A. Dietr.</td>
<td>Darwin Botanic Garden (D970237)</td>
<td>1</td>
</tr>
<tr>
<td>Z105</td>
<td>Zingiber montanum (J. König) Link ex. A. Dietr.</td>
<td>Southern Cross University (NCM04-122)</td>
<td>2, 5</td>
</tr>
<tr>
<td>Z17</td>
<td>Zingiber spectabile</td>
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<td>Z50</td>
<td>Zingiber spectabile</td>
<td>Southern Cross University</td>
<td>1</td>
</tr>
<tr>
<td>Z55^</td>
<td>Zingiber ‘Dwarf Apricot’</td>
<td>Southern Cross University</td>
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<tr>
<td>Z19*</td>
<td>Zingiber/Etlingera ‘Aniseed Ginger’</td>
<td>Darwin Botanic Garden (D95331)</td>
<td>1</td>
</tr>
</tbody>
</table>

* refer to Section 6.2.2 for details of extraction methods; ^: cultivated in tubs at Southern Cross University; #: cultivated in the grounds of Southern Cross University; *: extract prepared from frozen rhizome material; +: ex George Brown Botanical Garden, Darwin; $: collected in the wild under permit.
6.2.2 Extraction methods

Although it would have been ideal for all extracts to have been prepared in the exact same manner, this was not possible for practical reasons. Plant material was obtained over a period of several years from various sources, in fresh, frozen or dried form. In some situations it was also decided to compare extracts made with different solvents. This section details the extraction methods employed.

6.2.2.1 Extraction method 1 (ethanolic)

All extracts were prepared from frozen rhizome material, except that of *Curcuma australasica*, which was prepared from freshly harvested material.

Where possible, material that had been grown at Southern Cross University under uniform conditions was used, but in cases where plants did not grow successfully, a frozen sample of the material grown in Darwin was used (Table 1).

Rhizomes were cleaned of dirt and old leaf bases, where present, were removed. A healthy tissue sample comprising a transection of the rhizome was chopped finely into pieces approximately 1 mm$^3$ in size. To chopped samples weighing between 0.9 g and 7.0 g was added double the mass of 99% ethanol. Samples were then sonicated for 20 minutes (Ultrasonic Cleaner 50 Hz, Unisonics Pty Ltd, Manly Vale, Australia) and subsequently centrifuged for 10 minutes at 4000 rpm (Hettich Universal 16A Centrifuge, Tuttlingen, Germany). The supernatant was retained and filtered through Zymark/Millipore Automation Certified Filters (glassfiber APFB 1.0 μm prefilter, hydrophilic PTFE membrane 0.45 μm).

One mL aliquots of the extracts were taken to dryness on a rotational vacuum concentrator (Christ Alpha 2-4, Osterode, Germany) 1 h at 35°C, 240 mbar; 1 h at 35°C, 150 mbar; 1 h at 35°C, 90 mbar; 4.5h at 45°C, 15 mbar). The resulting dry extracts were dissolved in DMSO to a final concentration of 50 mg/mL.
6.2.2.2 Extraction method 2 (ethanolic)

Fresh rhizomes were sliced and dried at 40°C (except sample Z109 which was dried at 90°C in order to examine the effect of drying temperature). The dried rhizomes were ground to a coarse powder in a Retsch SM 2000 hammermill (Haan, Germany) fitted with a 4 mm mesh and extracted with 10 parts (by mass) ethanol. The plant material was macerated for 24 hours in a stoppered conical flask while being agitated on a Bioline Orbital Shaker BL 4236. The resulting extract was filtered through filter paper (Whatman No. 1) in a Buchner apparatus and the solvent removed under vacuum on a Buechi rotary evaporator (Rotavapor R114, Buechi Labortechnik, Flawil, Switzerland). The dry extract was weighed, redissolved in ethanol to a known concentration and stored at –20°C until use. The dry extract yield as percentage of dry herb mass was calculated.

6.2.2.3 Extraction method 3 (ethanolic)

Fresh rhizomes were sliced and dried at 40°C. The dried rhizomes were ground to a coarse powder in a Waring blender and extracted with 4 parts (by mass) ethanol. The plant material was macerated for 24 hours in a stoppered conical flask while being agitated on a Bioline Orbital Shaker BL 4236. The resulting extract was filtered through filter paper (Whatman No. 1) in Buchner apparatus and stored –20°C. Prior to testing in bioassays a small quantity of extract was dried under a nitrogen stream and resolved to a known concentration.

6.2.2.4 Extraction method 4 (ethanolic)

Approximately 100 g fresh rhizome material (from 3-4 different rhizomes and incorporating different rhizome parts) was chopped finely (approximately 1 mm³ cubes) and macerated with 2 parts (by mass) ethanol in an agitated conical flask for 24 hours as described above. The resulting extract was filtered through filter paper (Whatman No. 1) in Buchner apparatus and stored –20°C. Prior to testing in bioassays a small quantity of extract was dried under a nitrogen stream and resolved to a known concentration.
6.2.2.5 Extraction method 5 (aqueous)

Dried milled rhizome material (5.0 g) was macerated with 25 mL Milli-Q water in a stoppered conical flask placed on an orbital shaker for 6 hours. The resulting extracts were stored at –20°C. Prior to use, the extracts were spun down in a centrifuge, and filtered through a double-layered glass filter-paper and a Millipore Millex-HN Nylon 0.45 μm filter.

6.2.2.6 Extraction method 6 (aqueous)

Freshly harvested rhizome and root tuber (5:1) were crushed in a mortar with a little water and left to macerate for 15 min. The resulting aqueous juice was stored and filtered as described for Extraction method 5.

6.2.3 Bioassay methods

6.2.3.1 Inhibition of PGE2 production

The bioassay was carried out in 96-well plates using Swiss albino mouse embryo fibroblast cells 3T3 (American Type Culture Collection (ATCC), Manassas, VA) and a PGE2 enzyme immuno-assay kit (Prostaglandin E2 EIA Kit – Monoclonal, Cayman Chemical Company, Ann Arbor, MI, Catalog No. 514010).

Cell culture. Murine 3T3 fibroblasts were grown in 96-well plates (Nunclon, Thermo Fisher Scientific, Rochester, NY). The growth medium consisted of Dulbecco’s Modified Eagle Medium (colourless) containing 2 mM L-glutamine, 100 mM pyruvate, 4.5 g/L glucose, 10% (v/v) foetal bovine serum and 100 U/mL each of penicillin G and streptomycin. The concentration of the cell suspension was determined using a cell counter (Beckman Coulter ActDiff Haematology Analyser, Fullerton, CA) and the suspension diluted with growth medium to give a cell concentration of $2 \times 10^5$ cells/mL. 90 μL of this suspension was pipetted into each well and the plate incubated at 37°C and 5% CO2 (Sanyo CO2 MCO-17 AIC Incubator). After incubation for approximately 20 hours, plant extracts and controls were added to the plate. In experiment series A plant extracts were tested at two concentrations (50 mg/mL and 0.5 mg/mL, giving final well concentrations of 5000 μg/mL and 500 μg/mL. In experiment series B plant extract were tested at three concentrations (1
mg/mL, 0.5 mg/mL and 0.1 mg/mL, giving final well concentrations of 100 µg/mL, 50 µg/mL and 10 µg/mL). Ethanolic plant extracts were diluted in the growth medium to an ethanol concentration of 1% before being added to the wells.

Acetylsalicylic acid (Sigma A5376) was used as positive control. After addition of samples and controls the plate was incubated for a further 3 hours. After 3 hours incubation, 1 µL calcium ionophore A21387 (calcimycin; Sigma C7522) solution (5 mM in DMSO) was added to each well to stimulate PGE_2 synthesis, the plate shaken gently on an orbital shaker for 20 seconds and incubated for a final 20 minutes. Immediately following incubation, the plate was centrifuged (Hettich Universal 16A Centrifuge, Tuttlingen, Germany) for 3 minutes at 1000 RCF and the supernatant transferred to a clean 96-well plate, sealed and frozen at –80°C until the enzyme immunoassay was performed.

**Enzyme immunoassay.** The PGE_2 enzyme immunoassay was carried out using the thawed cell culture supernatant, which was diluted 500-fold with the buffer provided with the kit. 50µL of diluted sample was added to each well. In experiment series A samples and controls were run in duplicate, in experiment series B in triplicate. A PGE_2 standard was run at 8 concentrations in duplicate on each plate in all experiments. PGE_2 acetylcholine esterase tracer and PGE_2 monoclonal antibody were added (50 µL of each to each well) and the sealed plate incubated at 4ºC for 18-20 hours. Following incubation the plate was washed 5 times with the wash buffer provided in the kit, dried and Ellman’s Reagent was added (200 µL per well). The plate was sealed, covered in alfoil and placed on an orbital shaker (moderate speed) for 75-90 minutes, after which the absorbance at 405 nm of the developed plate was read in a Wallac Victor 2 Plate Reader (Perkin Elmer, Waltham, MA). All samples, standards and controls were assayed in triplicate.

**Data analysis.** Raw data were obtained as concentrations of PGE_2 (pg/well). Standard curves for PGE_2 were plotted and R^2 values calculated to confirm linearity. Mean, standard deviation and coefficient of variance were calculated for replicates of samples, standards and controls. Inhibition of PGE_2 release by stimulated 3T3 cells was reported as percentage inhibition compared with stimulated cells treated with solvent controls.
6.2.3.2 Oxygen Radical Absorbance Capacity (ORAC)

This assay is based on the work of Cao and Prior (Cao & Prior, 1999).

Ethanolic plant extracts were taken to dryness under a nitrogen stream and resolved and diluted in AWA solvent (acetone 70.0 : Milli-Q water 29.5 : acetic acid 0.5) to give concentrations of 100 µg/mL, 50 µg/mL, 25 µg/mL and 12.5 µg/mL.

Trolox was used as a standard and BHT as a positive control; stock solutions of both in 75 mM phosphate buffer pH 7.4 were diluted in AWA solvent to give Trolox concentrations of 100 µM, 50 µM, 25 µM, and 12.5 µM and butylated hydroxytoluene (BHT) concentrations of 500 µM, 250 µM, 125 µM, and 62.5 µM.

The assay was carried out in black 96-well fluorescence assay plates (Perkin Elmer Optiplate). Into each well was added 10 µL fluorescein solution (6.0 x 10^{-7} M in 75 mM phosphate buffer [NaH2PO4 17.25 g : Na2HPO4 86.25 g, adjusted to pH 7.4]); 20 µL sample, Trolox, BHT or solvent control solution; and 170 µL AAPH (2,2’-azobis-2-methylpropanimidamide, dihydrochloride; Cayman) solution (20 mM in 75 mM phosphate buffer, pH 7.4). Fluorescein control wells received 10 µL fluorescein solution and 190 µL phosphate buffer. Immediately after the addition of the AAPH solution, the assay plates were placed in Wallac Victor 2 Plate Reader (Perkin Elmer, Waltham, MA) and the fluorescence recorded at 37°C every minute for 35 minutes with the integral of these readings representing the total antioxidant capacity. All samples, standards and controls were assayed in quadruplicate.

Data analysis. Mean and standard deviation were calculated for replicates. Standard curves for Trolox standards were plotted and R^2 values calculated to confirm linearity.

6.2.3.3 Inhibition of nitric oxide production

This assay measures the release of nitric oxide by murine monocytes stimulated by lipopolysaccharide (LPS).

Cell culture. RAW264 murine leukemic monocyte-macrophages (ATCC, Manassas, VA) were grown in clear 96-well plates (Nunclon). The growth medium was the same as that
used in the PGE₂ assay (see above). 100 µL of cell suspension (10⁶ cells/mL) was added to each well and the plate incubated at 37°C and 5% CO₂ (Sanyo CO₂ MCO-17 AIC Incubator). After incubation (approx. 20 hours), 15 µL of plant extracts and controls were added to the wells and the plate incubated for a further 1 hour. Ethanolic plant extracts were diluted in the growth medium to an ethanol concentration of 1% before being added to the wells. Extracts were screened at 10% and 20% of stock extract concentrations, which ranged from 35 – 50 mg/mL. One active extract was also assayed at 2% and 1% of stock extract concentration. After incubation (1 hour), 5 µL of LPS solution (1 µg/mL in growth medium) was added to each well and the plate returned to the incubator for a further 18 – 20 hours. At the completion of the incubation period, the plate was centrifuged (3 min. at 1500 RCF) and 50 µL of the supernatants transferred to a clear assay plate (Spectra Plate, PerkinElmer).

**Nitrite assay.** Nitrite standards (7) were prepared by serial dilution of sodium nitrite in Milli-Q water; standards were further diluted 10-fold in growth medium (final nitrite well concentrations ranging from 1 to 500 µM). 50 µL of each standard concentration was transferred to the designated wells. 50 µL of Griess Reagent (0.1% N-1-napthylethlenediamine dihydrochloride, 1% sulphanilic acid in 5% phosphoric acid) was added to all wells on the plate, the content mixed gently by placing the plate on an orbital shaker (ensuring no bubbles were present), and the plate incubated at room temperature for 15 – 20 min protected from light. Following incubation the absorbance at 550 nm was read in a Wallac Victor 2 Plate Reader. All samples, standards and controls were assayed in triplicate.

**Data analysis.** Mean and standard deviation were calculated for replicates. Standard curves for nitrite were plotted and R² values calculated to confirm linearity. The nitric oxide (nitrite) production in sample wells was calculated as a percentage of the production in solvent control wells.

### 6.2.3.4 Modulation of natural killer cell activity

**Target cells**

Target cells were K562 cells (ATCC, Monassas, VA, USA), a leukemic cell line derived from an individual with chronic myeloid leukaemia in terminal blast crisis. K562 cells lack
major-histocompatibility-complex class I and II antigens, which makes them a sensitive target for human NK cells. K562 cells were stored under liquid nitrogen and prior to use rapidly thawed in 37°C water bath, then incubated in medium (RPMI-1640 (Invitrogen, Auckland, New Zealand) 87%, FBS 10% (Invitrogen), L-glutamine (Invitrogen) 1%, Penicillin/Streptomycin (Invitrogen) 2%) for 1 hour at 37°C and 5% CO₂ (Sanyo CO₂ MCO-17 AIC Incubator). Following incubation, the cell suspension was centrifuged for 5 minutes at 120 RCF at room temperature, the supernatant discarded, and the cell pellet resuspended in 1 mL RPMI-1640. Cells were then stained with 5 µL of a fluorescent membrane dye (DiO Vybrant Cell Labelling Solution, Molecular Probes, Eugene, OR, USA, cat. no. V-22886), incubated for a further 5 minutes at 37°C and 5% CO₂, then centrifuged for 5 minutes at 120 RCT and washed twice with medium to remove excess dye before being resuspended in 1 mL medium. The cell concentration of the suspension was determined using an electronic cell counter (Beckman Coulter ActDiff Haematology Analyser, Fullerton, CA) and adjusted with medium to 0.1 × 10⁹ cells/L.

**Effector cells**

Human peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood collected by venesection into 4-mL Lithium-Heparin collection tubes. The whole blood was diluted 1:1 with sterile phosphate-buffered saline (PBS), layered in 15-mL Falcon tubes on a cushion of Isopaque-Ficoll (Amersham Biosciences, Uppsala, Sweden), and centrifuged for 20 minutes at 700 RCT (no brake, 0 deceleration). Following centrifugation, the upper serum layer was removed, the PBMC layer collected and transferred to a clean 15-mL Falcon tube, to which 12 mL of PBS was added. This cell suspension was centrifuged for 10 minutes at 250 RCF, the supernatant removed, and the PBMC pellet resuspended in 1 mL medium. The cell concentration of this suspension was determined and adjusted with medium to between 1 × 10⁹ and 5 × 10⁹ cells/L, dependent on the NK cell activity of the donor blood used.
Pre-incubation of effector cells with extracts

In the case of aqueous extracts, 196 µL of cell suspension was incubated with 4 µL extract. In order to minimise the solvent effects of the ethanolic extracts, 398 µL of PBMC suspension was incubated with 2 µL extract for 2 hours at 37°C and 5% CO₂.

Incubation of effector cells with target cells

Following the pre-incubation of effector cells with test extracts, effector and target cell suspensions were combined in 5-mL Falcon tubes and incubated for 2 hours at 37°C/5% CO₂. For the ethanolic extracts, 200 µL of the pre-incubated effector cell suspension was combined with 200 µL target cell suspension, resulting in a final effector to target cell ratio of approximately 25:1 (subsequently reduced to 12:1 due to high activity of NK cells) and a solvent concentration of 0.25%. For the aqueous extracts, 100 µL effector cell suspension was combined with 200 µL target cell suspension and 100 µL medium, resulting in an effector : target cell ratio of approximately 12:1 and a solvent concentration of 0.5%. No-treatment and solvent controls were included in each experiment. After incubation for 2 hours the tubes were placed on ice (protected from light) followed by the addition of 100 µL DNA stain (propidium iodide, Molecular Probes, Eugene, OR, cat. no. P-3566; 20 µL stock/mL in PBS, final propidium iodide concentration 4 µg/mL) to each tube, after which the tubes were again placed on ice for 5 minutes, then assayed within 30 minutes. Control tubes containing target cell suspension and test extract but no effector cells were run for all samples and the no-treatment and solvent controls in order to monitor any mortality of the target cells caused by the extracts and/or solvent, independent of effector cell activity. The kill rate of the control was subtracted from the kill rate of each test sample (see below).

Flow cytometric assay

The assay was carried out on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) flow cytometer linked to a MacIntosh computer OS 9.1 running CellQuest Pro Software (Becton
The percentage kill of target cells was based on a total count of 2500 target cells. The specific cytotoxicity was calculated as follows:

\[
\text{Specific cytotoxicity} \, (\%) = \frac{\text{Dead target cells(Sample)} \, (\%)}{\text{Dead target cells(Control)}}
\]

### 6.3 Results

#### 6.3.1 Inhibition of PGE₂ production

A total of 42 samples representing 41 taxa and 39 species from a total of 14 genera were screened for inhibition of PGE₂ production. Samples were tested at two or three concentrations. The data thus generated did not allow for accurate determination of IC₅₀ values, but estimates were made where possible based on the available data (Table 6-2). For the full data set, refer to Appendix B.

Only 9 of the 42 samples demonstrated 50% inhibition of PGE₂ in the concentration range tested. The species showing the most potent and dose-dependent inhibition were *Alpinia galanga*, *Boesenbergia rotunda*, *Curcuma australasica*, *C. longa*, *C. parviflora*, *Kaempferia galanga*, *Pleuranthodium racemigerum*, *Zingiber officinale* and *Z. montanum*. Most of these are known medicinal plants, but nothing has been reported previously about the biological activity of the two native Australian species, *C. australasica* and *P. racemigerum*. 
Table 6-2. Inhibition of PGE$_2$ production in 3T3 murine fibroblasts.

Estimated IC$_{50}$ values are shown. NA: the estimated maximum inhibition was less than 50%. Concentrations refer to final concentrations in assay well. n=3 expect: * (n=2), + (n=4) and ^ (n=6).

<table>
<thead>
<tr>
<th>Taxon</th>
<th>ID</th>
<th>Estimated IC$_{50}$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcuma longa$^*$</td>
<td>Z106</td>
<td>c. 10</td>
</tr>
<tr>
<td>Curcuma australasica$^*$</td>
<td>Z101</td>
<td>c. 50</td>
</tr>
<tr>
<td>Curcuma parviflora</td>
<td>Z118</td>
<td>c. 50</td>
</tr>
<tr>
<td>Alpinia galanga</td>
<td>Z103</td>
<td>&gt;92</td>
</tr>
<tr>
<td>Boesenbergia rotunda</td>
<td>Z104</td>
<td>c. 94</td>
</tr>
<tr>
<td>Pleuranthodium racemigerum$^*$</td>
<td>Z128</td>
<td>50-100</td>
</tr>
<tr>
<td>Zingiber officinale</td>
<td>Z108</td>
<td>50-100</td>
</tr>
<tr>
<td>Zingiber montanum</td>
<td>Z105</td>
<td>50-100</td>
</tr>
<tr>
<td>Zingiber montanum$^*$</td>
<td>Z02</td>
<td>c. 500</td>
</tr>
<tr>
<td>Kaempferia galanga$^*$</td>
<td>Z05</td>
<td>c. 500</td>
</tr>
<tr>
<td>Alpinia calcarata$^*$</td>
<td>Z49</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Alpinia mutica$^*$</td>
<td>Z08</td>
<td>&gt;600</td>
</tr>
<tr>
<td>Alpinia purpurea 'Eileen McDonald''</td>
<td>Z11</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Alpinia zerumbet$^*$</td>
<td>Z52</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Elettaria cardamomum$^*$</td>
<td>Z12</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Hedychium coronarium$^*$</td>
<td>Z10</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Scaphochlamys biloba$^*$</td>
<td>Z01</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Zingiber longipedunculatum$^*$</td>
<td>Z06</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Zingiber spectabile</td>
<td>Z17</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Zingiber/Etlingera (Aniseed ginger)$^*$</td>
<td>Z19</td>
<td>&gt;600</td>
</tr>
<tr>
<td>Alpinia arctiflora</td>
<td>Z129</td>
<td>NA</td>
</tr>
<tr>
<td>Alpinia caerulea</td>
<td>Z102</td>
<td>NA</td>
</tr>
<tr>
<td>Alpinia luteocarpa</td>
<td>Z111</td>
<td>NA</td>
</tr>
<tr>
<td>Alpinia malaccensis$^*$</td>
<td>Z48</td>
<td>NA</td>
</tr>
<tr>
<td>Alpinia modesta</td>
<td>Z127</td>
<td>NA</td>
</tr>
<tr>
<td>Alpinia spectabile 'Giant Orange''</td>
<td>Z53</td>
<td>NA</td>
</tr>
<tr>
<td>Costus barbatus</td>
<td>Z112</td>
<td>NA</td>
</tr>
<tr>
<td>Costus leucanthus</td>
<td>Z113</td>
<td>NA</td>
</tr>
<tr>
<td>Costus malortieanus</td>
<td>Z114</td>
<td>NA</td>
</tr>
<tr>
<td>Costus productus</td>
<td>Z116</td>
<td>NA</td>
</tr>
<tr>
<td>Costus pulverulentus</td>
<td>Z115</td>
<td>NA</td>
</tr>
<tr>
<td>Costus tappenbeckianus$^*$</td>
<td>Z15</td>
<td>NA</td>
</tr>
<tr>
<td>Curcuma cordata</td>
<td>Z117</td>
<td>NA</td>
</tr>
<tr>
<td>Etlingera australasica</td>
<td>Z107</td>
<td>NA</td>
</tr>
<tr>
<td>Etlingera elatior 'Burma torch''</td>
<td>Z04</td>
<td>NA</td>
</tr>
<tr>
<td>Hornstedtia scottiana</td>
<td>Z126</td>
<td>NA</td>
</tr>
<tr>
<td>Kaempferia rotunda</td>
<td>Z120</td>
<td>NA</td>
</tr>
<tr>
<td>Renealmia cernua</td>
<td>Z121</td>
<td>NA</td>
</tr>
<tr>
<td>Scaphochlamys kunstleri$^*$</td>
<td>Z122</td>
<td>NA</td>
</tr>
<tr>
<td>Tapeinochilos ananassae</td>
<td>Z125</td>
<td>NA</td>
</tr>
<tr>
<td>Zingiber ottensii</td>
<td>Z124</td>
<td>NA</td>
</tr>
<tr>
<td>Zingiber sp. 'Dwarf Apricot'$</td>
<td>Z55</td>
<td>NA</td>
</tr>
</tbody>
</table>
6.3.2 Oxygen Radical Absorbance Capacity (ORAC)

Fifteen species were tested for antioxidant activity in the ORAC assay (Table 6-3).

Table 6-3. Oxygen radical absorbance capacity (ORAC).

Antioxidant activity is expressed in micromol Trolox equivalents (TE). Values shown are means±SEM for between 2 and 4 concentrations; each concentration was done in 4 replicates. ^: Drying temperature for plant material.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>ID</th>
<th>TE (µmol) per g extract</th>
<th>TE (µmol) per g dry herb equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcuma longa</td>
<td>Z106</td>
<td>3504±1141</td>
<td>528±172</td>
</tr>
<tr>
<td>Zingiber officinale (40°C)^</td>
<td>Z108</td>
<td>4166±63</td>
<td>262±4</td>
</tr>
<tr>
<td>Zingiber montanum</td>
<td>Z105</td>
<td>2707±509</td>
<td>156±29</td>
</tr>
<tr>
<td>Boesenbergia rotunda</td>
<td>Z104</td>
<td>2889±338</td>
<td>155±18</td>
</tr>
<tr>
<td>Zingiber officinale (90°C)^</td>
<td>Z109</td>
<td>3323±65</td>
<td>131±3</td>
</tr>
<tr>
<td>Alpinia galanga</td>
<td>Z103</td>
<td>933±36</td>
<td>61±2</td>
</tr>
<tr>
<td>Curcuma australasica</td>
<td>Z101</td>
<td>2357±479</td>
<td>54±11</td>
</tr>
<tr>
<td>Hornstedtia scottiana</td>
<td>Z126</td>
<td>2284±275</td>
<td>47±6</td>
</tr>
<tr>
<td>Zingiber ottensii</td>
<td>Z124</td>
<td>757±37</td>
<td>40±2</td>
</tr>
<tr>
<td>Tapeinochilos ananassae</td>
<td>Z125</td>
<td>1257±125</td>
<td>37±4</td>
</tr>
<tr>
<td>Curcuma parviflora</td>
<td>Z118</td>
<td>1452±191</td>
<td>37±5</td>
</tr>
<tr>
<td>Etlingera Australasica</td>
<td>Z107</td>
<td>778±75</td>
<td>36±4</td>
</tr>
<tr>
<td>Pleuranthodium racemigerum</td>
<td>Z128</td>
<td>1418±221</td>
<td>34±5</td>
</tr>
<tr>
<td>Alpinia caerulea</td>
<td>Z102</td>
<td>390±36</td>
<td>23±2</td>
</tr>
<tr>
<td>Alpinia luteocarpa</td>
<td>Z111</td>
<td>1540±253</td>
<td>22±4</td>
</tr>
<tr>
<td>Scaphochlamys kunstleri</td>
<td>Z122</td>
<td>944±60</td>
<td>18±1</td>
</tr>
</tbody>
</table>

The samples most active in this assay were Curcuma longa, Zingiber officinale, Z. montanum and Boesenbergia rotunda. This was the case whether the samples were ranked by Trolox Equivalence (TE, µmol) per gram extract or TE per gram dried herb equivalent.
6.3.3 Inhibition of nitric oxide production

Eight species were tested for their ability to inhibit nitric oxide production in RAW264 cells (Table 6-4).

Table 6-4. Inhibition of nitric oxide production in LPS-stimulated RAW264 macrophages.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>ID</th>
<th>Concentration (µg/mL)</th>
<th>Percent inhibition of nitric oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alpinia galanga</em></td>
<td>Z103</td>
<td>4.6</td>
<td>57±4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.2</td>
<td>78±2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.0</td>
<td>100±2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>46.0</td>
<td>92±3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>92.1</td>
<td>97±2</td>
</tr>
<tr>
<td><em>Curcuma australasica</em></td>
<td>Z101</td>
<td>34.7</td>
<td>86±7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>69.4</td>
<td>95±1</td>
</tr>
<tr>
<td><em>Zingiber officinale</em></td>
<td>Z109</td>
<td>50.2</td>
<td>85±5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100.3</td>
<td>95±1</td>
</tr>
<tr>
<td><em>Boesenbergia rotunda</em></td>
<td>Z104</td>
<td>47.3</td>
<td>67±1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94.52</td>
<td>100±3</td>
</tr>
<tr>
<td><em>Alpinia caerulea</em></td>
<td>Z102</td>
<td>47.6</td>
<td>57±9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95.1</td>
<td>63±1</td>
</tr>
<tr>
<td><em>Zingiber montanum</em></td>
<td>Z105</td>
<td>50.2</td>
<td>35±2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100.3</td>
<td>72±1</td>
</tr>
<tr>
<td><em>Curcuma longa</em></td>
<td>Z106</td>
<td>49.7</td>
<td>35±1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>99.3</td>
<td>51±2</td>
</tr>
<tr>
<td><em>Etlingera australasica</em></td>
<td>Z107</td>
<td>49.7</td>
<td>0±0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>92.7</td>
<td>16±1</td>
</tr>
</tbody>
</table>

Several of the tested extracts showed potent inhibition on nitric oxide at the concentrations tested. At a concentration of 95-100 µg mL⁻¹ *Boesenbergia rotunda, Curcuma australasica* and *Zingiber officinale* caused almost complete inhibition of nitric oxide production. The most potent of the extracts was *Alpinia galanga*, which demonstrated dose-dependent inhibition in the range 5-92 µg mL⁻¹ with complete inhibition achieved at 23 µg mL⁻¹.
6.3.4 Modulation of natural killer cell activity

The results of the screening of 9 ethanolic extracts for modulation of NK cell activity are shown in Table 6-5.

Table 6-5. Effect of ethanolic extracts on natural killer cell activity against K562 leukaemia cells.

^: Drying temperature for plant material.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>ID</th>
<th>Sample conc. (µg/mL)</th>
<th>Conc. in pre-incubation tube with effector cells (µg/mL)</th>
<th>Increase in specific cytotoxicity compared with solvent control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td></td>
<td>88.6</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Solvent control (ethanol)</td>
<td></td>
<td></td>
<td>0.5%</td>
<td>0.0</td>
</tr>
<tr>
<td>Boesenbergia rotunda</td>
<td>Z104</td>
<td>188.4</td>
<td>0.94</td>
<td>18.5</td>
</tr>
<tr>
<td>Curcuma longa</td>
<td>Z106</td>
<td>198.0</td>
<td>0.99</td>
<td>10.8</td>
</tr>
<tr>
<td>Zingiber officinale (90°C)^</td>
<td>Z109</td>
<td>200.0</td>
<td>1.00</td>
<td>8.6</td>
</tr>
<tr>
<td>Etlingera australasica</td>
<td>Z107</td>
<td>184.8</td>
<td>0.92</td>
<td>6.8</td>
</tr>
<tr>
<td>Zingiber officinale (40°C)^</td>
<td>Z108</td>
<td>173.6</td>
<td>0.87</td>
<td>0.0</td>
</tr>
<tr>
<td>Zingiber montanum</td>
<td>Z105</td>
<td>200.0</td>
<td>1.00</td>
<td>-4.6</td>
</tr>
<tr>
<td>Curcuma australasica</td>
<td>Z101</td>
<td>138.4</td>
<td>0.69</td>
<td>-4.9</td>
</tr>
<tr>
<td>Alpinia caerulea</td>
<td>Z102</td>
<td>189.6</td>
<td>0.95</td>
<td>-4.9</td>
</tr>
<tr>
<td>Alpinia galanga</td>
<td>Z103</td>
<td>91.8</td>
<td>0.46</td>
<td>-28.4</td>
</tr>
</tbody>
</table>

None of the ethanolic extracts tested produced a strong increase in NK cell activity. The most effective extracts were *Boesenbergia rotunda* and *Curcuma longa*, which increased NK cell activity by 18.5% and 10.8%, respectively. *Alpinia galanga* (tested at half the concentration of the other extracts) caused an almost 30% decrease in activity.

Eight aqueous extract were also assayed for modulation of NK cell activity (Table 6-6).
Table 6-6. Effect of aqueous extracts on natural killer cell activity against K562 leukaemia cells.

*: percentage of stock extract. ^: Drying temperature for plant material.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>ID</th>
<th>Sample conc.*</th>
<th>Increase in specific cytotoxicity compared with solvent control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td></td>
<td></td>
<td>-2.0</td>
</tr>
<tr>
<td>Solvent control (ethanol)</td>
<td></td>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Curcuma longa</td>
<td>Z106</td>
<td>0.1%</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2%</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4%</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0%</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0%</td>
<td>8.0</td>
</tr>
<tr>
<td>Curcuma australasica</td>
<td>Z101</td>
<td>0.1%</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0%</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0%</td>
<td>-0.5</td>
</tr>
<tr>
<td>Etlingera australasica</td>
<td>Z107</td>
<td>0.1%</td>
<td>-0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0%</td>
<td>-9.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0%</td>
<td>1.2</td>
</tr>
<tr>
<td>Zingiber officinale (40°C)</td>
<td>Z108</td>
<td>0.1%</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0%</td>
<td>-11.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0%</td>
<td>7.0</td>
</tr>
<tr>
<td>Zingiber officinale (90°C)</td>
<td>Z109</td>
<td>0.1%</td>
<td>-1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0%</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0%</td>
<td>-0.7</td>
</tr>
<tr>
<td>Zingiber montanum</td>
<td>Z105</td>
<td>0.1%</td>
<td>-0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0%</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0%</td>
<td>-11.9</td>
</tr>
<tr>
<td>Boesenbergia rotunda</td>
<td>Z104</td>
<td>0.1%</td>
<td>-6.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0%</td>
<td>-3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0%</td>
<td>-18.8</td>
</tr>
<tr>
<td>Alpinia galanga</td>
<td>Z103</td>
<td>0.1%</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2%</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4%</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0%</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0%</td>
<td>-77.0</td>
</tr>
</tbody>
</table>
None of the aqueous extracts stimulated NK cell activity against K562 cells in a substantial or dose-dependent manner. *Alpinia galanga* and to a lesser extent *Boesenbergia rotunda* dramatically decreased the activity of natural killer cells at the highest concentration tested (10% of stock extract), while these concentrations did not kill the target cells.

**6.4 Discussion**

6.4.1 Inhibition of PGE₂ production

This study has for the first time demonstrated *in vitro* PGE₂ inhibition by extracts of the two native Australian Zingiberaceae, *Curcuma australasica* and *Pleuranthodium racemigerum*. The estimated IC₅₀ for the two extracts were c. 50 µg mL⁻¹ and c. 75 µg mL⁻¹, respectively, which was similar to that of *Zingiber officinale*. These findings suggested the presence of compounds with potent biological activity, and the extracts were therefore subsequently the subject of bioactivity-guided fractionation (see Chapter 7). This is also the first report of PGE₂ inhibition by the South-east Asian species *Curcuma parviflora*.

Panduratin A isolated from *Boesenbergia rotunda* (syn. *Kaempferia pandurata*) has previously been show to inhibit PGE₂ and nitric oxide production as well as iNOS and COX-2 expression in RAW264.7 cells (Yun et al., 2003), but the present study is the first to demonstrate PGE₂ inhibition by a crude extract of the plant.

PGE₂ inhibition has also previously been shown for an extract of *Zingiber montanum* (syn. *Z. cassumunar*) and several phenylbutanoid compounds isolated from this species (Jeenapongsa et al., 2003; Jiang et al., 2006b; Panthong et al., 1997).

Extracts of *Zingiber officinale*, *Curcuma longa* and to a lesser degree *Alpinia galanga* were included in this assay primarily as positive controls, as they are well established inhibitors of PGE₂ production *in vitro* and hence confirmed the validity of the assay.

The inhibitory effects of ginger (*Z. officinale*) and several ginger constituents on COX activity and subsequent PGE₂ production are well known and have been demonstrated in isolated enzyme assays, cell-based models and animals. The early work by Kiuchi and colleagues found that [6]-gingerol, [6]-gingerdione, [10]-gingerdione, [6]-dehydrogingerdione and [10]-dehydrogingerdione isolated from fresh ginger were potent
inhibitors of COX *in vitro* with IC$_{50}$ values comparable to that of indomethacin (Kiuchi *et al.*, 1982). Subsequent work by the same group reported potent inhibition by [10]-gingerol, [6]-shogaol, [10]-gingerol, [6]-acetylgingerol, [6]-shogaol, [6]-dehydroygingerdione, [10]-dehydroygingerdione, [6]-gingerdione and [10]-gingerdione, many of which had IC$_{50}$ values <2.5 µM (Kiuchi *et al.*, 1992), and [8]-paradol was shown to be a potent inhibitor of COX *in vitro* with an IC$_{50}$ of 4 µM (Nurtjahja-Tjendraputra *et al.*, 2003).

Although gingerols (in particular [6]-gingerol) are commonly referred to as the main active component in ginger extracts, there are clearly numerous other compounds with anti-inflammatory activity present. This was demonstrated by a recent study from the University of Arizona showing that no correlation existed between PGE$_2$ inhibitory activity of ginger extracts and their gingerol content (Jiang *et al.*, 2006b). This study found IC$_{50}$ values for methanolic ginger extracts ranging from 0.058 to 0.629 µg mL$^{-1}$, which is much lower than the estimated IC$_{50}$ obtained in the present study (50-100 µg mL$^{-1}$). This difference may be due to the different cell lines and assay methods used in the two studies (Jiang and colleagues used the human promonocytic cell line U937 and an immunoassay kit from R&D System, while the present study used murine 3T3 fibroblasts and the PGE$_2$ immunoassay kit from Cayman Chemical Co.). The magnitude of difference seems unlikely to be due to phytochemical differences between the plant materials used in the two studies.

The same University of Arizona study also found methanolic extracts of *Alpinia galanga*, *Zingiber montanum* and *Z. spectabile* to be potent inhibitors of PGE$_2$ production (IC$_{50}$ values of 0.06, 7.68 and 1.17 µg mL$^{-1}$, respectively (Jiang *et al.*, 2006b). In the present study *A. galanga* and *Z. montanum* were both found to have some inhibitory activity (albeit again at much higher concentrations), while *Z. spectabile* was not active.

*Curcuma longa* was the most potent inhibitor of PGE$_2$ activity in the present study (IC$_{50}$ = c. 10 µg mL$^{-1}$). Rhizome extracts have been shown to inhibit PGE$_2$ production in HL-60 cells (IC$_{50}$ = 0.92 µg mL$^{-1}$) (Lantz *et al.*, 2005), and the principal constituent, curcumin, is a well documented inhibitor of COX expression and PGE$_2$ production (Funk *et al.*, 2006; Hong *et al.*, 2004; Ireson *et al.*, 2001).
6.4.2 Oxygen Radical Absorbance Capacity (ORAC)

The most potent plant materials in terms of oxygen radical absorbance capacity (ORAC) were turmeric (Curcuma longa) and ginger (Zingiber officinale) with values of 528 µmol TE and 262 µmol TE per gram dry herb equivalent, respectively. The antioxidant properties of both of these plants are well known (refer to Chapter 2, Sections 2.2.1.3.1 and 2.3.1.2.2.1).

Another study from the same laboratory reported a very similar ORAC value (557 µmol TE per gram dry herb equivalent) for a turmeric extract prepared using a sequential three-solvent extraction process (Wojcikowski et al., 2007). Another study reported an ethanolic extract of fresh turmeric as having an ORAC value of 19.5 µmol TE per gram fresh weight (Tilak et al., 2004). With fresh turmeric rhizome typically having a water content of about 75% (data not shown), this is equivalent to approximately 80 µmol TE per gram dry herb. This value is considerably lower than the one obtained in the present study (528 µmol TE), but differences in extraction method and efficiency (stirring versus sonication) may at least partly explain the discrepancy. Quantitative phytochemical differences between the two plant samples may also have contributed.

One study has reported an ORAC value of 148 µmol TE per gram for fresh ginger (Zingiber officinale) (Ninfali et al., 2005). Given that ginger rhizome typically has a water content close to 90% (data not shown), this is equivalent to an ORAC value of approximately 1500 µmol TE per gram dry herb equivalent, which is an order of magnitude greater than the value obtained in the present study (262 µmol TE per gram for ginger dried at 40° C). This difference may reflect that the fresh rhizome has significantly greater antioxidant capacity than the dried, but phytochemical differences in the plant material assayed and differences in experimental methodology could also be factors.

It is interesting to note the difference in ORAC value for the two ginger samples, which were prepared from the same raw material. Z108 was dried at 40° C, while Z109 was dried at 90° C. Z108 had twice the ORAC value of Z109 (262 versus 131 µmol TE per gram dry herb equivalent), clearly demonstrating that antioxidant activity is lost when plant material is dried at high temperature. This is not unexpected, as oxidative processes catalysed by high temperature are likely to deplete antioxidant capacity.
Curcuminoids isolated from Zingiber montanum (syn. Z. cassumunar) have been reported to possess potent antioxidant activity (Masuda & Jitoe, 1994; Nagano et al., 1997). In the present study the crude rhizome extract was found to be quite potent in the ORAC assay (ORAC value of 156 µmol TE per gram dry herb equivalent).

Boesenbergia rotunda had an ORAC value similar to that of Z. montanum. B. rotunda (syn. B. pandurata) has previously demonstrated potent antioxidant activity in a rat brain homogenate assay, and several antioxidant compounds including panduratin A have been isolated (Shindo et al., 2006).

Galangal (Alpinia galanga) has been shown to exert antioxidant activity in raw minced beef (Cheah & Abu, 2000) but has not previously been tested in the ORAC assay. The observed activity (61 µmol TE per gram dry herb equivalent) was quite modest.

None of the other species tested have been reported as having antioxidant activity. The two native Australian species that displayed good activity in the PGE₂ assay, Curcuma australasica and Pleuranthodium racemigerum, had only modest antioxidant activity in the ORAC assay (54 and 34 µmol TE per gram dry weight equivalent, respectively).

6.4.3 Inhibition of nitric oxide production

The ethanolic extract of Alpinia galanga was found to be a potent inhibitor of nitric oxide production. At a concentration of 4.6 µg mL⁻¹ it caused 57% inhibition, which corresponds well with a reported IC₅₀ = 7.3 µg mL⁻¹ for an 80% aqueous acetone extract in LPS-activated mouse peritoneal macrophages (Morikawa et al., 2005). 1’S-1’-acetoxychavicol acetate isolated from A. galanga was found to be active in a peritoneal macrophage assay (Matsuda et al., 2005).

Inhibition of nitric oxide production in vitro has been demonstrated for several other Alpinia species and a considerable number of compounds isolated from them. These include diarylheptanoids, a curcuminoid and flavonoids from A. blepharocalyx (Kadota et al., 1996; Prasain et al., 1998), diarylheptanoids from A. officinarum (Lee et al., 2006; Matsuda et al., 2006) and sesquiterpenes from A. oxyphylla (Muraoka et al., 2001). Inhibition of inducible
nitric oxide synthase (iNOS) has been reported for isolates from *A. katsumadai* (Hong et al., 2002), *A. officinarum* (Yadav et al., 2003) and *A. oxyphylla* (Chun et al., 2002).

A methanolic extract of *Curcuma longa* (10 µg mL⁻¹) was reported to cause 88% inhibition of nitric oxide production in LPS-stimulated RAW264.7 cells (Hong et al., 2002). In comparison, the present study found only 35% inhibition by *C. longa* at a concentration of 50 µg mL⁻¹ (and 51% inhibition at 99 µg mL⁻¹).

Inhibition of nitric oxide production and suppression of iNOS expression has previously been demonstrated for methanolic extracts of *C. longa*, *C. comosa* and *C. zedoaria* (Camacho-Barquero et al., 2007; Hong et al., 2002; Jang et al., 2004; Jantaratnotai et al., 2006). Active compounds identified from these species include curcumin from *C. longa* and two sesquiterpenoids from *C. zedoaria*. The sesquiterpenoid xanthorrhizol isolated from *C. xanthorrhiza* has also been shown to inhibit iNOS activity and expression (Chung et al., 2007; Lee et al., 2002).

In the present study ginger (*Zingiber officinale*) extract was a potent inhibitor of nitric oxide at the concentrations tested (50 and 100 µg mL⁻¹), while *Z. montanum* was less potent. While inhibitory effects on nitric oxide production have not previously been reported for the latter, this activity has been described for *Z. officinale* in osteoarthrotic sow chondrocytes (Shen et al., 2005), and [6]-gingerol (the major pungent compound in ginger) has shown dose-dependent inhibition of nitric oxide production and reduction in iNOS in LPS-stimulated J774.1 mouse macrophages (Ippoushi et al., 2003). From other *Zingiber* species, sesquiterpenoids from *Z. zerumbet* and the labdane diterpene aframodial from *Z. mioga* have been shown to inhibit the expression of iNOS (Kim et al., 2005a; Murakami et al., 2002).

### 6.4.4 Modulation of natural killer cell activity

Only modest increases in NK cell activity were observed in this study for certain extracts, while others were inactive. Best results were seen for the ethanolic extracts of *Boesenbergia rotunda* (18.5% increase compared with solvent control) and *Curcuma longa* (10.8% increase). The aqueous extract of *C. longa* also produced a modest increase (about 10%), but this was not dose dependent in the concentration range tested. In contrast, both the ethanolic extract of *Alpinia galanga* and the aqueous extract of fresh *Boesenbergia rotunda* caused
marked inhibition of NK cell activity (by 28.4% and 18.8%, respectively). There are no previous reports concerning the effects of *A. galanga* or *B. rotunda* on NK cell activity.

Curcumin (0.01 µg mL\(^{-1}\)) has previously been shown to significantly increase the cytotoxicity of human NK cells *ex vivo*, using K562 cells as a target (Yadav *et al.*, 2005). However, in that study effector cells were incubated with curcumin (solvent unknown) for 18 hours as opposed to 2 hours in the present study, and the effector cells were subsequently incubated with the target cells for 4 hours compared with 2 hours in the present study. Curcumin was also found to partially reverse breast tumour exosome-mediated inhibition of NK cell tumour cytotoxicity *in vitro* (Zhang *et al.*, 2007). However, NK cell activity did not change significantly compared with controls in rats fed dietary curcumin (1, 20 or 40 mg per kg bodyweight) for 5 weeks (South *et al.*, 1997).

In the present study the presence of ethanol even at a very low concentration (0.25%) in the target cell suspension caused a significant (47%) decrease in specific NK cell cytotoxicity compared with the untreated control. The sizeable effect of the solvent may have affected the results of this assay for the ethanolic extracts. Further work should explore the use of alternative extraction solvents (less polar than water) that may interfere less with the assay than does ethanol.

### 6.4.5 Conclusion

This study has confirmed the Zingiberaceae as a rich source of compounds with interesting biological and pharmacological actions. Of some 40 species tested in at least one assay, the established medicinal species turmeric (*Curcuma longa*) and ginger (*Zingiber officinale*) emerged as the most active. Two other plants with traditional medicinal uses, galangal (*Alpinia galanga*) and *Boesenbergia rotunda* also showed good activity in several assays.

Of the 41 taxa tested turmeric was the most potent inhibitor of PGE\(_2\), and it was also the best antioxidant in the ORAC assay. It caused only moderate inhibition of nitric oxide production, but other studies suggest that this may be an aberrant result. The well known ability of ginger to inhibit PGE\(_2\) production *in vitro* was confirmed, and ginger also showed good antioxidant capacity, although rhizome material dried at 90° C had only half the activity of material dried at 40° C. Ginger was also a potent inhibitor of nitric oxide
production, but like turmeric did not affect NK cell activity significantly. *Boesenbergia rotunda* inhibited PGE$_2$ production and showed antioxidant activity. The ethanolic extract of the dried material caused an increase in NK cell activity, while an aqueous extract of the fresh material caused a marked decrease. The explanation for this is unknown and warrants further investigation.

Of particular interest was the screening of seven native Australian species in the PGE$_2$ assay, as none of these had previously been investigated for pharmacological activity. Two of these species, *Curcuma australasica* and *Pleuranthodium racemigerum*, showed good activity in this assay and were selected for further investigations (see Chapter 7).
7. BIOACTIVITY-GUIDED FRACTIONATION OF TWO NATIVE AUSTRALIAN ZINGIBERACEAE

7.1 Introduction

In the previous chapter, two native Australian species, *Curcuma australasica* and *Pleuranthodium racemigerum*, were identified as having significant PGE$_2$ inhibitory activity *in vitro*. In this respect they demonstrated potency similar to well known medicinal species such as *Curcuma longa* and *Alpinia galanga*.

Due to the paucity of information about the chemistry and pharmacological activity of both *Curcuma australasica* and *Pleuranthodium racemigerum*, these species were selected for further work with a view to extending the knowledge of the Zingiberaceae and about native Australian plants.

The process of bioactivity-guided fractionation was applied and led to the isolation of pharmacologically active compounds from both species. Inhibition of PGE$_2$ was used as the primary bioassay in this process, but fractions with high activity in this assay were also tested for cytotoxic properties, primarily to ensure that the activity observed in the cell-based PGE$_2$ assay was not simply due to cytotoxic effects. Testing for cytotoxicity included the murine cell line P388D1, which in the laboratory’s experience is highly sensitive to cytotoxic agents. It should be noted that significant cytotoxic activity may potentially be of interest in itself.

From *C. australasica* two known bioactive sesquiterpene compounds, zederone and furanodien-6-one were isolated. In the case of *P. racemigerum* a novel curcuminoid compound with significant pharmacological activity was isolated and structurally characterised. The isolated compounds could potentially become candidates for further work and preclinical testing.
7.2 Materials and Methods

7.2.1 Plant material

Fresh rhizome of *Curcuma australasica* was obtained from the George Brown Botanic Gardens in Darwin (S 12° 27’; E 130° 50’) as a gift from Dr Greg Leach who also identified the material.

*Pleuranthodium racemigerum* was collected in rainforest (828 m above sea level) in the Wet Tropics Region of North Queensland in Gillies Range (S 17° 13’; E 145° 40’) under a permit issued by the Queensland Environmental Protection Agency. Voucher specimens have been deposited in the Medicinal Plant Herbarium at Southern Cross University (NCM05-047) and the Queensland Herbarium (AQ736263).

7.2.2 Extraction methods

7.2.2.1 *Curcuma australasica*

Fresh rhizomes were cleaned, sliced and extracted in double the mass of ethanol (99.7%) in a sonicating bath for 30 min. The extract was filtered through filter paper (Whatman No. 3) in a Buchner apparatus. The biomass was again covered with ethanol, left to steep for 22 hours at 5°C, sonicated for 10 min, then filtered. The two filtrates were combined, and the extract taken to dryness under vacuum on a Büchi Rotavapor R-114 (Switzerland) with the water bath temperature at 40°C. The resulting semi-dry extract was dried on a rotational vacuum concentrator for 48 hours at –88°C and 0.140 mbar (Christ Alpha 2-4, Osterode, Germany). The dry extract was stored at –20°C until use.

7.2.2.2 *Pleuranthodium racemigerum*

Fresh rhizomes were cleaned, sliced and dried at 40°C. The dried rhizomes were ground to a coarse powder in a Waring blender and extracted with 4 parts (by mass) ethanol. The plant material was steeped for 24 hours in a stoppered conical flask while being agitated on a Bioline Orbital Shaker BL 4236 (Edwards Instrument Company, Australia). The resulting
extract was filtered through a Quickfit glass filter and stored at –20°C. Prior to fractionation, the extract was dried under vacuum on a Büchi Rotavapor R-114 (Switzerland) with the water bath temperature at 45°C. The resulting extract, which was oily, was redissolved in 91% aqueous methanol and partitioned with hexane in a separating funnel. The methanolic phase was dried under vacuum. Prior to testing in bioassays a small quantity of extract was dried under a nitrogen stream and redissolved to a known concentration.

7.2.3 Fractionation by preparative HPLC

7.2.3.1 Curcuma australasica

The dry extract was redissolved in 75% aqueous methanol (1 part extract to 10 parts solvent by mass) and filtered through an Acrodisc CR PTFE 0.45 µm microfilter (Pall Gelman Lab., Ann Arbor, MI). Fractionation was carried out on a Gilson Preparative HPLC system fitted with a Gilson FC204 Fraction Collector and an Altima C-18 5 µm column (150 mm long; internal diameter 22 mm) (Alltech, Kentucky). Mobile phase A consisted of HPLC-grade water obtained from an in-house Milli-Q system (Waters, Milford, MA), mobile phase B consisted of HPLC-grade methanol (EM Science, Gibbstown, NJ); both contained 0.05% trifluoroacetic acid (TFA). The mobile phase gradient was A:B (70:30, v/v) to A:B (5:95, v/v) over 20 min followed by A:B (5:95, v/v) for 10 min, with a flow rate of 20 mL/min. UV-VIS detection was at 210 nm and 360 nm. Twenty-nine 0.8-minute fractions were collected, commencing at 2 min. Another fractionation of the same extract was carried out, this time collecting 18 0.5-min fractions, starting at 12 min (3 1-mL injections of sample were fractionated). Fractions were dried on a rotational vacuum concentrator for 48 hours at –88°C and 0.140 mbar (Christ Alpha 2-4, Osterode, Germany).

7.2.3.2 Pleuranthodium racemigerum

The methanolic extract residue (700 mg) was redissolved in 3.5 mL acetonitrile with a few drops of water and fractionated on a Gilson Preparative HPLC system as described in the previous section. Mobile phase A consisted of HPLC-grade water, mobile phase B consisted of HPLC-grade acetonitrile; both contained 0.05% TFA. The gradient eluting mobile phase
was A:B (70:30, v/v) to A:B (5:95, v/v) over 20 min followed by A:B (5:95, v/v) for 5 min, with a flow rate of 20 mL/min. UV-VIS detection was at 210 nm and 360 nm. Twenty-nine 1-minute fractions were collected, commencing at 1 min. Two 1-mL injections of the sample were fractionated. Another fractionation of the same extract was carried out, again collecting 29 1-min fractions. Fractions were dried on a rotational vacuum concentrator as described in the previous section.

### 7.2.4 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker AVANCE DRX500 ($^1$H at 500.13 MHz; $^{13}$C at 125.77 MHz; 5mm QNI probe) spectrometer with XWin-NMR software. The $^1$H and $^{13}$C NMR spectra were recorded using deuterated chloroform (CDCl$_3$) with the residual solvent peaks as reference (7.27 ppm for $^1$H and 77.2 ppm for $^{13}$C). In one case deuterated pyridine (pyridine-$d_5$) was used instead (residual solvent peaks 8.74 ppm for $^1$H and 150.35 ppm for $^{13}$C). The chemical shifts were expressed in parts per million (ppm) as δ values and the coupling constants ($J$) in Hertz (Hz). All experiments were carried out using the Bruker pulse programs.

#### 7.2.4.1 One-dimensional NMR spectroscopy

##### 7.2.4.1.1 $^1$H NMR

The $^1$H NMR spectra were obtained for the isolated compounds. The chemical shifts, coupling constants, peak intensities and splitting patterns of each of the proton signals provided information about the type of protons present in the molecule and their chemical environments (Williams & Fleming, 1989).

##### 7.2.4.1.2 J-modulated $^{13}$C NMR

The carbon resonances were distinguished according to their proton attachments (C, CH, CH$_2$, CH$_3$) using the APT pulse sequence. The $J$-modulated $^{13}$C NMR spectra displayed the
CH₃ and CH resonances arbitrarily pointing down and inverted with respect to the CH₂ and the quaternary carbons, which were arbitrarily pointing up (Sanders & Hunter, 1987).

7.2.4.2 Two-dimensional homonuclear correlation NMR spectroscopy

In the two-dimensional experiments, the ¹H NMR spectra were displayed along both axes and the proton correlations were indicated in the contour plot by cross-peaks along the diagonal.

7.2.4.2.1 ¹H-¹H Correlation spectroscopy (COSY)

This two-dimensional technique was used to correlate the chemical shifts of ¹H nuclei that were coupled to one another. A magnitude sequence with a final pulse angle of 45° (COSY45) was used to obtain a COSY spectrum. The cross-peaks produced indicated which ¹H nuclei were J-coupled.

7.2.4.3 Two-dimensional heteronuclear correlation spectroscopy

7.2.4.3.1 Heteronuclear single quantum correlation (HSQC) spectroscopy

This two-dimensional method was used to determine which ¹H nuclei were bonded to which ¹³C nuclei (¹J_CH) in a molecule using an INEPT pulse sequence.

7.2.4.3.2 Heteronuclear multiple-bond correlation (HMBC) spectroscopy

This two-dimensional method was used for determining long-range ¹H-¹³C connectivities and was useful in determining the C assignments in a molecule through its correlation with a proton.
7.2.5 Determination of accurate mass

A sample of the isolated Compound 3 from *Pleuranthodium racemigerum* was sent to the School of Molecular and Microbial Sciences, University of Queensland, where determination of accurate mass by high-resolution mass spectroscopy was carried out by Mr Graham Macfarlane.

Electron impact ionization (EI) experiments were conducted on a Kratos MS25 RFA instrument via a direct insertion probe at 70 eV and source temperature of 200° C. Perfluorokerosene (PKF) was used as reference for magnet scan accurate mass in EI.

Electrospray ionization (ESI) experiments were conducted on a Finnigan MAT 900 XL-Trap instrument with a Finnigan API III electrospray source, using MeOH as the solvent and polypropylene glycol and polyethylene glycol as references for accurate mass data, acquired by electric sector scan.

7.2.6 UV spectroscopy

UV absorbance data were obtained on a Hewlett-Packard Spectrophotometer 8453 (Palo Alto, CA) controlled by UV-Visible ChemStation software (Rev. A.0803; Dayton, Ohio). Absorbance data were acquired in the range 190-1100 nm at 1 nm intervals. The novel Compound 3 from *Pleuranthodium racemigerum* was dissolved in methanol at a concentration of 0.014 mg/mL (4.723 × 10⁻⁵ M). The determination of peak absorption (λmax) was based on the mean value of five measurements. The extinction coefficient (wavelength-dependent molar absorptivity coefficient), ε, was calculated from the Beer-Lambert Law, A = ε × c × d, where A is absorbance, c the molar concentration and l the distance (cuvette path) in cm.

7.2.7 Cytotoxicity assays

Cytotoxicity in 3T3 murine fibroblasts, P388D1 murine lymphoblasts, Caco-2 human colonic adenocarcinoma, PC3 human prostate adenocarcinoma, HepG2 human hepatocyte carcinoma, and MCF7 human mammary adenocarcinoma (all cell lines originating from the
American Type Culture Collection, ATCC) was assayed in 96-well plates using the ATPLite kit (PerkinElmer, Waltham, MA) with chlorambucil and curcumin as reference compounds. Cells were cultivated in the appropriate medium and test and reference compounds added at five different concentrations and incubated for 24 hours (5% CO₂). All samples were assayed in triplicate.

The ATPLite assay was carried out according to the manufacturer’s instructions. Briefly, the kit components were equilibrated to room temperature. The lyophilised substrate solution was reconstituted with buffer and this reagent added to the plate containing the cells incubated with test substances and standards. The plate was then shaken on an orbital microplate shaker (700 rpm) for 2 minutes before luminescence was measured on a Wallac Microbeta Scintillation and Luminescence Counter (PerkinElmer, Waltham, MA). ATP was quantified using an ATP standard curve based on luminescence measurements of ATP standards. Median lethal dose (LD₅₀) values were calculated using Excel 2003 (Microsoft Corporation, Redmond, WA) and GraphPad Prism 4 (San Diego, CA).

7.2.8 PGE₂ assay

Inhibition of PGE₂ production by 3T3 murine fibroblast cells (ATCC) was assayed using the Prostaglandin E₂ EIA Kit (Cayman Chemical, Ann Arbor, MI) as described in the previous chapter.

7.3 Results

7.3.1 Curcuma australasica

7.3.1.1 Activity-guided fractionation

The extract was profiled by LC-MS prior to fractionation (Fig. 7-1).
Fig. 7-1. LC-MS chromatogram of *Curcuma australasica* extract redissolved in methanol.
Top: 210 nm; centre: 280 nm; bottom: total ion chromatogram.

Based on the chromatograms of the whole extract and the individual fractions, some fractions were combined before being tested for their ability to inhibit PGE$_2$ production in murine fibroblasts. The results of this assay are shown in Table 7-1.
Table 7-1. Inhibition of PGE$_2$ production in 3T3 murine fibroblast cells by fractions of Curcuma australasica extract.

The final concentration of test substances in the assay well was 1mg/mL.

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Percent inhibition (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16±8</td>
</tr>
<tr>
<td>2</td>
<td>12±9</td>
</tr>
<tr>
<td>3-9</td>
<td>18±11</td>
</tr>
<tr>
<td>10</td>
<td>19±3</td>
</tr>
<tr>
<td>11-13</td>
<td>13±11</td>
</tr>
<tr>
<td>14</td>
<td>26±5</td>
</tr>
<tr>
<td>15</td>
<td>47±6</td>
</tr>
<tr>
<td>16</td>
<td>80±10</td>
</tr>
<tr>
<td>17</td>
<td>82±6</td>
</tr>
<tr>
<td>18</td>
<td>64±4</td>
</tr>
<tr>
<td>19</td>
<td>57±8</td>
</tr>
<tr>
<td>20</td>
<td>61±9</td>
</tr>
<tr>
<td>21-28</td>
<td>17±15</td>
</tr>
<tr>
<td>6-gingerol</td>
<td>94±4</td>
</tr>
<tr>
<td>8-gingerol</td>
<td>96±3</td>
</tr>
<tr>
<td>10-gingerol</td>
<td>92±2</td>
</tr>
<tr>
<td>8-shogaol</td>
<td>82±5</td>
</tr>
</tbody>
</table>

Two fractions, F16 and F17, showed strong inhibition of PGE$_2$ production (80% and 82% inhibition, respectively). F17 proved to contain a single major compound, Compound 1 (Figs. 7-2, 7-3), while F16 contained multiple compounds and included overlap with the compound in F17. On this basis it was decided to proceed with structural elucidation of the major compound in F17.
Fig. 7-2. LC-MS chromatogram for Compound 1 (Fraction 17) from *Curcuma australasica*.

Fig. 7-3. Mass spectrum (M+1; left) and UV spectrum (right) for Compound 1 (Fraction 17) from *Curcuma australasica*.

Another active fraction, F20, was found to consist of another almost pure compound (Compound 2), which was also the subject of structural elucidation, although it displayed less potent activity in the PGE₂ assay (61% inhibition). The LC-MS chromatogram for F20 is shown in Fig. 7-4 and the mass and UV spectra in Fig. 7-5.
Fig. 7-4. LC-MS chromatogram for Compound 2 (Fraction 20) from *Curcuma australasica*.

Fig. 7-5. Mass spectrum (M+1; left) and UV spectrum (right) for Compound 2 (Fraction 20) from *Curcuma australasica*.
7.3.1.2 Cytotoxic activity of Compound 1

In order to ensure that the observed activity in the PGE$_2$ was not simply due to cytotoxic effects, Compound 1 was also tested for cytotoxicity in the sensitive P388D1 murine lymphoma cell line and compared to a number of other compounds and extracts (Table 7-2).

Table 7-2. Cytotoxicity of Compound 1 from Curcuma australasica in P388D1 murine lymphoma cells.

Values are mean±SD percentage survival compared to DMSO-treated (n=3).

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Percent survival (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>500 µg/mL</td>
</tr>
<tr>
<td>DMSO (control)</td>
<td>100±8</td>
</tr>
<tr>
<td>Alpinia galanga</td>
<td>1±0</td>
</tr>
<tr>
<td>Alpinia caerulea</td>
<td>68±2</td>
</tr>
<tr>
<td>Zingiber officinale</td>
<td>48±6</td>
</tr>
<tr>
<td>Curcuma longa</td>
<td>28±1</td>
</tr>
<tr>
<td>Curcuma australasica</td>
<td>34±3</td>
</tr>
<tr>
<td>Compound 1</td>
<td>-</td>
</tr>
<tr>
<td>Curcumin</td>
<td>-</td>
</tr>
<tr>
<td>6-shogaol</td>
<td>-</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>-</td>
</tr>
</tbody>
</table>

Compound 1 was found to be considerably less cytotoxic than curcumin and [6]-shogaol. The cytotoxicity of the crude extract of Curcuma australasica was similar to that of Zingiber officinale and less than that of C. longa.

7.3.1.3 Structural elucidation of Compound 1 and 2

Compounds 1 and 2 were the subjects of structural elucidation experiments by NMR spectroscopy.
Compound 1 was identified as the sesquiterpene ketone zederone (Fig. 7-6) by comparison of its spectral data with those reported in the literature (Hikino et al., 1966; Shibuya et al., 1987). These data are presented in Table 7-3.

Fig 7-6. Structure of zederone (Compound 1).
Table 7-3. ¹H and ¹³C NMR spectral data of Compound 1 (zederone) from *Curcuma australasica*.

δ = chemical shift (ppm), int = integration, mult = multiplicity, J = coupling constant, s = singlet, d = doublet, dd = doublet of a doublet, ddd = doublet of a doublet of a doublet, dddd = doublet of a doublet of a doublet of a doublet, dt = doublet of a triplet, dq = doublet of a quartet, m = multiplet, br = broad

<table>
<thead>
<tr>
<th>Position</th>
<th>δc  126 MHz</th>
<th>δh  500 MHz (int, mult, J in Hz)</th>
<th>δh, 500 MHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>131.5</td>
<td>5.49, 1H, dd (11.9, 4.0)</td>
<td>5.48, dd (11.5, 4.0)</td>
</tr>
<tr>
<td>2</td>
<td>24.9</td>
<td>2.25, 1H, m</td>
<td>2.23, br d</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.53, 1H, m</td>
<td>2.52, dddd (11.5, 12.0, 3.5, 13.5)</td>
</tr>
<tr>
<td>3</td>
<td>38.2</td>
<td>1.29, 1H, m</td>
<td>1.29, ddd (13.5, 4.0, 13.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.31, 1H, dt (13.0, 3.5)</td>
<td>2.29, ddd (13.0, 3.5, 3.5)</td>
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<td>66.8</td>
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<td>6</td>
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<tr>
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<td>123.5*</td>
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</tr>
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<td>8</td>
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</tr>
<tr>
<td>9</td>
<td>42.1</td>
<td>3.73, 2H, dd (16.5)</td>
<td>3.69, 3.75 (16.5)</td>
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<td>10</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>122.5*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>138.3</td>
<td>7.10, 1H, s</td>
<td>7.08, q (1.2)</td>
</tr>
<tr>
<td>13</td>
<td>10.5</td>
<td>2.13, 3H, d (1.2)</td>
<td>2.11, d (1.2)</td>
</tr>
<tr>
<td>14</td>
<td>15.4</td>
<td>1.35, 3H, s</td>
<td>1.34, br s</td>
</tr>
<tr>
<td>15</td>
<td>15.9</td>
<td>1.61, 3H, s</td>
<td>1.60, br s</td>
</tr>
</tbody>
</table>

* Assignment could have been interchanged.

Compound 2 was identified as another sesquiterpene ketone, 1(10)E,4E-furanodien-6-one, also by comparison with spectral data from the literature (Dekebo et al., 2000; Hikino et al., 1975; Makabe et al., 2006). These data are presented in Table 7-4.

Differences greater than 1 ppm exist between the observed δC values and those reported by Dekebo et al. (2000) for C-6 (1.2 ppm), C-7 (1.8 ppm), C-9 (1.3 ppm) and C-11 (1.2 ppm), but these can be reasonably explained by the differences in instrumentation. It should be
noted that one of the coupling constants of the olefinic proton H-1 was found to be 11.5 Hz. Dekebo et al. (2000) and Hikino et al. (1975) reported coupling constants of 6.6 and 4.5 Hz, and 7.5 and 7.5 Hz, respectively. The large \( J \) value observed with this compound (2) has to be a coupling of H-1 to one of the protons at the 2-position (H-2), probably the proton resonating at 2.32 ppm (CDCl\textsubscript{3}) and for the \( J \) value to be that high, both H-1 and one of the methylene protons must have assumed an axial-axial orientation. With the methyl group at the 10-position \textit{trans} to H-1, and the methyl group at the 4-position \textit{trans} to H-5, the structure could have formed a rigid configuration making H-1 almost axial to one of the methylene protons of H-2.
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
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<td>Compound 2</td>
<td>Compound 2</td>
<td>Compound 2</td>
<td>Compound 2</td>
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<td>4</td>
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<td></td>
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<td>5.15</td>
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</tr>
<tr>
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<tr>
<td>8</td>
<td>8.10</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Table 7-4. 1H and 13C NMR spectral data of Compound 2 (1H, (7)F, (4)E-terminated-gone) from Curnuma asystatica.
The observed $^1$H and $^{13}$C chemical shift assignments for this compound (2) agree with the literature values for 1(10)$E,4E$-furanodien-6-one as shown in Table 7-4. The structure of 1(10)$E,4E$-furanodien-6-one is shown in Fig. 7-7.

There are other reported isomers of this compound such as the 1(10)$E,4Z$ isomer, which is isofuranodienone (Hikino et al., 1975) and the 1(10)$Z,4Z$ isomer (Brieskorn & Noble, 1983). The NMR spectral data for these two isomers are summarised in Table 7-5. It is evident from inspection of Table 7-4 and Table 7-5 that the NMR data of the isolated compound (2) are consistent with those of the $E,E$ isomer.
Table 7-5. $^1$H NMR spectral data from the literature for the isomers isofuranodienone and 1(10)$Z$,4$Z$-furanodien-6-one.

$\delta =$ chemical shift (ppm)

<table>
<thead>
<tr>
<th>Position</th>
<th>Isofuranodiene Hikino et al. (1975) CDCl$_3$</th>
<th>1(10)$Z$,4$Z$-furanodien-6one Brieskorn &amp; Noble (1983) CDCl$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.20</td>
<td>5.29</td>
</tr>
<tr>
<td>5</td>
<td>6.10</td>
<td>5.88</td>
</tr>
<tr>
<td>9</td>
<td>3.13, 3.49</td>
<td>3.34</td>
</tr>
<tr>
<td>12</td>
<td>7.00</td>
<td>7.08</td>
</tr>
<tr>
<td>13</td>
<td>1.91</td>
<td>2.20</td>
</tr>
<tr>
<td>14</td>
<td>1.89</td>
<td>1.76</td>
</tr>
<tr>
<td>15</td>
<td>1.55</td>
<td>1.65</td>
</tr>
</tbody>
</table>
7.3.2 Pleuranthodium racemigerum

7.3.2.1 Activity-guided fractionation

An HPLC chromatogram of the extract redissolved in 90% aqueous methanol is shown in Fig. 7-8.

![HPLC Chromatogram](Z:\CHROMO~1\001-0101.D)

Fig. 7-8. LC-MS chromatogram of Pleuranthodium racemigerum extract redissolved in 90% aqueous methanol.

Top: 210 nm; centre: 280 nm; bottom: 360 nm.

Based on the chromatograms of the whole extract and the individual fractions, fractions were combined prior to testing for their ability to inhibit PGE$_2$ production in murine fibroblasts. The results of this assay are shown in Table 7-6.
Table 7-6. Inhibition of PGE₂ production in 3T3 murine fibroblast cells by combined fractions of *Pleuranthodium racemigera* extract.

Fractions and reference compounds were dissolved in DMSO at a concentration of 10mg/mL prior to being assayed. * Concentrations are final concentrations of test substance in assay well.

<table>
<thead>
<tr>
<th>Combined fraction no. (fractions)</th>
<th>Percent inhibition (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1μg/mL*</td>
</tr>
<tr>
<td></td>
<td>5μg/mL*</td>
</tr>
<tr>
<td></td>
<td>10μg/mL*</td>
</tr>
<tr>
<td>C1 (1-8)</td>
<td>-35±3</td>
</tr>
<tr>
<td></td>
<td>-20±3</td>
</tr>
<tr>
<td></td>
<td>-33±3</td>
</tr>
<tr>
<td>C2 (9-13)</td>
<td>4±0</td>
</tr>
<tr>
<td></td>
<td>17±2</td>
</tr>
<tr>
<td></td>
<td>27±1</td>
</tr>
<tr>
<td>C3 (14-16)</td>
<td>10±1</td>
</tr>
<tr>
<td></td>
<td>24±6</td>
</tr>
<tr>
<td></td>
<td>28±3</td>
</tr>
<tr>
<td>C4 (17-18)</td>
<td>15±1</td>
</tr>
<tr>
<td></td>
<td>41±9</td>
</tr>
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<td>47±4</td>
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<td>C5 (19-22)</td>
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<td>19±2</td>
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<td></td>
<td>-4±0</td>
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<td>C6 (23-29)</td>
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<td></td>
<td>13±1</td>
</tr>
<tr>
<td></td>
<td>-11±2</td>
</tr>
<tr>
<td>Aspirin (0.05mM*)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30±8</td>
</tr>
</tbody>
</table>

The most potent combined fraction, C4, was found to consist of a pure compound (Compound 3). At a concentration of 10 μg/mL (subsequently calculated to be equivalent to 33.7 μM), this compound inhibited PGE₂ production in 3T3 cells by close to 50%.

The LC-MS chromatogram for Compound 3 is shown in Fig. 7-9 and the mass and UV spectra in Fig. 7-10.
Compound 3 was subjected to further testing for cytotoxic activity and structural elucidation.
7.3.2.2 Cytotoxic activity of Compound 3

Compound 3 was screened for cytotoxic effect in 3T3 murine fibroblast cells. As considerable cytotoxicity was detected (LD$_{50}$ = 52.8 µM) after incubation for 3 hours (Fig 7-11), further testing was undertaken.

![Cytotoxicity of Compound 3 against 3T3 cells after 3 h](chart)

**Fig. 7-11. Cytotoxic effect of fraction Compound 3 from Pleuranthodium racemigerum on 3T3 murine fibroblasts.**

Compound 3 was tested for cytotoxic activity against one other murine cells line (P388D1 lymphoblast) and four human cell lines (Caco-2 colonic adenocarcinoma, PC3 prostate adenocarcinoma, HepG2 hepatocyte carcinoma, and MCF7 mammary adenocarcinoma). The cytotoxicity of Compound 3 was compared with that of the related compound curcumin and the chemotherapeutic drug chlorambucil. The results are shown in Fig. 7-12.
Fig. 7-12. Dose-response curves for cytotoxic activity of Compound 3 from *Pleuranthodium racemigerum* against five cell lines.

Curcumin was included for comparison and chlorambucil as a positive control.
LD₅₀ values based on the dose-response curves were calculated for Compound 3 and curcumin (Table 7-7).

Table 7-7. LD₅₀ (µM) values for cytotoxic activity of Compound 3 and curcumin against five cancer cell lines.
95% confidence intervals are shown in brackets

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Compound 3</th>
<th>Curcumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caco-2</td>
<td>44.8 (32.6-61.8)</td>
<td>48.6 (38.9-60.4)</td>
</tr>
<tr>
<td>PC3</td>
<td>23.6 (20.6-26.9)</td>
<td>22.7 (17.7-28.9)</td>
</tr>
<tr>
<td>HepG2</td>
<td>40.6 (28.0-58.9)</td>
<td>43.8 (25.6-75.2)</td>
</tr>
<tr>
<td>MCF7</td>
<td>56.9 (45.3-71.1)</td>
<td>51.5 (34.8-76.0)</td>
</tr>
<tr>
<td>P388D1</td>
<td>117.0 (53.4-255.6)</td>
<td>75.7 (65.9-86.8)</td>
</tr>
</tbody>
</table>

The cytotoxic effects of Compound 3 closely reflected those of curcumin in the four human cancer cell lines. In the case of the murine P388D1 cell line the LD₅₀ for Compound 3 was 55% higher than for curcumin.

7.3.2.3 Structural elucidation of Compound 3

The NMR data obtained for Compound 3 from Z128 Pleuranthodium racemigerum are summarised in Table 7-8.
Table 7-8. 1H and 13C NMR data from one-dimensional (1H and J-modulated 13C NMR) and two-dimensional correlation (COSY, HSQC and HMBC) NMR spectroscopy experiments on Compound 3 from Pleuranthodium racemigerum.

δ = chemical shift (ppm), int = integration, mult = multiplicity, J = coupling constant, s = singlet, d = doublet, t = triplet, dt = doublet of a triplet, tt = triplet of a triplet

<table>
<thead>
<tr>
<th>Position</th>
<th>Chemical shift</th>
<th>1H (COSY)</th>
<th>HMBC (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>δH, 500 MHz (int, mult, J in Hz)</td>
<td>δC 126 MHz</td>
<td>1JCH</td>
</tr>
<tr>
<td>1</td>
<td>3.29 d (6.4)</td>
<td>38.3</td>
<td>H-2</td>
</tr>
<tr>
<td>2</td>
<td>5.55 dt (15.2, 6.4)</td>
<td>129.6</td>
<td>H-1, H-3</td>
</tr>
<tr>
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<td>5.51 dt (15.2, 6.4)</td>
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<td>H-2, H-4</td>
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<td>4</td>
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<td>1.43 tt (7.3, 7.7)</td>
<td>29.2</td>
<td>H-4, H-6</td>
</tr>
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<td>1.61 dt (7.7, 7.8)</td>
<td>31.4</td>
<td>H-5, H-7</td>
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<td>7</td>
<td>2.55 t (7.8)</td>
<td>35.0</td>
<td>H-6</td>
</tr>
<tr>
<td>1'</td>
<td>-</td>
<td>135.1</td>
<td>-</td>
</tr>
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<td>2'</td>
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<td>H-3'</td>
</tr>
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<td>6.76 d (8.5)</td>
<td>115.3</td>
<td>H-2'</td>
</tr>
<tr>
<td>4'</td>
<td>-</td>
<td>153.8</td>
<td>-</td>
</tr>
<tr>
<td>5'</td>
<td>6.76 d (8.5)</td>
<td>115.3</td>
<td>H-6'</td>
</tr>
<tr>
<td>6'</td>
<td>7.04 d (8.5)</td>
<td>129.6</td>
<td>H-5'</td>
</tr>
<tr>
<td>1&quot;</td>
<td>-</td>
<td>133.4</td>
<td>-</td>
</tr>
<tr>
<td>2&quot;</td>
<td>7.11 d (8.7)</td>
<td>129.6</td>
<td>H-3&quot;</td>
</tr>
<tr>
<td>3&quot;</td>
<td>6.86 d (8.7)</td>
<td>114.0</td>
<td>H-2&quot;</td>
</tr>
<tr>
<td>4&quot;</td>
<td>-</td>
<td>158.0</td>
<td>-</td>
</tr>
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<td>5&quot;</td>
<td>6.86 d (8.7)</td>
<td>114.0</td>
<td>H-6&quot;</td>
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<tr>
<td>6&quot;</td>
<td>7.11 d (8.7)</td>
<td>129.6</td>
<td>H-5&quot;</td>
</tr>
<tr>
<td>-OCH₃</td>
<td>3.82 s</td>
<td>55.5</td>
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</tr>
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</table>
The $^1$H NMR spectrum showed signals in the region of 6.7-7.2 ppm, indicative of aromatic protons. Signals were consistent with two aromatic rings.

**Ring A.** It was observed that the proton at 6.76 ppm (H-3’/H-5’) showed coupling to the proton at 7.04 ppm (H-2’/H-6’) with a $J$ value (coupling constant) of 8.5 Hz, suggesting ortho-coupling. These signals each integrated to two protons, suggesting two sets of identical protons in an aromatic ring.

The HSQC showed that the protons H-3’/H-5’ and H-2’/H-6’ were directly correlated to the carbon signals at 115.3 and 129.6 ppm, respectively. HMBC showed long-range correlations of the protons to the quaternary carbon signals at 153.8 ppm (C-4’) and 135.2 ppm (C-1’), which made up the aromatic ring system with the carbon at position 4’ having a hydroxyl substituent, as signified by its resonance in the downfield region (Fig. 7-13).

![Fig. 7-13. Heteronuclear correlations in Ring A.](image)

**Ring B.** Similar to what was observed for Ring A, the proton at 6.86 ppm (H-3”/H-5”) showed coupling to the proton at 7.11 ppm (H-2”/H-6”) with a $J$ value of 8.7 Hz, indicative of ortho-coupling. Again, these signals integrated to two protons each, suggesting the presence of a second aromatic ring.

Analogous to the findings for Ring A, the HSQC showed protons H-3”/H-5” and H-2”/H-6” to be directly correlated to the carbon signals at 114.0 ppm and 129.6 ppm, respectively, and HMBC showed long-range correlations to quaternary carbon signals at 158.0 ppm (C-4”) and 133.4 ppm. In contrast to Ring A, a singlet that integrated to 3H was observed in the $^1$H spectrum resonating at 3.82 ppm, consistent with the presence of a methoxy substituent. HMBC showed correlation of the methoxy protons to the quaternary carbon at 158.0 ppm (C-4”), placing the methoxy group at the C-4” position (Fig. 7-14).
Carbon bridge. The proton signals at 5.55 ppm (H-2) and 5.51 ppm (H-3) indicated olefinic protons coupling to each other in an $E$ position ($J = 15.2$ Hz). Five methylene proton signals (H-1, H-4, H-5, H-6 and H-7) were also present in the region from 1.4 ppm to 3.3 ppm, and the COSY experiment showed that H-1 and H-4 were vicinal to H-2 and H-3, respectively. H-5 also showed $J_{HH}$ correlation to H-4 and H-6, and H-6 to H-7, as shown in Fig. 7-15.

HMBC showed correlation of H-1 to the quaternary carbon at 133.4 ppm (C-1’”), which connected C-1 to C-1””. Likewise, H-7 showed correlation to the quaternary carbon at 135.1 ppm (C-1’), linking C-7 to C-1’.

The structure of Compound 3 as shown in Fig. 7-15 was given the systematic name 1-(4”-methoxyphenyl)-7-(4’-hydroxyphenyl)-2$E$-heptene. The APCI-MS data showed a $(M+1)^+$ value of 297.2, which is consistent with a molecular formula of $C_{20}H_{24}O_2$.  

Fig. 7-15. Chemical structure of Compound 3 from *Pleuranthodium racemigerum*, 1-(4”-methoxyphenyl)-7-(4’-hydroxyphenyl)-2$E$-heptene.
7.3.2.4 Accurate Mass

The accurate mass for the Compound 3 was experimentally determined to be 319.1683, which is consistent with the formula C\textsubscript{20}H\textsubscript{24}O\textsubscript{2}Na and the theoretical mass of 319.1674 as shown in Table 7-9. This confirmed the molecular formula of the novel Compound 3 as C\textsubscript{20}H\textsubscript{24}O\textsubscript{2}.

Table 7-9. Accurate mass determination for Compound 3.

<table>
<thead>
<tr>
<th></th>
<th>Mass determined by MS</th>
<th>Theoretical mass*</th>
<th>Difference (δ)</th>
<th>Exact mass of C\textsubscript{20}H\textsubscript{24}O\textsubscript{2}*</th>
<th>Molecular weight of C\textsubscript{20}H\textsubscript{24}O\textsubscript{2}*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C\textsubscript{20}H\textsubscript{24}O\textsubscript{2}Na</td>
<td>319.1683</td>
<td>C\textsubscript{20}H\textsubscript{24}O\textsubscript{2}Na</td>
<td>319.1674</td>
<td>0.0009</td>
<td>296.18</td>
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<tr>
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<td>296.40</td>
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</table>

* Data from ChemDraw Pro v. 4.01 (CambridgeSoft Corporation, Cambridge, Massachusetts, 1997)

7.3.2.5 UV spectrophotometric data and extinction coefficient

The UV spectrum of the novel Compound 3, 1-(4’-methoxyphenyl)-7-(4’-hydroxyphenyl)-2\textit{E}-heptene, is shown in Fig. 7-16. The peak absorption (λ\textsubscript{max}) of the compound was at 203 nm, with other absorption maxima at 225 and 278 nm.
The extinction coefficient (wavelength-dependent molar absorptivity coefficient), $\varepsilon$, was calculated for the novel Compound 3 from *Pleuranthodium racemigerum* using $\varepsilon = A/(c \times l)$, where $A$ is the absorbance, $c$ the molar concentration ($4.7233468 \times 10^{-5}$ M), and $l$ the distance (cuvette path; 1 cm). The peak absorbance readings and extinction coefficients $\varepsilon$ for Compound 3, 1-(4”-methoxyphenyl)-7-(4’-hydroxyphenyl)-2E-heptene at 203 nm, 225 nm and 278 nm are shown in Table 7-10.

**Table 7-10. Peak absorption ($\lambda_{\text{max}}$) and extinction coefficients ($\varepsilon$) of Compound 3 at 203 nm, 225 nm and 278 nm.**

<table>
<thead>
<tr>
<th></th>
<th>Abs$\lambda_{203\text{nm}}$</th>
<th>Abs$\lambda_{225\text{nm}}$</th>
<th>Abs$\lambda_{278\text{nm}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>0.82368</td>
<td>0.16267</td>
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<td>1.19610</td>
<td>0.82355</td>
<td>0.16243</td>
</tr>
<tr>
<td>3</td>
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<td>4</td>
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<td>0.16282</td>
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<tr>
<td>5</td>
<td>1.19570</td>
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<td>0.16307</td>
</tr>
<tr>
<td>Mean</td>
<td><strong>1.19480</strong></td>
<td><strong>0.82447</strong></td>
<td><strong>0.16274</strong></td>
</tr>
<tr>
<td>SD</td>
<td>0.00301</td>
<td>0.00105</td>
<td>0.00023</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>$2.53 \times 10^4$</td>
<td>$1.75 \times 10^4$</td>
<td>$3.45 \times 10^3$</td>
</tr>
<tr>
<td>$\log(\varepsilon)$</td>
<td><strong>4.40</strong></td>
<td><strong>4.24</strong></td>
<td><strong>3.54</strong></td>
</tr>
</tbody>
</table>

Fig. 7-16. UV spectrum of 1-(4”-methoxyphenyl)-7-(4’-hydroxyphenyl)-2E-heptene (Compound 3).
7.4 Discussion

Biologically active compounds were successfully isolated from two native Australian Zingiberaceae species using bioactivity-guided fractionation. Bioactivity-guided fractionation is a widely used method in natural products research. It is an effective means of isolating the most active major compounds in a complex mixture, while it might be less suitable for the identification of highly active compounds that occur in very low concentrations. As the present work was of a preliminary nature, carried out on species that had never been studied previously, bioactivity-guided fractionation was deemed an appropriate methodology.

7.4.1 Curcuma australasica

From Curcuma australasica the sesquiterpene ketones zederone (Compound 1, Fig. 7-6) and furanodien-6-one (Compound 2, Fig. 7-7) were isolated for the first time. Zederone has previously been reported from the rhizomes of C. zedoaria (Christm.) Roscoe (Hikino et al., 1966; Makabe et al., 2006) and C. phaeocaulis Valeton (Hou et al., 1997), and from Chloranthus serratus (Thunb.) Roem. et Schult. (Chloranthaceae) (Kawabata et al., 1985), while furanodien-6-one has been reported from C. zedoaria (Hikino et al., 1975; Makabe et al., 2006), Commiphora molmol Engler (myrrh) (Brieskorn & Noble, 1983) and other Commiphora species (Dekebo et al., 2000).

Zederone was isolated from the almost pure fraction of Curcuma australasica that most potently inhibited the production of PGE₂, suggesting this compound confers anti-inflammatory activity to C. australasica. Zederone was found to be moderately cytotoxic against P388D1 cells, but its toxicity was less than that of both curcumin and [6]-shogaol. Compared with zederone, furanodien-6-one was a less potent inhibitor of PGE₂. These findings are in contrast to those of Makabe et al. (2006), who tested 11 sesquiterpenoids isolated from C. zedoaria for in vivo anti-inflammatory activity in the 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear oedema model and found furanodien-6-one but not zederone to be a potent topical anti-inflammatory agent. As oedema induced by TPA is reduced or delayed by COX inhibitors (Young et al., 1983), an anti-inflammatory effect of zederone in this model would have been expected, given the
good *in vitro* activity in the PGE₂ seen in the present study. Given the purity of Fraction 17 from which zederone was isolated, it seems unlikely that a compound other than zederone contributed significantly to the inhibition of PGE₂. It is possible that the seemingly incongruent results obtained in the two studies reflect differences between an *in vitro* model and an *in vivo* situation, or that stability issues played a role.

### 7.4.2 *Pleuranthodium racemigerum*

From *Pleuranthodium racemigerum*, the extract of which was a potent inhibitor of PGE₂ production (see Chapter 6), a novel bioactive curcuminoid compound was isolated (Compound 3, Fig. 7-15). This compound, 1-(4‴-methoxyphenyl)-7-(4‴-hydroxyphenyl)-2E-heptene, also strongly inhibited PGE₂ production (IC₅₀ ≅ 34 µM). In addition, it displayed considerable dose-dependent cytotoxicity against four human and two murine cells lines. Its potency as a cytotoxic agent was similar to that of the related curcuminoid compound, curcumin. These findings suggest this novel compound could warrant further investigations, both as a potential anti-inflammatory agent and as a cytotoxic agent. Whether its cytotoxicity precludes it from being used as an anti-inflammatory agent would need to be determined in the first instance by animal studies, but the fact that the cytotoxic potency resembled that of curcumin suggests that it may exert anti-inflammatory activity *in vivo* at sub-toxic concentrations.

This is the first report concerning the chemistry and pharmacology of *Pleuranthodium racemigerum*. As this species grows in rainforest and has a limited distribution, it is doubtful that wild stock would be able to sustain a potential development of this plant as a source of a new medicinal substance, but the plant could potentially be cultivated in tropical areas, should further work indicate that an efficacious and safe medicine can be derived from it.
8. CONCLUDING REMARKS

This chapter discusses the findings in terms of the research questions and aims of the project (refer to Chapter 2, Section 2.16). It also identifies areas of future research arising from the present work.

8.1 Phytochemical investigations of ginger (Zingiber officinale)

This part of the work made a significant and original contribution to the knowledge of the phytochemistry of ginger. It included the first extensive survey of Australian commercial and experimental ginger clones in terms of the main pungent compounds, and also provided the first survey of steam-distilled Australian ginger oils for more than three decades.

Seventeen clones of ginger were examined for gingerol and shogaol content and in terms of essential oil composition (Chapters 4 and 5). The aim of identifying a clone yielding particularly high levels of pharmacologically active gingerols was achieved. The cultivar known as ‘Jamaican’ produced significantly higher concentrations of [6]-, [8]- and [10]-gingerol than did any other clone, and it contained these compounds in a somewhat different ratio. A linear relationship existed between the content of [6]-, [8]- and [10]-gingerol, which occurred in the approximate ratio of 3:1:1 in all clones except ‘Jamaican’, which contained relatively higher levels of [8]- and [10]-gingerol.

Given that gingerols appear to confer many of the pharmacological effects of ginger (refer to Chapter 2, Section 2.2.1.3), ‘Jamaican’ may well prove to be of particular pharmaceutical interest, although agronomic studies would be required to confirm its suitability as a viable source of raw material for pharmaceutical use.

‘Jamaican’ also yielded an essential oil that was distinct from that of other clones. It was characterised by a relatively high content of sesquiterpene hydrocarbons (incl. zingiberene, β-sesquiphellandrene and ar-curcumene) and a relatively low content of monoterpenoids such as geranial, neral and geranyl acetate, compared with the other clones. The combination of high gingerol levels (pungency) with distinct essential oil composition (aroma) should make ‘Jamaican’ a cultivar of interest also to the flavour and fragrance industry.
The question of whether significant phytochemical differences that are genetically determined exist between genotypes of ginger was answered in the affirmative with the identification of the cultivar ‘Jamaican’ and its distinct phytochemical profile, which set it apart from 16 other clones that were grown, harvested and extracted under identical conditions.

Apart from the ‘Jamaican’, none of the clones stood out phytochemically. This included the experimental tetraploid clones, which did not differ significantly from their parent cultivar in terms of gingerol content or essential oil composition. This is noteworthy, because a previous study in Japan found tetraploid gingers to contain increased levels of gingerols (Nakasone et al., 1999), and it demonstrates that the effects of polyploidy on secondary metabolites are unpredictable.

With the exception of ‘Jamaican’, the steam-distilled essential oils were characterised by very high levels (>50%) of citral (neral + geranial) and correspondingly low levels of sesquiterpenes, when compared with published ginger oil analyses generally and the previous survey of Australian ginger essential oil (Connell & Jordan, 1971) in particular. These findings confirm the reputation of Australian ginger as having a ‘lemony’ aroma due to its high citral content. Neral and geranial occurred in a fixed ratio of 2:3 in all clones.

Both aqueous and ethanolic extracts of ginger were shown to inhibit COX-1 in vitro (Chapter 3), which is in accordance with the literature (refer to Chapter 2, Section 2.2.1.3.2). A strong positive correlation existed between inhibitory activity and the content of both [6]- and [8]-gingerol, confirming that these compounds inhibit COX-1. Ethanol extracted gingerols more effectively than did water. Hot water was more effective than cold water, but when ethanol was used as a solvent, temperature did not affect the extraction efficiency, suggesting that ethanol at ambient temperature is a highly efficient solvent of gingerols.

Most of the phytochemical investigations of ginger in the present study were carried out on extracts prepared from fresh rhizomes at ambient temperature. Shogaols were not detected in these extracts; [6]-shogaol was identified only in hot Soxhlet extracts. This would appear to confirm that shogaols are not native constituents of ginger rhizome, but form as degradation products of gingerols.
8.2 Screening Zingiberaceae for pharmacological activity

In this part of the work 41 taxa were screened for *in vitro* inhibition of PGE₂ production, and a number of the samples were also tested for antioxidant activity, inhibition of nitric oxide production, and for modulation of natural killer cell activity. The work succeeded in making a substantial and original contribution to the knowledge of the Zingiberaceae as medicinal plants and a source of pharmacologically active compounds.

The hypothesis that the combination of ethnobotanical and taxonomic information is a productive strategy to identify previously unrecognised plants with therapeutic potential was supported by the work, which identified two native Australian species that had not previously been known to possess pharmacological activity.

The work also provided new insights into the pharmacological activity of several known medicinal plants. Inhibition of PGE₂ was demonstrated for the first time for extracts of the South-east Asian medicinal plants *Boesenbergia rotunda* and *Curcuma parviflora*. This supports the traditional use of *B. rotunda* in the treatment of rheumatism and muscular pains (bin Jantan *et al.*, 2001). The inhibition of nitric oxide by *Zingiber montanum* was shown for the first time. This property supports the use of the species in traditional Thai medicine for the treatment of asthma (Piromrat *et al.*, 1986), given that nitric oxide acts as an inflammatory mediator in the lung, and concentrations of nitric oxide correlate with airway inflammation in this condition (Stewart & Katial, 2007). Few Zingiberaceae species have previously been tested for modulation of natural killer cell activity, and the finding that both *Alpinia galanga* and the aqueous extract of fresh *B. rotunda* caused inhibition of natural killer cell activity is novel and warrants further investigation. It would also be desirable to conduct further work to confirm whether ethanolic extracts of *B. rotunda* and *C. longa* do in fact significantly increase the activity of NK cells, as suggested by the present work.

Two native Australian species, *Curcuma australasica* and *Pleuranthodium racemigerum*, were identified in this work as being potent inhibitors of PGE₂ (IC₅₀ values being similar to that of *Zingiber officinale*). Neither species has a recorded history of use as a medicinal plant, nor has either of them been the subject of previous pharmacological or phytochemical investigations. From *C. australasica* two known sesquiterpene ketones, zederone and furanodienone, were isolated. Both inhibited PGE₂ production, zederone more potently so. These compounds have previously been reported from other *Curcuma* species. From *P.*
racemigerum a novel curcuminoid compound, 1-(4”-methoxyphenyl)-7-(4’-hydroxyphenyl)-2E-heptene, was isolated. This compound was a potent inhibitor of PGE\textsubscript{2} production (IC\textsubscript{50} \cong 34 \, \mu M), and its structure was successfully elucidated using NMR techniques. It also displayed dose-dependent cytotoxicity against six different cell lines, its potency being similar to that of the related compound, curcumin. Curcuminoids and other diarylheptanoids are widespread in some Zingiberaceae genera, such as Curcuma, Zingiber and Alpinia.

Thus this work has contributed to the understanding of the pharmacology of the Zingiberaceae. It has also successfully identified species with previously unrecognised pharmacological activity from genera with recognised medicinal species (Pleuranthodium racemigerum was previously placed in the genus Alpinia) and isolated active compounds that were chemically related to other active compounds in related species.

### 8.3 Direction of future research

The Zingiberaceae, with about 1100 species in more than 50 genera, clearly represents a rich source of secondary metabolites. For most of these species little or no information about their phytochemistry or pharmacological properties exist, so there is vast scope for further investigations of the family as a whole. In terms of future research arising more directly from the present work, there is also significant scope.

The ginger cultivar ‘Jamaican’ was identified as being phytochemically unique among the clones investigated, but whether it would prove a viable source of raw material for the pharmaceutical and/or flavour and fragrance industries would depend on its agronomic attributes. Agronomic trials with ‘Jamaican’ would be able to determine its suitability as a commercial crop in terms of yield, resistance to disease, etc.

Although none of the tetraploid clones included in this work proved to have altered phytochemical characteristics, it would be worthwhile to monitor new experimental polyploid clones, in particular with respect to increased production of gingerols.

It would also be of interest to investigate the environmental effects, in particular climate and latitude, as well as the effect of growth period and harvest time, on the phytochemistry of
ginger. Such work could clarify, for example, if any of these parameters impact on the citral content of the essential oil.

With respect to the pharmacological activity of the species included in this work, there is clearly much more to be done. The PGE\textsubscript{2} assay employed was expensive and time consuming to the point of being limiting. With the initial screening done, the species that showed inhibition greater than 50% should be subjected to further work in order to establish accurate IC\textsubscript{50} values. Several other bioassays relevant to potential anti-inflammatory activity could be applied. These include inhibition of phospholipase A\textsubscript{2} (PLA\textsubscript{2}), 5-lipoxygenase (5-LOX) and TNF-\textalpha, and effects on COX-1 and COX-2 expression and on the key transcription factor nuclear factor kappa B (NF\kappa B).

The most promising samples in these assays could be targeted for bioassay-guided fractionation provided the active compounds were not already identified. As only a subset of species were included in the assays for antioxidant activity, inhibition of nitric oxide and modulation of natural killer cell activity, the remainder could be screened in these assays. It would also be desirable to screen them for cytotoxic activity.

In terms of the two native Australian species that were found to possess good PGE\textsubscript{2} inhibitory activity, further pharmacological investigations as outlined above would be highly desirable. Both species should also undergo further phytochemical studies in an attempt to isolate and characterise more novel bioactive compounds. Given the paucity of information on any Australian Zingiberaceae, it would be highly desirable to carry out a comprehensive survey using bioassay-guided fractionation of the remainder 12 native species.
<table>
<thead>
<tr>
<th>Study Design</th>
<th>Main Outcome Measures</th>
<th>Design</th>
<th>Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**A P P E N D I X A:** **CLINICAL EFFICACY TRIALS OF GINGER PREPARATIONS**

VAS: Visual analogue scale; V- Western Ontario and McMaster Universities Osteoarthritis Index of Osteoarthritics, L/D: twice daily; 4d/L: four times daily.

<table>
<thead>
<tr>
<th>Post-operative nausea and vomiting</th>
<th>( p &gt; 0.05 )</th>
<th>( p &lt; 0.05 )</th>
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<td>( p &gt; 0.05 )</td>
<td>( p &gt; 0.05 )</td>
</tr>
<tr>
<td>Postoperative nausea and vomiting</td>
<td>( p &lt; 0.05 )</td>
<td>( p &lt; 0.05 )</td>
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<tr>
<td>Postoperative nausea and vomiting</td>
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<td>( p &gt; 0.05 )</td>
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<td>( p &lt; 0.05 )</td>
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<td>( p &gt; 0.05 )</td>
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<td>( p &lt; 0.05 )</td>
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<td>( p &lt; 0.05 )</td>
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<td><strong>Immunologic Monitoring</strong></td>
<td><strong>Safety Monitoring</strong></td>
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<td>----------------------</td>
<td>---------------------------</td>
<td>----------------------</td>
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<td>Dobutamine, phenylephrine, epinephrine</td>
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<tr>
<td>Treatment</td>
<td>VAS: *</td>
<td>Severity of nausea</td>
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<td>-----------</td>
<td>--------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Placebo</td>
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<td>10</td>
</tr>
<tr>
<td>Treatment A</td>
<td>60</td>
<td>5</td>
</tr>
</tbody>
</table>

* VAS: Visual Analogue Scale
**APPENDIX B: QUALITY ASSESSMENT OF CLINICAL TRIALS OF GINGER IN OSTEOARTHRITIS**

Assessment of the quality of the reporting of two randomised trials of ginger in osteoarthritis (with ginger extract as the sole active intervention).

Checklist based on the revised CONSORT Statement (Moher et al., 2001) except items marked with an asterisk (*), which were adapted from the CONSORT Statement elaborated for the reporting of herbal interventions (Gagnier et al., 2006). Each item was given a score of 0, 0.5 or 1, depending on whether the information was provided not at all or to an unsatisfactory extent (0), to some extent (0.5), or to a satisfactory or mostly satisfactory extent (1).

<table>
<thead>
<tr>
<th>PAPER SECTION And topic</th>
<th>Item</th>
<th>Description</th>
<th>Bliddal et al. 2000</th>
<th>Wigler et al. 2003</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE &amp; ABSTRACT</td>
<td>1</td>
<td>How participants were allocated to interventions (e.g., &quot;random allocation&quot;, &quot;randomized&quot;, or &quot;randomly assigned&quot;).</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>INTRODUCTION Background</td>
<td>2</td>
<td>Scientific background and explanation of rationale.</td>
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<td>0</td>
</tr>
<tr>
<td>METHODS Participants</td>
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<td>Eligibility criteria for participants and the settings and locations where the data were collected.</td>
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<td>0.5</td>
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<tr>
<td>Interventions</td>
<td>4</td>
<td>Precise details of the interventions intended for each group and how and when they were actually administered.</td>
<td>Elaborated in 4A-4F</td>
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<tr>
<td>Herbal medicinal product name</td>
<td>4A*</td>
<td>E.g. Latin binomial, extract name, brand name, name of manufacturer</td>
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<td>1</td>
</tr>
<tr>
<td>Characteristics of the herbal product</td>
<td>4B*</td>
<td>Incl. plant parts, fresh or dried or extract, solvent details, method of authentication, lot number of raw material, details of voucher specimen or retention sample</td>
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</tr>
<tr>
<td>Dosage regimen and qualitative description</td>
<td>4C*</td>
<td>Dosage, duration of administration, how these were determined; content of quantified herbal constituents per dosage unit; details of additives; for standardised products, the quantity of each active/marker per dosage unit</td>
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</tr>
<tr>
<td>Qualitative testing</td>
<td>4D*</td>
<td>Chemical fingerprint and methods for obtaining this; description of any special/purity testing; standardisation: what to standardise and how</td>
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<td>0</td>
</tr>
<tr>
<td>Placebo/control group</td>
<td>4E*</td>
<td>The rationale for the type of control/placebo used</td>
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<td>1</td>
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<td>Practitioner</td>
<td>4F*</td>
<td>A description of the practitioners (e.g., training and practice experience) that are a part of the intervention</td>
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<td>Specific objectives and hypotheses.</td>
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<tr>
<td>Outcomes</td>
<td>6</td>
<td>Clearly defined primary and secondary outcome measures and, when applicable, any methods used to enhance the quality of measurements (e.g., multiple observations, training of assessors).</td>
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<td>1</td>
</tr>
<tr>
<td>Sample size</td>
<td>7</td>
<td>How sample size was determined and, when applicable, explanation of any interim analyses and stopping rules.</td>
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<td>Code</td>
<td>Description</td>
<td>Score</td>
<td>Correct Score</td>
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<tr>
<td>Randomization - Sequence generation</td>
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<td>Method used to generate the random allocation sequence, including details of any restrictions (e.g., blocking, stratification)</td>
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<td>1</td>
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<tr>
<td>Randomization - Allocation concealment</td>
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<td>Method used to implement the random allocation sequence (e.g., numbered containers or central telephone), clarifying whether the sequence was concealed until interventions were assigned.</td>
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<tr>
<td>Randomization - Implementation</td>
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<td>Who generated the allocation sequence, who enrolled participants, and who assigned participants to their groups.</td>
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<td>Blinding (masking)</td>
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<td>Whether or not participants, those administering the interventions, and those assessing the outcomes were blinded to group assignment. When relevant, how the success of blinding was evaluated.</td>
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<td>0.5</td>
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<tr>
<td>Statistical methods</td>
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<td>Statistical methods used to compare groups for primary outcome(s); Methods for additional analyses, such as subgroup analyses and adjusted analyses.</td>
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<tr>
<td>RESULTS Participant flow</td>
<td>13</td>
<td>Flow of participants through each stage (a diagram is strongly recommended). Specifically, for each group report the numbers of participants randomly assigned, receiving intended treatment, completing the study protocol, and analyzed for the primary outcome. Describe protocol deviations from study as planned, together with reasons.</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Recruitment</td>
<td>14</td>
<td>Dates defining the periods of recruitment and follow-up.</td>
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<td>Baseline data</td>
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<td>Baseline demographic and clinical characteristics of each group.</td>
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<td>1</td>
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<tr>
<td>Numbers analyzed</td>
<td>16</td>
<td>Number of participants (denominator) in each group included in each analysis and whether the analysis was by &quot;intention-to-treat&quot;. State the results in absolute numbers when feasible (e.g., 10/20, not 50%).</td>
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<td>1</td>
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<tr>
<td>Outcomes and estimation</td>
<td>17</td>
<td>For each primary and secondary outcome, a summary of results for each group, and the estimated effect size and its precision (e.g., 95% confidence interval).</td>
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<td>1</td>
</tr>
<tr>
<td>Ancillary analyses</td>
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<td>Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those pre-specified and those exploratory.</td>
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<td>-</td>
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<tr>
<td>Adverse events</td>
<td>19</td>
<td>All important adverse events or side effects in each intervention group.</td>
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<td>1</td>
</tr>
<tr>
<td>DISCUSSION Interpretation</td>
<td>20</td>
<td>Interpretation of the results, taking into account study hypotheses, sources of potential bias or imprecision and the dangers associated with multiplicity of analyses and outcomes.</td>
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<td>1</td>
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<tr>
<td>Generalizability</td>
<td>21</td>
<td>Generalizability (external validity) of the trial findings.</td>
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<tr>
<td>Overall evidence</td>
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<td>Total score</td>
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**Total score:** 17.5
# APPENDIX C: INHIBITION OF PGE$_2$ IN 3T3 CELLS

Concentrations shown are final concentrations in assay well. Mean value±SEM; n=3 except * (n=2), + (n=4) and ^ (n=6).

<table>
<thead>
<tr>
<th>Taxon</th>
<th>ID</th>
<th>Concentration (µg/mL)</th>
<th>Percent inhibition of PGE$_2$</th>
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</thead>
<tbody>
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<td><em>Alpinia arctiflora</em></td>
<td>Z129</td>
<td>100.0</td>
<td>-18.51±1.81</td>
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<td>50.0</td>
<td>12.08±1.39</td>
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<tr>
<td></td>
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<td>10.0</td>
<td>1.41±0.26</td>
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<tr>
<td><em>Alpinia caerulea</em></td>
<td>Z102</td>
<td>94.8</td>
<td>33.81±8.99</td>
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<td></td>
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<td>47.4</td>
<td>35.37±5.61</td>
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