Phytochemical and Biochemical Investigations of

*Ochna macrocalyx*

and

*Bupleurum fruticosum*

- Searching for NF-κB inhibitory compounds

Thesis presented by

Sharon Shuk Lan Tang

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Centre for Pharmacognosy and Phytotherapy

The School of Pharmacy

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ABSTRACT

As part of a continuing search for natural product NF-κB inhibitors, the medicinal plants *Ochna macrocalyx* and *Bupleurum fruticosum* were selected for phytochemical and biochemical investigations. *Ochna macrocalyx* compounds were additionally tested for antibacterial and cytotoxic activity. The transcription factor NF-κB regulates the expression of genes involved in the immune and inflammatory responses.

*Ochna macrocalyx* Oliv. (Ochnaceae) is a medicinal Tanzanian tree used for gastrointestinal and gynaecological disorders, which was collected during an ethnobotanical study. Fractions of the crude ethanolic extract inhibited NF-κB at 200 μg/ml in an electrophoretic mobility shift assay. Fractionation of the extract and compound isolation were performed using Sephadex LH-20, silica gel (thin layer, vacuum liquid and flash chromatography) and reverse phase C-18 (in HPLC). Compounds were identified using 1- and 2-D {H and {C NMR, FAB and EI mass spectroscopy. Six distinctive biflavanoidal compounds were isolated from the bark – two isoflavanone dimers, one of which is new, and four chalcone dimers. None of the isolated compounds showed NF-κB inhibitory activity in an IL-6 luciferase reporter gene assay, used with HeLa cells.

Antibacterial broth dilution minimum inhibitory concentration assays were performed with three strains of multi-drug resistant *Staphyloccocus aureus*, and two of the compounds calodenin B and dihydrocalodenin B showed good antibacterial activity. In cytotoxicity assays (using MTT reduction) against MCF-7 breast cancer cells, calodenin B showed cytotoxic activity (IC₅₀ = 7 ± 0.5 μM).

*Bupleurum fruticosum* L. (Umbelliferae) is a shrub distributed in the Mediterranean area and has been traditionally used as an anti-inflammatory and antiseptic. The ethyl acetate extract of the aerial parts at 100 μg/ml strongly inhibited NF-κB in the IL-6 luciferase assay. Bioassay guided fractionation led to the isolation of three related phenylpropanoids, two of which also showed good activity with IC₅₀'s of approximately 30 μM for both compounds.
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>1-D / 2-D</td>
<td>1- / 2- dimensional</td>
</tr>
<tr>
<td>δ^*</td>
<td>chemical shift</td>
</tr>
<tr>
<td>δ_H</td>
<td>chemical shift (H NMR spectrum)</td>
</tr>
<tr>
<td>δ_C</td>
<td>chemical shift (13C NMR spectrum)</td>
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<td>ppm</td>
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<td>COSY</td>
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<td>HMQC</td>
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<td>double doublet</td>
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<td>NF</td>
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<td>Description</td>
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<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
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<td>p</td>
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<td>ultra-violet</td>
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<td>VLC</td>
<td>vacuum liquid chromatography</td>
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CHAPTER ONE: INTRODUCTION

1. IMMUNITY, INFLAMMATION AND NF-κB AS A MOLECULAR TARGET

Medical problems involving inflammation vary widely, ranging from relatively mild cases such as muscular pain to more serious and difficult to manage conditions such as rheumatoid arthritis. Plant extracts have been used to treat inflammatory conditions for centuries, and in conventional western medicine non-steroidal anti-inflammatory drugs belong to one of the most widely distributed and frequently used drugs.

1.1 Inflammation

Inflammation is the first response of tissue against injury and is fundamental in the survival of the organism. It forms part of the immune response system, providing protection against noxious external stimuli and enabling repair of damaged tissue. The inflammatory response can be divided into microcirculatory and cellular. The microcirculatory response involves the vasodilation of blood vessels and increased permeability of cell walls. The cellular response involves the active migration of inflammatory cells such as neutrophils, macrophages, lymphocytes and plasma cells present in the blood to the area of injury. The physical symptoms can be described by the ‘cardinal’ signs of inflammation, where the tissue becomes hot, red, swollen and painful, with loss of function. The redness and heat are due to an increase in blood flow and the swelling due to the accumulation of fluid at the inflammation site. Pain results from an accumulation of chemicals which stimulate nerve endings.
Fig. 1 Mediators of inflammation

1. Tissue injury
   - Production of inflammatory mediators
     - **Vasoactive mediators**
       - Histamine
       - Serotonin
       - Bradykinin
       - Anaphylatoxins
       - Leukotrienes/prostaglandins
       - Platelet activating factor
       - Nitric oxide
     - Vasodilatation and increased vascular permeability
       - **Oedema**
     - Chemotactic factors
       - C5a
       - Lipoxigenase products (LTB₄)
       - Formylated peptides
       - Chemokines
     - Recruitment and stimulation of inflammatory cells
       - Acute inflammation
         - Polymorphonuclear leukocytes
         - Platelets
         - Mast cells
       - Chronic inflammation
         - Macrophages
         - Lymphocytes
         - Plasma cells

Fig. 2 The arachidonic acid cascade.

Certain inflammatory mediators are derived from phospholipids and fatty acids.

- Phospholipids
  - **Phospholipase-A₂**
  - Arachidonic acid
    - Cyclo-oxygenase
      - Endoperoxides
        - Prostaglandins
        - Thromboxane-A₂
        - Prostacyclin
    - Lipoxygenase
      - Hydroperoxy-/hydroxy-fatty acids
        - Leukotrienes

Source: Rubin & Farber 1999
1.2 Chemical mediators of the inflammatory and immune response

There are many endogenous mediators of inflammation in the body, including direct acting mediators (histamine, bradykinin, prostaglandins, lymphokines, cytokines), lytic enzymes which cause direct damage to the tissue and may facilitate the migration of cells, and chemotactic factors which stimulate cell migration to the site of inflammation (Fig. 1). These mediators are released in response to tissue damage and invasion by microorganisms (Rubin & Farber 1999).

The immune response is a complex series of cellular interactions activated by foreign antigenic materials. The major effectors of the immune system are the lymphocytes which are responsible for antibody formation in response to the foreign antigens.

| Table 1. Cytokines: a group of low molecular weight proteins secreted by macrophages |
|-----------------------------------|----------------------------------|
| • interleukins (IL)               | growth factors, colony stimulating factors |
| • TNF-α                            | interferons                        |
| • chemokines                       |                                   |

Rubin & Farber 1999

1.3 Disorders of the inflammatory and immune response

Unfortunately there are medical disorders which arise from the immune response. Allergic reactions and hayfever, which are milder disorders, are very common ailments where the body can overreact to moderate traces of an otherwise harmless foreign substance. These are attributed mostly to the release of histamine and are commonly treated with anti-histamines. Chronic auto-immune diseases, such as rheumatic fever, rheumatoid arthritis, and disseminated lupus erythematosus are characterised by diffuse inflammatory reactions which appear to provide no protection to the host. Here the antigen is the host cell molecule, which is perceived as foreign
by the host’s own immune system, with resulting cell necrosis, fibrosis, and lymphocytic and plasma cell infiltration of the tissue. Currently symptomatic drug treatment for pain in chronic inflammatory disease is in almost all cases with non-steroidal anti-inflammatory drugs (NSAIDs). The NSAIDs inhibit cyclo-oxygenase (COX) (Fig. 2), and the resulting inhibition of prostaglandin synthesis is largely responsible for their therapeutic effect. The main side effect of NSAIDs is gastrointestinal irritation which may result in ulceration, thought to be caused by the inhibition of COX-1. This is a serious problem especially when they are taken in high doses on an everyday basis, which is the case for patients with chronic inflammatory disease. This has resulted in the co-prescribing of gastro-protective agents such as misoprostol, or ranitidine to reduce gastric irritation. More recently a new class of NSAID has emerged, called the COX-2 inhibitors, which include rofecoxib and celecoxib, which exert an anti-inflammatory effect by selective inhibition of the COX-2 enzyme (Kuritzky & Lewis 2003; Laine 2003)

1.4 NF-κB and its role in defence and disease

NF-κB is one of the principal inducible transcription factors in mammalian cells, with a pivotal role in regulating the expression of genes involved in the immune and inflammatory responses.

The name Nuclear factor-κB originates from when it was first identified in 1986 as a regulator of the kappa light chain gene in murine B lymphocytes. It has subsequently been found in many different cells, and is now considered to be ubiquitous in mammalian cells, with an analogous system in the fruit fly Drosophila which has also been studied (Barnes & Karin 1997; May & Ghosh 1998).
Activators of NF-κB include cytokines (interleukins (IL), tumour necrosis factor (TNF)), protein kinase C activators, viruses, bacterial products (lipopolysaccharide), oxidants and ultra-violet light (Table 2). The genes NF-κB regulates includes those for inflammatory cytokines, chemokines, enzymes that generate inflammation mediators, immune receptors, adhesion molecules, nitric oxide synthase, nitric oxide, and prostaglandin synthase (COX, especially COX-2). In chronic inflammatory and auto-immune diseases, NF-κB is responsible for the increased expression of genes for cytokines, enzymes, adhesion molecules and nitric oxide synthase, which are present in increased amounts in patients with asthma, ulcerative colitis and rheumatoid arthritis (Makarov 2000; May & Ghosh 1998). NF-κB frequently functions together with other transcription factors (activator protein-1, nuclear factor of IL-6) in the regulation of these genes.

NF-κB is also important in neuronal and glial cell function (O'Neill & Kaltschmidt 1997). It is involved in brain function, particularly following injury and in neurodegenerative conditions such as multiple sclerosis and Alzheimer’s disease.

Brain specific activators of NF-κB include glutamate and neurotrophins.

Certain viral infections also appear to involve NF-κB in either their transcription or viral clearance. This was first shown for HIV (Human Immunodeficiency Virus), which causes profound effects on the brain, including neurodegeneration and inflammation. HIV infects glial and neuronal cells, and in several brain cell lines cytokines such as IL-1 and TNF induce HIV expression through NF-κB activation (Baldwin 2001; O'Neill & Kaltschmidt 1997).
Table 2

<table>
<thead>
<tr>
<th>NF-κB binding sites are found in promotor regions of genes that code for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>inflammatory cytokines (IL-1, IL-6, IL-8 and TNF-α)</td>
</tr>
<tr>
<td>acute phase response molecules</td>
</tr>
<tr>
<td>anti-apoptotic factors</td>
</tr>
<tr>
<td>pro-apoptotic factors</td>
</tr>
<tr>
<td>cell adhesion molecules (chemokines)</td>
</tr>
<tr>
<td>IκB</td>
</tr>
<tr>
<td>immunoreceptors</td>
</tr>
<tr>
<td>haemotopoietic growth factors</td>
</tr>
<tr>
<td>transcription factors</td>
</tr>
<tr>
<td>granulocyte/macrophage colony stimulating factor</td>
</tr>
<tr>
<td>inducible nitric oxide synthase and nitric oxide</td>
</tr>
<tr>
<td>prostaglandin synthase (cyclooxygenase, esp. COX-2)</td>
</tr>
</tbody>
</table>

NF-κB activators
- Tumour necrosis factor (TNF-α)
- IL-1
- Phorbol 12-myristate 13 acetate (PMA)
- oxidants (H₂O₂)
- UV light
- bacterial products (LPS)
- HIV-1
- viral proteins (EBNA-2 and LMP-1 from Epstein-Barr virus)
- oncoproteins (Ras, Raf, HTLV-I)
- glutamate
- neurotrophins

1.5 Role of NF-κB in cancer

NF-κB has been shown to be involved in tumour development, where it has been found constitutively activated in some types of cancer. A connection between inflammation and cancer has been suspected for some time, although the mechanistic link is not well understood, NF-κB is thought to be involved, being associated with both processes.

There is strong evidence for the role of NF-κB in inhibiting apoptosis, programmed cell death. Therefore in certain chronic inflammatory diseases or infections the persistent activation of NF-κB may give rise to the survival of abnormal precancerous cells which would ordinarily be eliminated by standard apoptotic processes.
An example which provides evidence for such a role is in gastrointestinal cancers. The involvement of the bacterium *Helicobacter pylori* in gastric ulcers is well known, and the prescribing of antibiotics along with proton pump inhibitors is standard treatment. A clear link has also been established between infection by *Helicobacter pylori* and gastric cancer, and is the first known example of a cancer caused by a bacterial infection. Another risk factor involved in gastric cancer includes increased expression of IL-1 due to single nucleotide polymorphisms in the IL-1 gene. *H. pylori* and IL-1 are both potent NF-κB activators (Karin, Cao, et al. 2002).

Cyclooxygenase-2 (COX-2), whose transcription is regulated by NF-κB, is responsible for prostaglandin synthesis in inflammation, and the link between COX-2 and colorectal cancer is strongly supported by epidemiological and experimental evidence. COX-2 is found overexpressed in colon adenomas and carcinomas, and COX2-null mice are resistant to colorectal cancer. Long-term consumption of aspirin or other COX inhibitors of over 10 years has been reported to reduce the risk of colorectal cancer, and the extensive consumption of curcumin, found in turmeric, in Asian countries such as India has been linked to their low incidence of colorectal cancer. Although curcumin is a relatively less potent and specific inhibitor of IKK (a kinase complex essential in NF-κB activation) in comparison to aspirin and sulindac, it has been shown to reduce colon carcinogenesis in several animal models (Karin, Cao, et al. 2002).

Constitutive activation of NF-κB has been associated with several aspects of tumourigenesis, including proliferation, prevention of apoptosis, angiogenesis and metastasis, where it is known to activate genes which are involved in these processes, detailed in Table 3.
As well as in the production of cytokines which stimulate cell proliferation, NF-κB has been shown to activate expression of G1 cyclins, with strong evidence that its induction of cyclin D1 drives the proliferation of mammary epithelial cells during pregnancy.

It has been shown that tumours which produce high levels of NF-κB are resistant to anti-cancer therapy, and inhibition of NF-κB results in their increasing sensitivity to therapy, which illustrates the possible role of NF-κB in apoptosis. NF-κB may also have a role in the emergence of neoplasms, where apoptosis is designed to induce cell death in abnormal cells that have undergone chromosomal rearrangements or DNA damage (Baldwin 2001; Karin, Cao, et al. 2002). Conversely, NF-κB has also been associated with pro-apoptotic mechanisms, where it has been shown to induce the pro-apoptotic Fas ligand protein, in response to chemotherapy or T-cell activation signals. There is also evidence that NF-κB regulates the expression of p53, a pro-apoptotic tumour suppressor protein, and that NF-κB is also required for p53 to induce cell death. So despite strong evidence for NF-κB as an anti-apoptotic factor, its full role in apoptosis and cell proliferation still requires further investigation.

Chemokines are NF-κB target-gene products, and are chemotactic factors which induce cell migration. At least one chemokine, IL-8, which is controlled by NF-κB, has been shown to promote angiogenesis, and NF-κB activation has also been shown to stimulate angiogenesis.

Other cancers which also have strong evidence for the involvement of NF-κB are lymphoid malignancies and breast cancer, which is perhaps not so surprising given the role of NF-κB in the immune system and in the proliferation of mammary epithelium in pregnancy (Karin, Cao, et al. 2002).
### Table 3 Roles of NF-κB in tumorigenesis

<table>
<thead>
<tr>
<th>Role in</th>
<th>Target genes activated by NF-κB</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell proliferation</td>
<td>cytokines: IL-2, granulocyte-macrophage colony stimulating factor (GM-CSF), CD40 ligand</td>
<td>production of growth factors</td>
</tr>
<tr>
<td></td>
<td>G1 cyclins: cyclin D1</td>
<td>cyclin D1 drives the proliferation of mammary epithelial cells during pregnancy.</td>
</tr>
</tbody>
</table>
| inhibition of apoptosis         | cellular inhibitors of apoptosis (cIAPs)  
A1/BFL1  
BCL-X1  
c-FLIP                                                                 | inhibitors of apoptosis                                                |
| angiogenesis and metastasis    | chemokines: IL-8                                                                                               | chemotactic factors which induce cell migration.                        |
|                                 | matrix metalloproteinases (MMPs)                                                                                   | MMP: proteolytic enzymes which promote tumour invasion of surrounding tissue. |
|                                 | vascular endothelial growth factor                                                                                |                                                                        |

Source: Karin, Cao, et al. 2002

### 1.6 NF-κB signalling cascades

NF-κB is a family of heterodimers, each dimer consisting of two Rel protein subunits.

So far 5 mammalian Rel proteins (p50, p52, c-Rel, p65/Rel A, and RelB) have been identified, various combinations of which form the NF-κB dimers. The most commonly represented NF-κB is composed of the subunits p65 and p50, being the complex which was first identified and also the most abundant. All Rel proteins share a highly conserved region of 300 amino acids at the N-terminal known as the Rel homology domain, which is responsible for DNA-binding, dimerisation, interaction with IκB, and contains the nuclear localisation sequence (NLS). In unstimulated cells NF-κB is found in the cytoplasm, made inactive by being non-covalently bound to
Inhibitory-κB (IκB). IκB is a family of proteins which inhibit NF-κB by preventing its translocation into the nucleus by masking the NLS (Makarov 2000; May & Ghosh 1998).

Two of the most important immune system signalling cascades which involve the activation of NF-κB are those for IL / lipopolysaccharide (LPS) and TNF. Although there is no full understanding or agreement as to every aspect of the signalling pathways, the main steps have been characterised (Fig. 3) (Bremner & Heinrich 2002).

Stimulation of specific receptors on the cell surface by activators such as TNF-α and IL-1 activate their specific pathways of transduction controlled by various kinases which converge on the IκB kinase (IKK) complex (Fig. 3). IKK is a high molecular weight complex containing two catalytic kinase units (IKKa and IKKB) and a regulatory unit (IKKγ).

IKK is responsible for the phosphorylation of the inhibitory IκB protein, a critical step in NF-κB activation. The phosphorylation of IκB leads to its recognition by a pIκB specific ubiquitin protein ligase (E3) which polyubiquitinates the pIκB (Karin & Ben Neriah 2000).

The ubiquitin mediated protein degradation system was originally thought to degrade old, damaged or abnormal proteins, but has recently been implicated in controlling the abundance of many functional regulatory proteins. Ubiquitin is a small highly conserved protein, ubiquitinously expressed in all eukaryotes. Degradation of a protein via the ubiquitin pathway starts with the co-valent attachment of one or more ubiquitin polypeptides, followed by ubiquitin-ubiquitin conjugation to form ubiquitin polymers. The ubiquitin tagged protein is then rapidly degraded by the 26S proteasome complex.
Figure 3  
NF-κB activation, membrane to nucleus

1. Stimulation of specific receptors on the cell surface.
2. Pathways of transduction controlled by various kinases which converge on IKK.
3. Activated IKK phosphorylates κB.
4. Polyubiquitination of plκB by a specific ubiquitin protein ligase (E3).
5. Degradation of κB by 26S proteasome complex.
6. Freed NF-κB translocates into the nucleus and binds to specific sequences in promotor regions of target genes (Table 2), and promotes their expression.

Source: Brenner & Heinrich 2002
The degradation of the IκB exposes the NLS of the NF-κB, which leads to its translocation into the nucleus. The NF-κB then binds to specific sequences in the promoter regions of target genes.

IκB brings about the termination of NF-κB translational activity, where newly synthesised IκB binds to the NF-κB, dissociating it from the DNA and exporting it back out to the cytoplasm. In addition, activation of IKK is only transient, although the method of inactivation is not fully understood.

In this thesis NF-κB was selected as a target to search for anti-inflammatory natural products, but it also has a role in proliferating other serious diseases as well - multiple sclerosis, Alzheimer’s, HIV and some cancers, cures for which still remain to be found. New compounds which have NF-κB inhibitory activity may potentially be useful therapeutically, or provide clues in finding a therapeutically useful agent. Use of any NF-κB inhibitory agent may however need to be used with caution, as although NF-κB is implicated in serious diseases, it provides an essential function in inflammation and immune response. Where NF-κB is important in the protection of cells against apoptosis, in this case its suppression may be deleterious. Targeting kinases and ligands involved in the NF-κB activation pathway may also affect other signalling pathways which use the same enzymes. However, glucocorticoids are routinely used in acute asthma attacks, and are potent inhibitors of NF-κB, so brief or short term inhibition of NF-κB may not pose too much risk, and may be sufficient to revert a pathological condition (Barnes & Karin 1997). Another example of therapeutically used NF-κB inhibitors are the NSAIDs, including aspirin, which are regularly prescribed for long term use, and apart from gastro-intestinal toxicity are
generally well tolerated. Several dietary compounds including flavonoids, curcumin, vanilloids and resveratrol are also known to block NF-κB activation.

Table 4

<table>
<thead>
<tr>
<th>Examples of natural product NF-κB modulators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classes of natural products with many known NF-κB modulators</td>
</tr>
<tr>
<td>Diterpenoids</td>
</tr>
<tr>
<td>Sesquiterpene lactones</td>
</tr>
<tr>
<td>Triterpenoids</td>
</tr>
<tr>
<td>Vanilloids</td>
</tr>
<tr>
<td>Plant extracts and active compounds</td>
</tr>
<tr>
<td>Black tea <em>Thea sinensis</em> (Theaceae)</td>
</tr>
<tr>
<td>- theoflavin derivatives (theaflavin-3,3’-digallate)</td>
</tr>
<tr>
<td>- Epigallocatechin-3-gallate</td>
</tr>
<tr>
<td>Huang Qui <em>Scutellaria baicalensis</em> (Lamiaceae)</td>
</tr>
<tr>
<td>- wogonin, baicalin, baicelein, oroxylin A</td>
</tr>
<tr>
<td>St. John’s wort <em>Hypericum perforatum</em> (Clusiaceae)</td>
</tr>
<tr>
<td>- hypericin</td>
</tr>
<tr>
<td>Turmeric <em>Curcuma longa</em> (Zingiberaceae)</td>
</tr>
<tr>
<td>- curcumin</td>
</tr>
<tr>
<td>Milk Thistle <em>Silybum marianum</em> (Asteraceae)</td>
</tr>
<tr>
<td>- silybin</td>
</tr>
<tr>
<td>Red wine</td>
</tr>
<tr>
<td>- ethyl gallate</td>
</tr>
<tr>
<td>Cannabis <em>Cannabis sativa</em> (Cannabidaceae)</td>
</tr>
<tr>
<td>- cannabinol</td>
</tr>
<tr>
<td>Liquorice root <em>Glycyrrhiza glabra</em> (Fabaceae)</td>
</tr>
<tr>
<td>- glycercrizhin</td>
</tr>
<tr>
<td>Other plant extracts:</td>
</tr>
<tr>
<td>- <em>Urtica dioica, Gingko biloba, Uncaria tomentosa</em></td>
</tr>
</tbody>
</table>

Source: Bremner & Heinrich 2002

1.7 Examples of natural product modulators of the NF-κB pathway

1.7.1 Diterpenes

Some kaurene diterpenoids (Fig. 4.1) have been found to act upstream of IKK by inhibiting one of the regulatory kinases (NF-κB inducing kinase or NIK) involved in the transduction signalling pathway which activates IKK. They showed NF-κB inhibitory activity at 25 μM.
**Hypoestoxide** is a diterpene from *Hypoestes rosea* (Acanthaceae) which was discovered through an ethnobotanical study, and shows NF-κB inhibitory activity at 50 μM (EMSA). It has been found to specifically inhibit IKK (Bremner & Heinrich 2002).

1.7.2 Sesquiterpene lactones

These are the most widely published class of natural products with reported NF-κB inhibitory activity, which was first discovered through an ethnobotanical study (Bremner & Heinrich 2002). Sesquiterpene lactones have also enabled structure activity relationships to be studied due to their structural diversity. It has been shown that their NF-κB inhibitory activity is enhanced by the presence of (Fig. 4.3):

- an isoprenoid ring system
- a lactone ring containing an exo-methylene group (α-methylene-γ-lactone) - which has been found to be essential for activity in the sesquiterpene lactones.
- an α,β-unsaturated cyclopentenone or conjugated ester moiety group.

**Parthenolide**, from feverfew (*Tanecetum parthenium*) is a potent NF-κB inhibitor, acting at below 10 μM, and its mechanism of action has been shown to be via the inhibition of IKK, specifically by the modification of a cysteine group in IKKβ.

**Helenalin**, from *Arnicae flos*, has potent cytotoxic activity, and is thought to target and alkylate the NF-κB subunit p65.

Although sesquiterpene lactones are structurally diverse and have many associated therapeutic uses, as alkylating agents they have unspecific cytotoxicity which may exclude many of them from being therapeutically useful (Bremner & Heinrich 2002). One example of a medicinally used sesquiterpene lactone is artemisinin in the treatment of malaria, which is described later in section 2.
Fig. 4 Examples of natural product NF-κB inhibitors (I)

1 Foliol

2 Hypoestoxide

3 2β,5-epoxy-5,10-dihydroxy-6α-angeloyxy-9β-isobutylxygermacran-8α,12-olide

4 Parthenolide

5 Helenalin

6 Prednisolone

7 Oleandrin

8 Theoflavin-3,3'-digallate

9 Hypericin
1.7.3 Triterpenes

There is much documentation on the anti-inflammatory activity of triterpenes, a class of compounds which include the steroids. Glucocorticoids are potent inhibitors of NF-κB (Fig. 4.6), and some triterpenes have been found to have NF-κB inhibitory activity.

The cardiac glycoside oleandrin (Fig. 4.7), from Nerium oleander (Apocynaceae) was reported to have activity at less than 10 μM. It has been found to act on IKK, but it is also thought to be a general kinase inhibitor as it also inhibits another transcription factor AP-1, thus may not be therapeutically useful due to its unspecificity (Bremner & Heinrich 2002).

1.7.4 Phenolic compounds

Theaflavin-3,3'-digallate (Fig. 4.8), from Thea sinensis, showed potent NF-κB inhibitory activity at 30 μM in electrophoretic mobility shift assays, and was found to inhibit IKKα expression.

Hypericin from Hypericum perforatum has reported activity at less than 5 μM. It is thought that hypericin works upstream of the NF-κB pathway, in particular targeting protein kinases, which is implicated in NF-κB induction and is activated by PMA. Additionally, in assays using hydrogen peroxide to induce NF-κB it was found to have no activity, showing that hypericin does not act as an NF-κB inhibitor via radical scavenging activity.

Some flavonoids have been found to have NF-κB inhibitory activity, genistein is an isoflavone from soya bean, and has relatively mild NF-κB inhibitory activity,
requiring concentrations of more than 100 μM. Quercetin, a common flavonoid found in higher plants, was found to have NF-κB inhibitory activity at 50 μM. It is known to inhibit many enzymes and is a radical scavenger, and has also been found to have anti-inflammatory, anti-bacterial, anti-viral and anti-hepatotoxic activity. Genistein and quercetin were found to inhibit TNF-α induced NF-κB activation by halting the degradation of IκB.

Other flavonoids with reported activity include wogonin (10 μM) and oroxylin A (70 μM) from the Chinese herb Huang Qui (Scutellaria baicalensis) (Fig. 5) (Bremner & Heinrich 2002).

Curcumin (Fig. 5) from Curcuma longa has been reported to inhibit NF-κB by targeting the IKK complex and arresting IκB phosphorylation at 10 μM. It has been shown to inhibit activation by IL-1, TNF-α, PMA and H₂O₂.

There are some concerns about anti-oxidant compounds showing NF-κB inhibition only exerting their effect through radical scavenging activity, as compounds which do not have specific activity targeting the NF-κB pathway are not considered to be of industrial interest. Phenolic compounds being proton donating also have anti-oxidant activity, which would enable them to reduce reactive oxygen species (ROS) such as H₂O₂, superoxide or hydroxyl radical. However, since the proposal of the oxidative stress model in 1992 which hypothesised the central role of intracellular ROS in NF-κB activation, there have been several studies whose findings refute this model. In addition there is no obvious involvement or requirement for any ROS in the NF-κB activation pathways for IL-1 or TNF.
Fig. 5 Examples of natural product NF-κB inhibitors (II)
Examples of NF-κB activation by H$_2$O$_2$ and NF-κB inhibition by anti-oxidants have been shown to be cell and stimulus specific, as there are several cell lines which do not respond to H$_2$O$_2$, or where anti-oxidants such as pyrrolidine dithiocarbamate (PDTC) have been ineffective. NF-κB inhibitory compounds including PDTC have also been found to have unexpected, or multiple targets on the activation pathway. Vitamin C, a well known general anti-oxidant was found to inhibit NF-κB activation by TNF by activating p38 mitogen-activated protein kinase, which inhibits IKK. Therefore the effects of oxidants/antioxidants on NF-κB can not be assumed to be always due to redox modulation, nor cell-independent (Bowie & O'Neill 2000).

1.7.5 Vanilloids

Capsaicin (Fig. 5) is the major spicy ingredient present in red peppers from the genus Capsicum. Capsiate and its dihydroderivatives are the major capsaicinoids present in sweet pepper. Capsaicin and capsiate are structurally very similar (although with very different 3D structures), both having a vanillyl core bound to a branched fatty acid, and the remarkable difference between their sensory properties (capsiate is not spicy to the taste buds) is due to the presence of the amide bond in capsaicin. A recent study used a synthetic capsiate derivative, nordihydrocapsiate (Fig. 5), and found it and capsaicin to have similar NF-κB inhibitory activity (50 %) at 50 μM using TNF-α as the inducer. The study also reported that nordihydrocapsiate inhibited IKK activation, and thus IκB degradation (Sancho, Lucena, et al. 2002).
1.8 Deducing mechanisms of action

The different classes of NF-κB inhibitory compounds described are diverse and clearly several different mechanisms of action are involved, although many appear to inhibit IKK. Deductions can be made as to which part of the pathway the compounds may be targeting from results obtained from different assays.

Different NF-κB inducers have different signalling cascades, but all lead down to the activation of IKK, which is common to all the signalling pathways. If an inhibitor is able to affect all inducers, then it can be deduced that it is acting at either on IKK, or downstream from it. If an inhibitor is only able to affect one or two different inducers, then it is likely that its target is upstream of IKK in the signalling cascade specific to the inducer.

There are various ways as to how a compound might inhibit IKK, including directly targeting the enzyme or IκB, and preventing its phosphorylation.

If an inhibitor is acting by impairing IKK activity or by preventing degradation of IκB, this can be seen with immune complex kinase assays using recombinant IκB protein as a substrate, and Western blots to study IκB steady state levels, respectively. This enables deductions as to whether an inhibitor is acting on IKK or downstream from it.

Further steps involved in IκB degradation include ubiquitination of phosphorylated IκB by E3, and degradation of IκB by 26S, although these are part of the ubiquitin mediated protein degradation system which is not specifically unique to the NF-κB signalling pathway.

Steps downstream of IκB degradation are the translocation of the NF-κB into the nucleus and its interaction with DNA. Electrophoretic mobility shift assays (EMSA)
can be used to test for inhibition of NF-κB binding to DNA. NF-κB is mixed in the presence of the inhibitor with radiolabelled oligonucleotides containing the specific gene sequences NF-κB binds to. Polyacrylamide gel electrophoresis is used to check for the presence of NF-κB-DNA complexes (Sancho, Lucena, et al. 2002).

As there are so many different targets within the NF-κB signalling pathway, there is still much work to be done in deducing the mechanisms of action of the different classes if inhibitors. The main steps of the signalling pathway have only recently been characterised, and more work is still needed for its full elucidation. Further investigation is also required into the different kinases and adaptor molecules which participate in the cascade.
2. ROLE OF ETHNOPHARMACOLOGY IN THE SELECTION OF LEAD MEDICINAL PLANTS

Documentation of popular and indigenous plant uses has a long tradition in botany. The term ethnobotany is used to describe the study of plant uses by people, and is interdisciplinary, using methods from anthropology and botany. Ethnobotany has also been defined as the direct interrelation between humans and plants by R. I. Ford (1978). It is often assumed that ethnobotany is restricted to specific indigenous populations, but in fact includes all human societies. It is a broad subject, as plants play an important role in most aspects of human activity with many uses such as food, construction material, dyes, and medicinal uses being the most frequently recorded, sometimes these sources date back to centuries ago. Knowledge of medicinal plants is part of the culture of indigenous societies, even in modern times herbal treatment is of major importance, which is also partly due to the high costs and unreliable supply of conventional pharmaceuticals (Heinrich 2000; Heinrich, Ankli, et al. 1998).

Medicinal plants have always been one of the main research interests in ethnobotany, but ethnopharmacology is a more precise term. Ethnopharmacology has been defined as the interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by man (Bruhn & Holmstedt 1981), although this term is not restricted to plants and includes all natural products (Heinrich & Gibbons 2001).

In this chapter the role of ethnopharmacology in drug development is discussed, but a major part of modern ethnopharmacological studies are also directed towards better understanding of the pharmacological effects of medicinal plants with an objective to
contribute to better health care in marginalised communities. Recently issues of alleged biopiracy have been raised, where in some instances biologically active natural products have been commercially developed by pharmaceutical companies without fair compensation given to the people they obtained the ethnopharmacological information from, or the nation in whose territory the material was originally discovered. Ethnopharmacological research now requires agreements and treaties based on international and bilateral treaties, the most important one of which is the **Convention on Biological Diversity** (CBD), to prevent the exploitation of a country’s biological resources. The CBD formed part of the Rio Summit in 1992, and recognises the sovereign rights of States over their own biodiversity and gives them the authority to determine access to these resources. The Bonn guidelines (formed at the Sixth Conference of the Parties in Hague, April 2002) cover important aspects of how the CBD should be implemented. The areas the guidelines cover include access and benefit sharing, and also the conservation and sustainable use of resources accessed (Heinrich, Ankli, et al. 1998; Simmonds 2002).

Many drugs used today were developed from medicinal plants, some well known examples are regularly used in pharmaceutical preparations to treat important medical conditions. Chloroquine (a derivative of **quinine** which is obtained from the bark of *Cinchona succirubra*) is still one of the main drugs used to treat malaria and as a chemopreventative despite growing resistance. **Digoxin** (from *Digitalis purpurea*) is the main drug used in the treatment of heart failure and atrial fibrillation. **Morphine** (from *Papaver somniferum*) is an important drug in pain control, from which the opioid analgesics are derived. These are powerful analgesics, regularly used in terminal patients, or in patients with severe pain. **Colchicine** (from *Colchicum*
Autumnale) and pilocarpine (from Pilocarpus jaborandi) are prescribed regularly for acute gout attacks and glaucoma respectively. Acetylsalicylic acid (Aspirin?) is derived from salicylic acid, originally obtained from the bark of the Willow tree which was often used as an analgesic. Aspirin is now also an important prophylactic drug used to prevent strokes in patients who have had ischemic strokes or transient ischemic attacks, and is known to improve mortality in these patients (Elwood 2001).

Catharanthus roseus actually has a different ethnobotanical background from its alkaloids' commercial chemotherapeutic use, and was originally investigated for its anti-diabetic properties. During World War II it had been used as an oral insulin substitute in the Phillipines when insulin had become unobtainable. When the plant extract was injected into rats it was found to produce very toxic effects by destroying white blood cells, causing the rats to die from bacterial infection. This observation led to the isolation of the vinca alkaloids vincristine and vinblastine, which are used as anti-cancer agents to treat leukemias, lymphomas and some solid tumours (Chadwick & Marsh 1990).

Ethnopharmacology in drug discovery fell out of favour in the mid 20th century with the advance of technology and pharmaceutical and chemistry research, and during this time few new commercially successful plant derived drugs were developed. But interest in and the use of natural products and herbal remedies has experienced a recent revival. Reasons for this include a possible growing distrust in conventional medicine in terms of efficacy and side effects, and the misguided belief that natural products are free from side effects. Also in life threatening diseases in which cures are being searched for, for example cancer and HIV/AIDS, all methods of drug
development are being looked at, particularly if conventional methods are not providing any leads.

Recently pharmaceutical companies have taken up high-throughput screening programmes, where they can screen 500,000 random samples, including natural products, a week in the search for a natural product with bioactivity against a specific target (Heinrich & Gibbons 2001). The term used to describe this is bioprospecting which is sometimes mistaken for ethnopharmacology. The highly profitable anticancer agent paclitaxel (*Taxus brevifolia*) was developed in this way. However such projects have had limited success and low hit rates, in addition requiring huge financial input, available only to pharmaceutical companies who have such capital and who may only invest in projects in which they consider will produce financial profit.

Bioprospecting is industry driven, whose focus is purely on the development of new drugs for commercial use in a relatively short period of time. Ethnopharmacological studies do not specifically set out to look for potential new active compounds, which is only considered a sideline with its central objectives of studying indigenous medicinal plant uses and contributing to safety and efficacy of herbal remedies at a local level. These studies are performed at a much smaller scale as well, carried out by trained ethnobotanists who will typically live and spend time with the indigenous people, leading to the documentation of a small but select group of medicinally used plants. The studies may be followed up with phytochemical and/or bioactivity investigations in a laboratory, the results of which may improve the understanding or the rationale behind the use of these plants.
Many of the medicinal plants investigated often show biological activity which support their traditional use. The ‘hit’ rate percentage for identifying active samples in ethnopharmacologically selected plants is significantly higher than that seen among randomly selected samples.

The technique of using ethnopharmacology to search for leads in drug development is useful and promising. Although this is less the case for anti-cancer agents as few medicinal plants are known to be used to treat cancer successfully, in searching for anti-inflammatory compounds, amongst many other disease treatments, its use may be ideal (Heinrich & Gibbons 2001).

Whilst pharmaceutical companies have invested in bioprospecting, very few are willing to invest in the use of ethnopharmacology. One reason for this may be due to the complexities involved in obtaining agreements and contracts in order for their work to be carried out, with fears of being accused of biopiracy and the lawsuits which may ensue. It is also a different discipline, requiring the employment of trained ethnobotanists who may need to work in countries rich in biodiversity. However it is clear that they would benefit from ethnopharmacological leads.

Conversely companies or university projects which specialise in ethnopharmacology may also employ the use of simple bioassays for screening random plant samples in the selection of a lead plant.

Examples of companies which invest in ethnopharmacological methods include Shaman Botanicals (formerly Shaman Pharmaceuticals) and Phytopharm.

Some promising new drugs which are currently being developed have their origins in ethnobotanical work. The phorbol ester prostratin is a promising new anti-HIV drug
which is currently undergoing trials. It was isolated from the Samoan plant *Homolanthus nutans*, used traditionally to treat hepatitis. Prostratin is also the basis of a landmark agreement announced by the AIDS Research Alliance of America whereby 20% of any commercial revenue that may be generated from prostratin would be returned to the Samoans who aided the researchers. It is the first compound ever licensed by the National Cancer Institute for development by a non-profit research institution (Cox 2001).

Another example is an obesity drug called ‘P57’ which is currently under development by Phytopharm and Pfizer, obtained from the South African *Hoodia* (Asclepiadaceae) through ethnobotanical knowledge provided by the Aboriginal San people, who use the cactus to suppress their appetite whilst hunting (Simmonds 2002). An agreement has been reached where the San have been promised a share of the royalties from the drug, which shows potential in becoming very successful. Pfizer, who paid Phytopharm $32 million for the rights to develop the drug, hopes to make the product available on prescription by 2007.

*Artemisinin*, a sesquiterpene lactone, was isolated from *Artemisia annua*, or Qinghao, a plant used in traditional Chinese medicine to treat fever and malaria. Artemisinin has antiplasmodial activity against chloroquine resistant strains of *Plasmodium falciparum*.

*Galantamine* is an example of a newly approved drug that was originally obtained from a medicinally used natural source, and is currently in use as an anticholinesterase inhibitor to improve cognitive function in moderate Alzheimer’s disease (Dewick 2002). It is an Amaryllidaceae alkaloid, its main natural sources are the species of the genera *Galanthus*, *Narcissus* and *Leucojum* (Amaryllidaceae), from Bulgaria and the
Caucasus region. First isolated from the Caucasian snowdrop (\textit{Galanthus} spp.) in the 1940's (Hanks 2002), the decoction of the bulbs was traditionally used to treat pain and polyneuritis (Shellard 2000). It was originally developed by Sanochemia, an Austrian Pharmaceutical company, who obtained the first patent on the synthetic process for galantamine, which is itself difficult to obtain from the natural source. Sanochemia later collaborated with Janssen Pharmaceutica and British-based Shire Pharmaceuticals, and galantamine obtained its first European registration in 2000 as Reminyl\textsuperscript{TM}. It has recently been approved for use in the EU and USA in 2001-2.

The European Union funded \textbf{Anti-Inflammatory Natural Products} (AINP) project is co-ordinated at the London School of Pharmacy Centre for Pharmacognosy and Phytotherapy. The aim of the project is to identify new natural product NF-\(\kappa\)B inhibitors and employs the screening of plant extracts mostly collected on an ethnobotanical basis. The work is undertaken by a team of 8 laboratories. Each laboratory offers a different but complementary field of expertise, ranging from ethnobotanical and pharmacological knowledge to in vitro and in vivo bioassay testing. The majority of the plant material collected was selected on the basis of ethnobotanical use against inflammation. Plant extracts were screened by three of the laboratories, based in Freiburg, Córdoba and at the London School of Pharmacy. So far (Jan 2003) a total of 200 plant species (829 plant extracts) have been screened over a two year period, from which 5 have shown potent and specific NF-\(\kappa\)B inhibitory activity at 100 \(\mu\)g/ml, and are worth further investigation. Contracts have been drawn up agreeing that if any commercially successful products are generated as a result of research performed under the AINP project, 50 \% of commercial revenue would go to the active plant's region of origin.
In the drug discovery process, ethnopharmacology is one approach amongst others which can and has provided valuable leads. It is complementary to bioprospecting, and also has other objectives in the study of medicinal plants and in supporting their traditional uses by indigenous people.
3. SELECTION OF *OCHNA MACROCALYX* AND *BUPLEURUM FRUTICOSUM*

*Ochna macrocalyx* bark was collected in an ethnobotanical study performed on the Washambaa, one of the main indigenous populations inhabiting the Western Usambara Mountains of Tanzania. It was one of 328 taxa collected during this study, where selection was based on the number of medicinal use reports each one had according to the healers interviewed. These were subsequently screened for NF-κB inhibitory activity using electrophoretic mobility shift assays. From the screening of the Tanzanian plants, *Ochna macrocalyx* was selected for further study as the crude bark extract was found to show activity. And although there have been phytochemical and biological work published in a number of papers on related species and other members of the Ochnaceae, which have shown interesting pharmacological profiles, no work has been published on this specific species. Thus, *Ochna macrocalyx* was selected based on its interesting ethnobotanical background, the activity it showed in NF-κB assay, the known pharmacological activity of related species, and in addition it had not been investigated before.

*Bupleurum fruticosum* was selected as part of the Anti-inflammatory Natural Products (AINP) project. The AINP project, funded by the European Union, aims to identify new small molecule inhibitors of NF-κB from natural products, and is currently being co-ordinated at the Centre for Pharmacognosy and Phytotherapy at the School of Pharmacy. *Bupleurum fruticosum* was one of the plants collected from Spain whose selection was based on ethnobotanical use against inflammation. Plants collected were screened for NF-κB inhibitory activity using the IL-6 luciferase
reporter gene assay, and the Bupleurum extract was found to have potent activity.

There has been much phytochemical and biological work previously published on Bupleurum fruticosum, which is a well known shrub in the Mediterranean, but none have so far reported on its NF-κB inhibitory activity.
4. **OCHNA MACROCALYX**

4.1 Ochnaceae

The Ochnaceae is a relatively small family of trees and shrubs, distributed over warm parts of the world. About 35 genera belong to this family, with approximately 600 species. Genera which belong to this family include *Lophira, Brackenridgea, Ouratea* and *Ochna*. The family is rich in biflavonoids and chalcone derivatives, and several members are known for their pharmacological and toxic profiles (Nia & Gunasekar 1992). A number of related species are used in traditional medicine in Africa, India and South America. The genus *Ochna* Linn. consists of more than 85 species distributed in tropical Africa, Asia, America and Australia. Some members of the genus are used extensively in traditional Indian medicine in the treatment of epilepsy, menstrual complaints, lumbago, asthma, ulcers and as an antidote to snake bites (Rao, Sreeramulu, et al. 1997).

4.2 The Usambara Mountains and the Washambaa

Tanzania is part of an area which has a high degree of biological diversity, with some areas within it reported to contain 3,000 - 4,000 species per 10,000 km². The Usambara Mountains are situated in the northeastern part of Tanzania, and are part of the Eastern Arc Mountains. The mountains reach 2,200 meters above sea level, the climate is influenced by the Indian Ocean and is relatively humid. The Washambaa, together with two other main ethnic groups, inhabit the Lushoto district, the region encompassing the Western Usambara Mountains and the adjacent
lowlands. The population density of the Lushoto district is 80-130/km$^2$ with the population of the Washambaa approximately 400,000. Higher altitudes (approximately 1 km upwards) are their preferred habitat, although their fields can be found in the lowlands. Medicinal plants are an important local resource and play a key role in the preparation of their traditional medicinal remedies (Schlage, Mabula, et al. 2000).

4.3 Traditional uses of *Ochna macrocalyx*

In the ethnobotanical study which was performed by Christina Schlage et al. (Schlage, Mabula, et al. 2000), 27 Washambaan healers or specialists in traditional remedies were interviewed, and reports of ethnobotanical uses of medicinal plants were documented. Only plants which were cited as medicinal by five or more informants were included in the study, in which over 300 taxa were collected.

*Ochna macrocalyx* is a tree with a distinctive yellow bark, which grows at the foot of the Usambara Mountains. It is known as ‘Nkatakwa’ by the Washambaan, which is what they call both *Ochna macrocalyx* and *Ochna holstii*, two separate species which are similar enough to be used for the same purpose. *Ochna macrocalyx* is considered to be the more powerful of the two (C. Schlage, personal communication 2000).

Six of the healers interviewed spoke about the use of Nkatawa, where only the powdered yellow bark is used. The bark is either boiled in milk, or taken with tea, warm water or maize porridge, and then ingested. Its main use is for gastro-intestinal disorders, for which 6 use-reports were recorded (Table 5). Other uses include treating diarrhoea and bloody diarrhoea, haemorrhoids and gastric ulceration (C. Schlage,
personal communication 2000). Uses which also come under this category are 'chango' and 'ngiri', which are important gastrointestinal ailments according to the Washambaa. Chango is described as pain and cramps of the lower belly which extend to the back and may be caused by inadequate food intake, and ngiri is described as a strong variation of chango, which if left untreated may lead to death. One use-report is recorded for each use of one plant reported by one healer. Its other main use is for gynaecological disorders, such as dysmenorrhoea and infertility.

Table 5. Use reports recorded for Ochna macrocalyx.

<table>
<thead>
<tr>
<th>Number of use reports</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>'Female' disorders: dysmenorrhoea, stomach problems, fertility problems</td>
</tr>
<tr>
<td>3</td>
<td>Diarrhoea and/or bloody diarrhoea</td>
</tr>
<tr>
<td>2</td>
<td>Haemorrhoids</td>
</tr>
<tr>
<td>1</td>
<td>gastric ulcer</td>
</tr>
<tr>
<td>1</td>
<td>Anaemia</td>
</tr>
<tr>
<td>1</td>
<td>Chango</td>
</tr>
<tr>
<td>1</td>
<td>Ngiri</td>
</tr>
</tbody>
</table>

4.4 Previous phytochemical and pharmacological studies on the Ochnaceae

No work has been published specifically on Ochna macrocalyx, but there has been biological and phytochemical work performed on other members of the Ochnaceae. Previous phytochemical work has shown the family to be rich in biflavonoids and related chalcones.
Much phytochemical work has been performed on *Brackenridgea zanguebarica*, which is a small tree occurring in north-west South Africa (Northern Transvaal), Zambia and Tanzania. It has distinctive root bark which is bright yellow in colour. For traditional uses, one paper describes it as having magical powers by the Venda people (indigenous to the east of the Soutpansberg in the Northern Province of South Africa) (Drewes, Hudson, et al. 1987), who believe that digging up the roots by an adult may lead to sterility.

Another paper written by a different research group had inadvertently ascribed the plant they were working on as *Cordia goetzei* (Marston, Zagorski, et al. 1988) (personal comment made by J. R. Ioset and K. Hostettman 2001). In this paper, the authors had performed anti-fungal bioassays on four isolated compounds (of which three were isolated in our phytochemical study from *Ochna macrocalyx*), which all had anti-fungal activity against spores of the plant pathogenic fungus *Cladosporium cucumerinum*. The same research group produced another article on the use of counter current chromatography on a *Brackenridgea zanguebarica* extract (Hostettmann & Marston 2001). This paper describes chromatography work, and does not go into phytochemical detail, but is significant as one of the compounds isolated from *Ochna macrocalyx* described nowhere else is illustrated here. The compounds isolated from *B. zanguebarica* are unusual and distinctive biflavonoids and dichalcone derivatives, some of which are strongly coloured pigments responsible for the colouring of the bark (Bombardelli, Bonati, et al. 1974; Drewes & Hudson 1983; Drewes, Hudson, et al. 1984; Drewes, Hudson, et al. 1987).

There has been extensive phytochemical work and a number of papers published on *Ochna calodendron*, by a research group at the University of Yaounde in Cameroon.
(Messanga, Pegnyemb, et al. 1998; Messanga, Kimbu, et al. 2001; Messanga, Kimbu, et al. 2002). They describe *Ochna calodendron* as a large tree with a yellowish stem bark widely distributed in the rain forests of South Cameroon where it is commonly used in traditional medicine (Messanga, Ghogomu, et al. 1998; Messanga, Tih, et al. 1994). Only one of their papers mentions details on traditional use, where the powdered dry stem bark is used for antimicrobial properties (Messanga, Pegnyemb, et al. 1998). From the stem bark they isolated a number of biflavonoids, one of which is the main principle responsible for the bark colouring (calodenin B) which was also isolated from *Brackenridgea zanguebarica*, and from *Ochna macrocalyx* in our study. In addition to calodenin B they also isolated three other pigmented biflavonoids (Messanga, Tih, et al. 1994), one of which is also present in *Lophira alata* and *L. lanceolata*, both also from the Ochnaceae.

There has been much phytochemical work on *Lophira lanceolata* and biological work published on *Lophira alata*. A lot of the phytochemical work was carried out by the same research group who worked on *Ochna calodendron* (Tih, Tih, et al. 1994; Tih, Tih, et al. 1999; Tih, Martin, et al. 1992; Tih, Tih, et al. 1994), who isolated a number of biflavonoids from the stem bark of these two species, one of which (lophirone F, Fig. 7), a chalcone dimer, is very similar to one of the compounds isolated in our study (ochnone) from *Ochna macrocalyx* (Tih, Sondengam, et al. 1990). The papers describes the genus *Lophira* as being distributed throughout tropical Africa, with *L. alata* and *L. lanceolata* from the forest and the savannah respectively used as medicinal plants. There are three papers published on biological work carried out on *L. alata* by a group in Japan (Murakami, Ohigashi, et al. 1991; Murakami, Tanaka, et al. 1992; Murakami, Tanaka, et al. 1992), who describe it as a medicinal plant used
for analgesia in tropical West Africa. They found the extract to have potent inhibitory activity against Epstein-Barr virus-early antigen (EBV-EA) induction, which they used as an assay to search for inhibitors of tumour promotion. Epstein-Barr virus (EBV) is a DNA tumour virus which has been implicated in Burkitt’s and Hodgkin’s lymphomas, and in glandular fever. The results reported in this study can be related to our work on NF-κB, as EBV is capable of inducing persistent NF-κB activation. The results of their bioassay subsequently led to their isolation of bi- and tetraflavonoids from the bark, which also showed inhibitory activity in their assays, as well as anti-inflammatory activity on mouse skin and are thus possible anti-tumour promotors.

Phytochemical work on Ouratea hexasperma was carried out by a group at the Universidade Federal Rural do Rio de Janiero in Brazil, who isolated isoflavanone and flavone dimers from the stem bark, roots and leaves, one of which (hexaspermone C) was isolated in our study from Ochna macrocalyx (Moreira, De Carvalho, et al. 1999; Moreira, Sobrinho, et al. 1994). The paper does not describe traditional uses of Ouratea hexasperma, but does state that it is a Brazilian plant, and that several Ouratea species are employed for the extraction of edible oil and are also used as medicinal plants.

A biological study has been carried out on the bark extracts of Ochna obtusata (synonyms: O. jabotapita, O. squarrosa), which was investigated for analgesic and anti-inflammatory activity based on its traditional use (Sivaprakasam, Viswanathan, et al. 1996). Some phytochemical work was also performed, isolating biflavonoids and isoflavones (Nia & Gunasekar 1992; Rao & Gunasekar 1989; Rao, Sreeramulu, et al. 1997). Ochna obtusata is described as a small tree or shrub found widely distributed
throughout Southern India. The plant is used in traditional medicine for pain, fever, inflammation, cholera, dysentery, dry cough and bronchitis. The root is used in menstrual complaints and asthma, the bark as a digestive tonic, and the boiled leaves are used as an emollient and medicated poultice. The pharmacological study showed that the bark extract had potent analgesic effect (on acetic acid induced writhing) on mice and significant anti-inflammatory effect in rat paw oedema.


**Flavonoids** are among the most widely distributed natural products in plants, occurring both in the free state and as glycosides. Their chemical structures are based on a C6-C3-C6 carbon skeleton. There are a huge number of published studies on flavonoids showing a variety of different bioactivities, such as anti-microbial and anti-inflammatory. Biosynthesis of flavonoids involves the phenylpropanoid intermediate p-coumaroyl CoA, and the elongation of its side chain with three malonyl CoA units. Closure of ring A forms the chalcone structure, closure of ring B forms the flavanone structure (Fig. 6) (Torssell 1983).
1. Cinnamic and hydroxy cinnamic acid, which also serve as precursors of phenylpropanoids, are synthesised from phenylalanine and tyrosine, which in turn are synthesised by the shikimic acid pathway. 2. The basic flavonoid skeleton is formed from three malonyl CoA units and a cinnamoyl CoA unit. 3. Chalcone is the first intermediate from which flavanone and all other flavonoids are formed. 4. Closure of the central ring forms the familiar and basic flavanone skeleton.

source: Torssell, 1983

From the literature research it can be seen that several members of the Ochnaceae are used medicinally for a wide range of traditional uses, but significant uses which seem to feature commonly include uses for anti-inflammatory, analgesic and antimicrobial conditions. Dysmenorrhoea is one of the uses reported for Ochna macrocalyx, and
there are at least three other members of *Ochna* which are said to be used for menstrual complaints (Table 6). However the term 'menstrual complaints' is a vague term which could just mean dysmenorrhea, and in this case the remedies may simply be employed for their anti-inflammatory or analgesic effects. *O. pumila*, *O. obtusata* and *O. afzelii*, whose traditional uses include menstrual complaints are also used for pain and inflammatory conditions, such as toothache and lumbago. The other main use for *O. macrocalyx* is diarrhoea, *O. obtusata* and *O. afzelii* also include dysentry in their list of traditional uses, possibly suggesting a link of some kind of common anti-diarrheal activity. Like *O. macrocalyx*, *O. afzelii* is also said to be used for infertility.

The literature shows the interesting ethnobotanical background, phytochemical and pharmacological profiles of species in the Ochnaceae and the genus *Ochna*. Previous phytochemical work shows a diversity in biflavonoids and polyflavonoids present in this family, several of which are found in a number of different species, but few are found outside of the Ochnaceae. Some of the phytochemical information related directly to *Ochna macrocalyx*, and aided in the identification of compounds isolated from it. The biochemical work which has been carried out on some of the species show interesting activity, such as the analgesic and anti-inflammatory activity found in *Ochna obtusata* which support its traditional use. Some of the information on traditional uses could also be related to those of *Ochna macrocalyx* and the biological results obtained in this study.
Table 6. Members of the Ochnaceae with published phytochemical/biochemical work.

<table>
<thead>
<tr>
<th>name</th>
<th>Origins</th>
<th>traditional uses</th>
<th>compounds isolated</th>
<th>biological work</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brackenridgea zanguebarica 'nxitavhatsindi'</td>
<td>Northern Transvaal, Zambia, Tanzania</td>
<td>magical powers</td>
<td>biflavonoids and chalcone dimers</td>
<td>antifungal activity</td>
</tr>
<tr>
<td>Lophira alata</td>
<td>Africa (forest)</td>
<td>analgesic/anti-inflammatory</td>
<td>polar polyflavonoids</td>
<td>possible anti-tumour promotor activity</td>
</tr>
<tr>
<td>Lophira lanceolata</td>
<td>Africa (savannah)</td>
<td></td>
<td>biflavonoids, chalcone dimers, tetraflavonoid</td>
<td></td>
</tr>
<tr>
<td>Ochna calodendron</td>
<td>Cameroon</td>
<td>antimicrobial</td>
<td>flavones, biflavonoids, triflavonoids</td>
<td></td>
</tr>
<tr>
<td>Ouratea hexasperma</td>
<td>Brazil</td>
<td>edible oil</td>
<td>isoflavanone and flavone dimers</td>
<td>one biflavonoid was previously found to potently inhibit cellular growth, DNA and protein synthesis</td>
</tr>
<tr>
<td>Ochna pulchra 'umnyelenyele, muparamhosva'</td>
<td>Central and Southern Africa</td>
<td>anti-blood parasitic agent, skin disease</td>
<td>vismiones, an anthrone and a bianthrone</td>
<td></td>
</tr>
<tr>
<td>Ochna pumila 'champa baha'</td>
<td>India</td>
<td>snake bites, menstrual complaints, tuberculosis, asthma, lumbago, ulcers</td>
<td>biamentoflavone derivatives, ochnaflavone and derivative</td>
<td></td>
</tr>
<tr>
<td>Ochna atropupurea</td>
<td></td>
<td></td>
<td>ochnaflavone</td>
<td></td>
</tr>
<tr>
<td>Ochna obtusata</td>
<td>India</td>
<td>pain, fever, inflammation, cholera, dysentery, bronchitis, asthma, menstrual complaints, digestive, emollient, medicated poultice</td>
<td>ochnaflavone, flavonoids, isoflavones, biflavonoids</td>
<td>analgesic and anti-inflammatory effects in mouse experiments</td>
</tr>
<tr>
<td>Ochna afzelii</td>
<td>Cameroon</td>
<td>jaundice, toothache, female infertility, menstrual complaints, lumbago, dysentery</td>
<td>isoflavonoids, biflavonoids</td>
<td></td>
</tr>
<tr>
<td>Ochna beddomei</td>
<td>India</td>
<td></td>
<td>biflavanone, flavonoids</td>
<td></td>
</tr>
<tr>
<td>Ochna integerrima</td>
<td>Thailand</td>
<td>digestive tonic, anthelmintic</td>
<td>biflavonoids (dihydroochnaflavone derivatives), flavonoids</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 7 Compounds isolated from the Ochnaceae

calodenin A

lophiron C

lophiron F

lophiron A

ochnaflavone

lophiroflavan A

lophirachalcone
5. **BUPLEURUM FRUTICOSUM**

5.1 Umbelliferae and Bupleurum

The **Umbelliferae** (also known as the **Apiaceae**) is one of the largest families of flowering plants, with around 400 genera and approximately 3,000 species. The family mostly consists of herbs, and can be found in northern temperate regions and tropical highlands throughout the world. The family is defined by its distinctive umbrella-like inflorescence, the umbel, from which its name is derived from. Many species in it are biennial, and produce vegetables which can be eaten by humans. It is an important and well-known family, whose more well-known members include the carrot and the parsley. Other examples include celery, parsnip, caraway, dill and coriander.

The Umbelliferae has a rich history, its distinctive inflorescences made it one of the first families to be recognised as a distinct group, which occurred towards the end of the 16th century. Several species are employed for medicinal purposes, for example fennel was used for intestinal purposes, *Cicuta* and *Conium* are used in homeopathy. *Conium maculatum*, also known as poison hemlock is famously known to have been used to kill Socrates.

The genus **Bupleurum** L. comprises about 200 species, and is primarily located in the northern hemisphere, Eurasia and North Africa. The better investigated and well-known taxa comes from China and Japan, where the roots of *B. falcatum* and *B. chinense* (syn.: Chai hu, Saiko, Hare's ear, Thorowax, *B. radix*) are used (Massanet, Guerra, et al. 1997). These are considered to be one of the most important herbs in
Chinese and Japanese herbalism, used in a tonic to improve liver and digestive function, and used in hepatitis, liver cirrhosis and irritable bowel syndrome. Recent research in Japan have shown that the roots have hepatoprotective effects, confirming its traditional use.

A number of plants from the genus *Bupleurum* have been subject to phytochemical investigations, from which have revealed the presence of saponins, terpenoids, coumarins, flavonoids, polyacetylenes and lignans.

5.2 Literature background and traditional uses of *Bupleurum fruticosum*  

*Bupleurum fruticosum* L. is an evergreen shrub often found localised in shaded holm oak forests on calcareous grounds. It grows spontaneously in parts of Italy and Spain (Massanet, Guerra, et al. 1997; Pistelli, Bertoli, et al. 1996). The plant has a strong, distinctive aroma, which contains aromatic compounds in abundance. The fruits which are said to have a fennel like aroma are used as a spice. Apparently animals have a certain repulsion to the shrub, also thought to be due to the aroma. The plant is known to be rich in essential oil, containing approximately 1-3 % v/w (Manunta, Tirillini, et al. 1992).

Traditionally, the aerial parts of the shrub are used for anti-inflammatory and antiseptic purposes (Guinea, Parellada, et al. 1994). In Sardinia it is used as an anti-rheumatic remedy (Pistelli, Bertoli, et al. 1996).
Table 7. Results for composition of essential oil from *Bupleurum fruticosum* obtained from two studies (Lorente et al. 1989, Manunta et al. 1992) (t = trace).

<table>
<thead>
<tr>
<th>Essential oil</th>
<th>composition (%v/v)</th>
<th>Lorente et al</th>
<th>Manunta et al</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>stems</td>
<td>leaves</td>
<td>stems</td>
</tr>
<tr>
<td><strong>Hydrocarbons</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>α-pinene</td>
<td>41</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>β-pinene</td>
<td>36</td>
<td>-</td>
<td>t</td>
</tr>
<tr>
<td>myrcene</td>
<td>3</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>α-phellandrene</td>
<td>3</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>β-phellandrene</td>
<td>-</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td>limonene</td>
<td>4</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>α-terpinine</td>
<td>-</td>
<td>-</td>
<td>t</td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>3</td>
<td>49</td>
<td>-</td>
</tr>
<tr>
<td>p-cymene</td>
<td>3</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
<td>12</td>
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</tr>
<tr>
<td>bornylene</td>
<td>-</td>
<td>t</td>
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<td>-</td>
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<tr>
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<td>-</td>
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</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>t</td>
</tr>
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<td>linalool</td>
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</tr>
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<td>borneol</td>
<td>t</td>
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</tr>
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<td>nerol</td>
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<td>-</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>elemicin</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td><strong>Esters</strong></td>
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</tr>
<tr>
<td>hexyl-2-methylbutyrate</td>
<td>-</td>
<td>t</td>
<td>-</td>
</tr>
<tr>
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<td>1.6</td>
<td>-</td>
</tr>
<tr>
<td>cinnamyl isovalerate</td>
<td>-</td>
<td>1.3</td>
<td>t</td>
</tr>
<tr>
<td>cinnamyl valerate</td>
<td>-</td>
<td>2.7</td>
<td>-</td>
</tr>
</tbody>
</table>
5.2.1 Essential Oil

Various studies have been performed on the essential oils of *B. fruticosum*, the composition of which has been examined by Italian chemists as far back as 1913. In a recent paper the composition of the essential oil from the stems and the leaves were studied using GC/MS. It reported a high percentage of almost 50% of γ-terpinene from the stems, and almost 40% of both sabinene and β-phellandrene from the leaves (Table 7) (Manunta, Tirillini, et al. 1992).

Another study investigated the oil composition of the flowering tops, as well as the anti-inflammatory effects of the oil and its components. This study found the main components of the oil to be α- and β-pinene (approx. 40% each, Table 7). The oil was found to have potent anti-inflammatory activity when administered orally and parenterally in carrageenan-induced oedema in rat paw. The activity was shown to be attributed in part by α- and β-pinene, and the presence of minor components in the oil were also shown to potentiate their activity (Lorente, Ocete, et al. 1989).

5.2.2 Aerial parts

Phytochemical investigations carried out on the aerial parts of *Bupleurum fruticosum* have been published in two papers by two separate research groups, from Spain and Italy (Pistelli, Bilia, et al. 1995, Massanet, Guerra, et al. 1997), who isolated a series of phenylpropanoids, two coumarins, spinasterol and erythrodiol.

The main *Bupleurum fruticosum* phenylpropanoids belong to a class of their own, they are methoxylated on the aromatic ring, and a number of them have the same unusual diester side chain which appears to be formed from the condensation of two 2-methyl-but-2-enenoic acid units (Fig. 8). They are not common in nature, and
similar phenylpropanoids have only been found in *Blumea lacera* (Asteraceae) and *Anthriscus sylvestris* (cow parsley, Fabaceae) (Pistelli, Bilia, et al. 1995).

**Phenylpropanoids**

Phenylpropanoids are a large group of compounds derived from the amino acids phenylalanine and tyrosine. Phenylpropanoids are so named as their basic structure contains a phenyl ring attached to a three carbon propane side chain, and are devoid of nitrogen. Various categories of phenylpropanoids include the hydroxycinnamic acids, phenylpropenes, coumarins and chromones. Lignans are formed from the fusion of two phenylpropanoids, and flavanoids are formed from a mixed synthetic pathway, where one of their rings are made up of a phenylpropanoid. Phenylpropanoids also serve as building blocks in the formation of the high molecular weight polymers lignins and tannins.

The principal precursors of phenylpropanoids are cinnamic acid and hydroxycinnamic acid. These two precursors arise from phenylalanine and tyrosine, which themselves are synthesised via the shikimic acid pathway.

Phenylpropenes are formed from the hydroxycinnamoyl alcohols, and are volatile, lipid soluble compounds usually isolated with terpenes in volatile oils, which contribute to the odour and flavour of the plant. Coumarins are lactones derived from hydroxycinnamic acids, which undergo ortho hydroxylation, and then ring closure between the ortho hydroxyl group and the carboxyl group.

The major phenylpropanoids isolated from *Bupleurum fruticosum* are unusual, the path of biosynthesis of the side chain is not obvious. The compounds isolated would appear to be of mixed biosynthetic pathways, with the basic phenylpropanoid
structure present, and then the diester side chain may possibly be formed from the condensation of two short chain fatty acids (Torssell 1983).

Erythrodiol (Fig. 8.15) is a fairly widely distributed triterpenoid aglycone. Some triterpenoids have a structure similar to steroidal compounds, and are often investigated for anti-inflammatory activity. In two separate studies (one of the studies was designed to show the anti-inflammatory activity of the constituents of virgin olive oil) erythrodiol was found to have anti-inflammatory activity in PMA induced chronic inflammation in mouse ear (De la Puerta, Martinez-Dominguez, et al. 2000; Mâñez, Carmen, et al. 1997). In another study which investigated the bioactivity of the triterpene diols and triols from chrysanthemum flowers, erythrodiol was found to have inhibitory activity against Epstein-Barr virus early antigen activation in an anti-tumour promoting assay (Ukiya, Akihisa, et al. 2002).

α-Spinasterol (Fig. 8.14) is a less commonly occurring phytosterol which is also found in spinach and alfalfa. One pharmacological study has been performed on α-spinasterol isolated from *Symplocos spicata*, which showed it to have anti-inflammatory activity in carrageenin induced acute paw oedema in rats (Frotan, Acharya, et al. 1984).

### 5.2.3 Roots

There has been much phytochemical and biochemical work performed on the roots of *Bupleurum fruticosum*. Two papers by the same research group (Pistelli, Bertolli et al. 1996, Pistelli, Bilia et al. 1993) have been published on the constituents from the roots, which include triterpenoid saponins and coumarins (Fig. 9). Three pharmacological studies have been performed on the root constituents.
Fig. 8 *Bupleurum fruticosum* isolates from aerial parts

1, 2, 3: 3-(methoxy/dimethoxy/trimethoxyphenyl)-2E-propenyl 2-2[2-methyl-2Z-butenoyloxy)methyl]-2Z-butenooate; 4: dimethoxy-4-(1-methoxy-2-propenyl)benzene; 5: 1,2-dimethoxy-4-(3-methoxy-1-propenyl)benzene; 6: 3,4-dimethoxycinnamaldehyde; 7: 7-(3,3-dimethylallyloxy)-6-hydroxy coumarin; 8: 1,2,3-trimethoxy-5-(3-ethoxy-1E-propenyl)benzene; 9: 1,2-dimethoxy-4-(3-ethoxy-1E-propenyl) benzene; 10: 1-(3,4,5-trimethoxyphenyl)-2-propyl-2-(2methyl-2Z-butenoyloxy)methyl]-2Z-butenooate; 11: 1,2,3-trimethoxy-5-(1-ethoxy-2-propenyl)-benzene; 12: 1-(3,4,5-trimethoxyphenyl)-2-propyl-2-(2-methyl-2Z-butenoyloxy)methyl]-2Z-butenooate; 13: 1-(3,4-dimethoxyphenyl)-propyl-2-(2-methyl-2Z-butenoyloxy)methyl]-2Z-butenooate; 14: spinasterol; 15: erythrodiol.
The methanol extract of the roots showed haemolytic activity, and crude saponin fractions showed immunostimulating and hepatoprotective effects. Buddlejasaponin IV, one of the compounds isolated from the roots, was also shown to have hepatoprotective effect, comparable to silybin, against galactosamine cytotoxicity in rat hepatocytes (Guinea, Parellada, et al. 1994).

Another study investigated the genotoxic, cytotoxic and anti-mitotic activity of three of the triterpenoid saponins and one phenylpropanoid from *B. fruticosum*. The study found them to have no genotoxicity, and only two of them showed toxicity against phytohaemaglutinin-stimulated lymphocytes (although only at above 200 μM) with no antimitotic activity (Scarpato, Pistelli, et al. 1998). This work was followed on in a second paper by the same research group, which investigated the effects of two of the saponins on the clastogenicity and cytotoxicity of the two anticancer drugs mitomycin C and bleomycin. One of the compounds was found to have a protective affect against mitomycin C induced DNA damage. It was also found to potentiate the mutagenic effects of bleomycin. The second compound was found to enhance the cytotoxicity of bleomycin (Scarpato, Bertoli, et al. 1998).

Literature research into other members of *Bupleurum* show a large number of phytochemical studies carried out on the roots. Their chemical profile is mostly of 'saikosaponins', oleanane type saponins which were first isolated from Bupleurii radix. Bupleurii radix is the root of *Bupleurum falcatum* and other related species (*B. chinensis, B. scorzonerifolium*), and is the key ingredient of a traditional Japanese herbal medicine (Kampo) formula known as 'sho-saiko-to', which itself is based on a
traditional Chinese formula (xao-chai-hu-tang). Sho-saiko-to and saikosaponins have well documented anti-inflammatory and hepatoprotective activity (Evans, 1996).

In conclusion, it can be seen that there has already been much phytochemical and biological research performed on *Bupleurum fruticosum*. Its traditional use as an anti-inflammatory agent is already supported in one previous *in vivo* study on its essential oils (Lorente, Ocete, et al. 1989), although the molecular target was not elucidated in the study. Erythrodiol and α-spinasterol which have been isolated from the aerial parts, have previously reported anti-inflammatory activity. Erythrodiol is also a triterpenoid, a class of compound which has shown NF-κB inhibitory activity. However erythrodiol and α-spinasterol are found in a number of different plants and are widely distributed. The phytochemical study which reported their isolation from *B. fruticosum* gave no detail on what percentage was found in the plant extract (Massanet, Guerra, et al. 1997), so it would be difficult to say how much they would contribute to the plant's overall anti-inflammatory effect.

Previous pharmacological studies on the roots have shown hepatoprotective properties, which complement the biological studies which have been performed on other *Bupleurum* species' roots, especially the well known *B. falcatum* and *B. chinensis*.

These studies show that *Bupleurum fruticosum* has an interesting biological profile, and are significant in supporting its therapeutic importance as a medicinal plant.

No anti-inflammatory studies have been performed specifically on aerial part extracts of *Bupleurum fruticosum*, and with the published biological studies on it and related species as well as the results of the AINP screening project, it seems to be a promising candidate for NF-κB investigations.
Fig. 9  *Bupleurum fruticosum* root isolates

Triterpenoid saponins

![Chemical structures of triterpenoid saponins](image)

| 1 | Buddlejasaponin IV | R₁ | R₂ | R₃ |
| 2 | Malonylbuddlejasaponin IV | H | CO-CH₂-COOH |
| 3 | | H | OMe | OH |
| 4 | | H | OH | OH |

Coumarins

![Chemical structures of coumarins](image)

7-(2-hydroxy-3-methyl-3-butenyloxy)-6-methoxycoumarin

5,7-dihydroxy-6-methoxy-8-(3-methyl-2-butenyl)coumarin

Others:
- scopoletin
- scoparone
- prenyletin
- capensin
- fraxatin
- aesculetin
- 7-(3-methyl-2-butenyloxy)-6-methoxycoumarin

Source: Pistelli, Bilia, et al. 1993
Pistelli, Bertoli, et al. 1996
6. ANTIBACTERIAL AND CYTOTOXICITY ASSAYS

Other biological assays were also performed on the isolated compounds from *Ochna macrocalyx*.

**Antibacterial activity**

The ability for pathogenic bacteria to continually acquire resistance to antibiotics makes the constant search for new anti-microbial agents important and necessary. The prolonged exposure of bacteria to various different antibiotics subsequently results in the development of their resistance to them.

The occurrence of methicillin, vancomycin and multi-drug resistant *Staphylococcus aureus* (SA) is a source of great concern particularly in clinical hospital environments, from which they have been isolated. Currently the group of antibiotics which are used as the last line of treatment against MRSAs are the glycopeptides, typically vancomycin, the use of which is reserved for resistant infections where all other antibiotics prove ineffective. Unfortunately the presence of vancomycin resistant strains of SA has already been reported in Japan and the United States back in 1997, and more recently in the UK in 1999 (Sieradzki, Roberts, et al, 1999).

One of the main mechanisms bacteria possess to enable them to be resistant to antibiotics is the efflux protein, which actively pumps antibiotic out of the organism. In SA, efflux mechanisms have been demonstrated to confer resistance to macrolides, fluoroquinolones and tetracyclines. The efflux proteins have also been characterised: *Msr(A)*, *Nor(A)* and *Tet(K)*, which efflux macrolides, norfloxacin and tetracycline, respectively. Some SAs have more than one type of efflux protein, or multi-drug
efflux proteins, as well as other mechanisms of resistance and are called multi-drug resistant (mdr) (Adcock 2002; Gibbons & Udo 2000).

Being linked to another research group working in the same department who are searching for antibacterial natural products, the facilities to test compounds in antibacterial assays were readily available. Compounds were tested against three strains of multi-drug resistant *Staphylococcus aureus* (mdr SA): XU212, RN 4220 and 1199-B, which possess the Tet(K), Msr(A) and Nor(A) efflux proteins respectively (Gibbons, Oluwatuyi, et al, 2003). They also have other types of resistant mechanisms, so are resistant to more than one type of antibiotic.

**Cytotoxicity MTT reduction assays**

Cytotoxicity assays were undertaken when the crude extract and fractions from *Ochna macrocalyx* were observed to have cytotoxicity against the HeLa cells used in the NF-κB assays, particularly in the luciferase assay where the cells were incubated with the extract for 7 hours. The object was to assess how toxic the extracts and compounds were, and the collection of toxicity data could enable the assessment of whether the NF-κB inhibition seen in the assays was due to cytotoxicity, or intrinsic NF-κB inhibitory activity.

The MTT assay is a simple, rapid, colourimetric assay which allows the indirect measurement of proliferation and cytotoxicity in cells. The assay employs the reduction by live cells of the yellow water soluble tetrazolium salt MTT [3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], which is one of many heterocyclic organic compounds that form coloured insoluble formazans after being
reduced. It is believed that the tetrazolium ring of the MTT is cleaved by succinate dehydrogenase, one of the enzymes found in the mitochondria which is involved in the production of electrons needed for ATP production. The reduction of the MTT results in the production of MTT formazan, which is insoluble in aqueous solutions and precipitates out as dark blue/purple crystals. The amount of formazan produced correlates with the number of viable cells present, and thus the optical density of the resulting solution is measured after the crystals have been solubilised (Hansen, Nielsen, et al. 1989; Mosmann 1983).

MCF-7 breast cancer cells were used in the cytotoxicity assays which were performed at the Centre for Toxicology at the School of Pharmacy, where the system is well established. The hormone responsive cells were derived from a breast cancer patient in 1970, developed at the Michigan Cancer Foundation, and are a standard cell line used in laboratories around the world (Levenson & Jordan 1997).
7. AIMS AND OBJECTIVES

*Ochna macrocalyx*

*Ochna macrocalyx* has had no phytochemical work published on it, and whose extract showed NF-κB inhibitory activity. The aim of my project was to isolate and identify as many compounds as possible from the bark extract, as well as specific NF-κB inhibitors, in the search for new bioactive compounds.

Because cytotoxicity was observed in the biological assays, I also wanted to determine whether or not the NF-κB inhibitory activity observed was not simply due to the cytotoxicity effects, thus cytotoxicity assays were performed. Other bioactivity was also tested for in anti-bacterial assays, the facilities for which were readily available to us.

With the phytochemical and biological information obtained, I could also discuss how they might support the importance of the plant in traditional medicine and how it is used in Washambaan medicine.

*Bupleurum fruticosum*

As a plant which has had much phytochemical work performed on it, here my objective was more specifically to isolate NF-κB inhibitors from the plant extract.
CHAPTER TWO: MATERIALS AND METHODS

PHYTOCHEMICAL METHODS

7. PLANT MATERIAL

*Ochna macrocalyx* was collected from the foot of the Western Usambara mountains in 1997-8 (research permit no. 97-165-NA-97-35) and identified by Christina Schlage. A voucher specimen (CS 42, 2) is held in the Centre for Pharmacognosy and Phytotherapy at the School of Pharmacy, London.

*Bupleurum fruticosum* was collected from Sierra de Segura, Spain in 2000-1 and identified by Diego Rivera (Universidad de Murcia). A voucher specimen (Rivera and Obón AINP 139) is held at the Universidad de Murcia, Spain.

8. CHROMATOGRAPHIC TECHNIQUES

The chromatographic techniques used in my phytochemical work are described in this section, and are categorised according to the different stationary phases which were used with the technique. More detailed information on specific solvent systems and isolation scheme of individual compounds is given in the results section.

8.1 Using silica gel as the stationary phase.

Silica gel was used as the main stationary phase material for adsorption chromatography. It is suitable for many compounds and can provide good separation results. For preparative purposes it is less suitable for very polar compounds which have a high affinity for silica, which itself is a polar stationary phase. Silica gel consists of a 3-D matrix of Si-O-Si bridges, with silanol (SiOH) groups throughout.
The stronger the ability for a compound to form hydrogen bonding, the stronger its interaction with silica gel becomes. Polar compounds tend to 'stick' to the silica due to interaction with the SiOH groups, and either stay at the origin after elution, or require very polar solvents to make them move, and recovery is poor. For polar compounds or compounds which may react with silica, an alternative stationary phase should be considered, such as Sephadex LH-20 or reverse phase carbon chain coated silica.

Silica gel was used in the following methods of chromatography:

8.1.1 Thin Layer Chromatography (TLC)

Analytical TLC plates: Merck Si gel 60 with F\textsubscript{254} indicator on aluminium sheets.

Preparative TLC plates: Merck Si gel 60 with F\textsubscript{254 +366} indicator on glass plates (20 X 20 cm).

Analytical TLC plates were used for analysis of extracts, and were used in TLC tanks with solvent saturated filter paper (Stahl 1988; Wagner 1984).

Both preparative and analytical plates were used in the isolation of compounds (Cannell 1998; Hostettmann, Hostettmann, et al. 1986). Analytical plates were used when the quantities of material were small, or when the resolution on the preparative plates was not sufficient. Usually multiple developments in one or more different solvent systems were employed, where the plate is developed once in one solvent system, then taken out and allowed to dry before being developed again in the same or a different solvent mixture. Compounds were viewed under UV light at 254 nm and
365 nm, and visualised by spraying with 4% vanillin in concentrated sulphuric acid and heating.

Scans show TLC plates which have been sprayed and heated, UV activity is marked out, with hatching indicating UV activity under short-wave UV light.

**Loading of sample**

In the isolation of compounds, depending on the complexity of the extract, generally about 20 mg of plant extract may be loaded onto one analytical TLC plate (20 cm x 20 cm), and about 80 - 100 mg onto a preparative TLC plate. Reducing of the loading quantity is required if the plate is overloaded, which can be seen as abnormal looking bands with little or no separation that follow the solvent front. More extract may be loaded if the bands look faint or thin.

The extract is dissolved in a small volume of appropriate solvent, and then applied as a thin line across the plate, approximately one inch from the bottom of the plate.

**Recovering the compound**

After development, the required compound band is marked out with a pencil, or with the sharp edge of a spatula. If the compound needs to be visualised using the spray, for an analytical plate a strip along the edge of the plate is cut off, which can then be sprayed. For a preparative plate, the main body of the plate is covered with a glass plate, and only the edge of the plate is sprayed. Also a groove is scored into the silica along the edge of the glass plate to avoid the vanillin and sulphuric acid reagent from spreading into the main body of the TLC plate.

The required band is scraped off using a spatula, and the silica poured directly into a small glass column with a frit (or defatted cotton wool). Approximately 50 - 100 mls
of an appropriate solvent is poured into the column to desorb the compound from the silica, which is collected in a round bottom flask. The use of pure methanol to desorb compounds from silica is avoided as small amounts of silica are able to dissolve in methanol. If polar solvent mixtures are required, then a mixture of up to 50% methanol in ethyl acetate is used. The solvent is removed using a rotoryevaporator, and the compound is transferred to a glass vial for storage by redissolving in a small amount of solvent, which is dried using nitrogen. If there is silica still present in the compound, the compound is redissolved in some ethyl acetate or other appropriate solvent, and the mixture passed through a 0.45 μm Teflon filter. The purity of the isolated compounds are analysed on TLC or NMR and are repurified as required. HPLC grade solvents are used in the isolation of pure compounds.

8.1.2 Flash chromatography

Flash chromatography uses a basic silica column but enables fast elution by using nitrogen gas to apply positive pressure which effectively pushes the solvent through the column (Hostettmann, Hostettmann, et al. 1986). Advantage over VLC is that small fractions may be collected in test tubes, and the columns may be reused.

A Biotage Flash 40i\textsuperscript{TM} module using a column with internal diameter of 40 mm and 150 mm length was used for quick separation of extracts of up to one gram in weight. Columns are obtained prepacked with KP-Si\textsuperscript{TM} silica (silica gel, particle size 32-63 μm, average pore size 60 Å).

Loading the extract
The extract was either adsorbed onto silica (equal quantities) before being loaded into the system using the sample injection module, or dissolved in appropriate solvent and injected directly onto the top of the column. If the second method is used, the solvent used should preferably be non polar such as hexane, or less polar than the eluting solvent system to prevent the extract spreading too much into the column before elution.

**Elution of column**

The elution protocol is similar to that for Vacuum Liquid Chromatography. If the compounds in the extract have $R_f$ values close to each other on silica, then an isocratic solvent system is more appropriate. The solvent system used should be designed such that when used in TLC on the extract, the furthest travelling compound should only have an $R_f$ of approximately 0.3. If the extract mixture contains compounds with very different polarities, then a gradient system is required to enable the quicker elution of the slow moving polar compounds. Here elution is started off with a non-polar solvent, which is gradually changed to become more polar to increase its eluting power.

The larger Flash 75i™ radial compression module was also used in the crude separation of an extract of five grams. The sample was adsorbed onto 5 g of silica before being loaded into the system using the sample injection module.

8.1.3 Vacuum Liquid Chromatography (VLC)
VLC was used for the crude fractionation of an extract, like Flash chromatography it also uses a silica column but here fast elution is enabled by the application of negative pressure, creating a vacuum at the collection point which pulls the solvent through the column (Cannell 1998).

The advantages of using VLC over Flash chromatography is, as the silica is packed in a glass column, mainly in being able to see your extract as it travels through the column, enabling the collection of specific bands where possible and the adjustment of the polarity of the solvent as appropriate. With Flash, a certain amount of guess work may be required, or fixed incremental increasing in solvent polarity in gradient systems has to be used. Another advantage of VLC is that it is much cheaper. A disadvantage is that to enable the formation of a vacuum, collection vessels used have to have the Quick fit ™ necks such as round bottom flasks or buchner flasks, and collection of smaller fractions in test tubes is generally not possible or practical.

A glass column (60 mm X 120 mm) with frit and a Quick fit (24/29) opening at the bottom was used. A T-piece adaptor was used with the side arm connected to a vacuum line and the fractions collected in round bottom flasks. The column was packed using silica gel G for TLC with 13 % gypsum. This was found to work better than ordinary column chromatography silica where the column produced was too loose. The gypsum enabled the column to be made more compact.

The silica is packed into the column dry with the vacuum on (and a round bottom flask attached to the column to form the vacuum), and packed down firmly with the bottom of a beaker, and a spatula for the edges. Filter paper, tissue or cleaned cotton wool is placed on the top of the bed to keep it flat. The bed is washed with (non-polar) solvent before the extract is loaded onto the column, without allowing the bed to dry.
The extract is prepared by adsorbing onto an equal proportion of silica (mix extract with silica and a suitable solvent in a round bottom flask, and then evaporate off the solvent using a rotary evaporator until the mixture is dry). This is applied evenly onto the top of the bed as a flat layer. About 5-10 g of extract may be loaded.

The column is eluted with 100-200 ml portions of solvent which is rapidly pulled through the column, and collected in the round bottom flask. Generally a gradient system is used, where the first portion of eluting solvent used is non-polar, and subsequent portions are increasingly more polar, until the final stages where most of the compounds in the extract have come through leaving only the most polar compounds. The column is then further eluted with a more polar solvent mixture, usually a mixture of methanol and ethyl acetate. An isocratic system can also be used when there are many compounds present in the extract with close R_f values.

### 8.2 Reverse phase C-18 silica (RP-C18)

This was generally used as an alternative to silica for compounds which otherwise did not purify, or with very polar compounds for which silica is not suitable.

#### 8.2.1 TLC

Analytical TLC plates: Merck RP-18 F_{254S} on aluminium sheets.

RP-C18 TLC was useful for obtaining an idea of how compounds might separate in the HPLC column. In comparison to ordinary silica TLC plates RP-C18 plates were used much more sparingly due to it being far more expensive. Solvents used included methanol, water and acetonitrile.

#### 8.2.2 High Performance Liquid Chromatography (HPLC)
Using the analytical HPLC column was preferable to using RP-C18 TLC plates as the column can be reused, in addition it is used to develop suitable solvent systems to enable preparative isolation of compounds (Cannell 1998; Hostettmann, Hostettmann, et al. 1986; Hamilton & Sewell 1977).

Prepacked analytical and semi-preparative RP-C18 silica columns (Waters radially compressed model 25 mm) were used for analysis of extracts and isolation of compounds. A Waters 600 controller with 996 photo-diode array detector was used. HPLC solvents were degassed with helium. Extracts for injection were made up by dissolving in appropriate solvent (1 mg/ml) and filtering through a 0.45 μM filter. For the semi-prep column, up to 100 mg of extract was injected, this was dissolved in 2.5 - 3 ml of solvent and filtered before injection.

Solvent systems used included methanol, acetonitrile and water.

### 8.3 Sephadex LH-20

This was used in a conventional gravity column chromatography system. Sephadex LH-20 can be used for size exclusion chromatography (Cannell 1998), although there may be direct interaction between the compound and the Sephadex. The advantages of using Sephadex LH-20 are that recovery of the extract is usually good, and the packing material can be reused.

Sephadex was allowed to swell in solvent (CHCl₃ or MeOH), before the slurry was poured into the column.
As Sephadex floats in chloroform, the bed was packed by allowing the chloroform to run out of the column until the level of the solvent is equal to that of the top of the Sephadex bed. The extract was dissolved in the minimum amount of the same solvent which was evenly applied to the top of the bed. Filter paper or tissue was placed onto the top of the bed to keep it flat, and chloroform was slowly poured onto the top of the column in an almost drop wise fashion for elution. This is not required if using methanol, as Sephadex settles to the bottom and packing is straight forward.

For crude separation, approximately 100 g of Sephadex was used for 1 g of extract. For purification, about 10 g was used for 50 mg.

9 spectroscopical techniques

The first step in the identification of a compound was with the obtaining of a 1-D \(^1\)H NMR spectrum and a molecular weight using mass spectroscopy. Fast atom bombardment (FAB) is the best method for this purpose as it is a ‘soft’ method of ionisation and tends to produce good molecular ion peaks. Electron ionisation (EI) tended to break down the compounds, so the molecular ion of the compound is not always present. However EIMS is also useful as it shows the fragmentation pattern of a molecule, and can provide valuable information on the substructures within the compound.

Electron spray (ES) although also a soft ionisation method is not always suitable particularly for non polar compounds which do not ionise under this method. In addition pH adjustments may be required depending on the compound to make it more stable, where it may otherwise produce various signals.

Possible molecular formulas could also be obtained using accurate mass calculations.
If the compound has been previously isolated, then it may be identified by comparison of the spectral data with that published in the literature. Otherwise the structure of the compound was elucidated using 2-D $^1$H and $^{13}$C NMR spectra (COSY, HMQC, HMBC and NOESY). NOESY enabled the relative stereochemistry of the compounds to be elucidated, but no further steps were taken to establish absolute chemistry, which would require larger amounts of compound which was difficult to obtain (Williams & Fleming 1995).

NMR experiments were performed using Bruker AVANCE 400 and 500 MHz.

Chemical shifts used for reference deuterated solvents: $\delta_H$ Acetone-d$_6$: 2.05 ppm, $\delta_H$ CDCl$_3$: 7.26 ppm. FAB and EIMS were obtained using ZAB-SE from VG-Analytical. ESMS was obtained using Finnigan Navigator.
10 NC-κB assays

10.1 Electrophoretic mobility shift assay (EMSA)

EMSA is a DNA binding assay using polyacrylamide gel electrophoresis. Free NC-κB bound to radiolabelled DNA fragments containing NC-κB specific gene sequences retard the mobility of the fragments during electrophoresis, resulting in bands forming corresponding to individual protein-DNA complexes.

Media: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % Fetal Calf Serum (FCS) and 1 % penicillin/streptomycin.

Preparation of test extracts: 5 mg of plant extract was mixed with 25 ml of media and 125 μl of DMSO in falcon tubes. The extract was allowed to solubilise under refrigeration overnight. Plant extracts were warmed and centrifuged prior to being used in the assay. Only the supernatant was used.

EMSA

HeLa cells (5 x 10⁵) were grown overnight on 10 cm dishes with 5 ml of medium at 37 °C and 5 % CO₂. The medium was removed from the dishes, and the cells incubated with 5 ml of medium containing the dissolved plant extract for one hour. Cells were then stimulated by the addition of phorbol 12-myristate-13-acetate (PMA) (25 ng/ml final conc.) and incubated for a further 15 minutes.
One dish was used for a ‘positive’ or stimulated control, which was not exposed to a plant extract, and one dish for a ‘negative’ or unstimulated control, which was not exposed to neither PMA nor extract. Parthenolide as an NF-κB inhibitor was used as a control for assessing the inhibitory activity of natural products and extracts (results not shown).

Medium was removed from the plates and the cells washed with phosphate buffered saline (PBS). 1 ml of PBS was added into each dish, and the cells scraped off the dish and into the PBS using a rubber policeman. The harvested cells were transferred into cooled Eppendorf tubes, and then pelleted by centrifugation (3000 rpm for 3 min at 4 ºC). The supernatant was removed, and the cells resuspended in 60-80 µl lysing TOTEX buffer (20 mM Hepes/KOH pH 7.9, 0.35 M NaCl, 20 % v/v glycerol, 1 % NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT)). The unstable PMSF (approx 1 µl of saturated solution in ethanol) is added in separately into each tube, and the suspension mixed by pipetting. The suspensions are incubated on ice for 30 minutes, with occasional shaking.

The cell debris is pelleted by centrifugation (13000 rpm, 10 min, 4 ºC) and the supernatent transferred into another set of Eppendorf tubes, which is then tested for the presence of NF-κB.

10 µl of cell extract is mixed with 10 µl of radiolabelled oligonucleotide reaction mixture (4 µl 5 x binding buffer (20 % w/v Ficoll 400, 100 mM Hepes/KOH pH 7.9, 1 mM DTT, 300 mM KCl), 1 µl poly dI-dC (Pharmacia), 1 µl bovine serum albumin (10 µg/ml), 1 µl radiolabelled oligo, 3 µl purified H2O) which contain the specific sequence NF-κB binds to. This is left to react for 15 minutes at 4 ºC before being separated by electrophoresis on a 4 % polyacrylamide gel. The gel is dried and then exposed to film (Kodak XAR5). The gel shows two or three bands, belonging to free

**Preparation of radiolabelled oligo:**

- 5 μl oligo (Gibco, made to specification)
- 5 μl 10 x buffer (Promega)
- 1 μl T4 polynucleotide kinase enzyme (Boehringer Mannheim)
- 5 μl γ-[³²P]-ATP (NEN Life science)
- purified water to 50 μl

The oligonucleotide contains the NF-κB binding site found in the HIV long terminal repeat (HIV-1 LTR):

```
5'-AGTTGAGGGGACTTTCCCAGGC-3'
3'-TCAACTCCCCTGAAAGGGTCCG-5'
```

The pre-cooled ingredients are mixed together into one Eppendorf tube, the radioactive ATP is put in second to last, and the polynucleotide kinase enzyme last, which starts the reaction. The mixture is incubated (with warm water 37°C) for 20-30 minutes. The mixture is filtered through P10 (Biorad) to remove free unbound ³²P-ATP. P10 is allowed to swell in Hepes buffer (10 mM pH 7.9) and EDTA (1 mM pH 8) overnight. The filter is prepared by placing silanised glass wool into an eppendorf tube with a hole pierced through the bottom, and adding 1 ml of P10 gel. The mixture is passed through the filter and collected in a separate eppendorf tube by centrifugation (2000 rpm, 1 min).
Preparation of EMSA gel:

5.3 ml Acrylamide
4 ml 5 x Tris boric acid-EDTA (TBE)
31 ml water
300 µl APS (10% solution stored at 4 °C, 2 months)
30 µl TEMED

TEMED is added last to the mixture to initiate polymerisation. The mixture is poured between two glass sheets of the EMSA apparatus, and allowed to set without air bubbles. 0.5 x TBE is used as running buffer.

5 x TBE

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>54 g</td>
</tr>
<tr>
<td>Boric acid</td>
<td>27.5 g</td>
</tr>
<tr>
<td>EDTA (0.5 M)</td>
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</table>

Phosphate buffered saline

<table>
<thead>
<tr>
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<td>NaCl</td>
<td>8 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.44 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.24 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 1 L</td>
</tr>
<tr>
<td>HCl</td>
<td>to adjust pH to 7.4</td>
</tr>
</tbody>
</table>

10.2 IL-6 Luciferase reporter gene method

The luciferase assay is a reporter system used in molecular biology which employs the use of the firefly luciferase gene to determine the level of expression of a transfected gene. In the assay used for this project, HeLa cells are transfected with plasmids containing the interleukin IL-6 promoter fused to the gene which codes for luciferase, an enzyme found in fireflies and glow beetles which produces light by adenosine tri-phosphate (ATP) dependent oxidation of the substrate luciferin. IL-6 is
one of the inflammatory cytokines regulated by NF-kB. On stimulation by an NF-kB activator, the freed NF-kB then binds to the IL-6 promoters and induces the expression of luciferase. The cells are then lysed to release the luciferase. When assayed in the presence of excess luciferin, the light emission is proportional to luciferase concentration, which in turn is proportional to the degree of NF-kB activity.

Cells: HeLa cells stably transfected with the luciferase reporter gene controlled by the IL-6 promoter were grown overnight in 12 well plates.

Preparation of plant extract: Extracts were dissolved in DMSO (10 mg/ml).

Incubation of cells with plant extract: 10 μl of plant extract dissolved in DMSO was added to the medium in the well (1 ml of medium per well) to obtain a final concentration of 100 μg/ml, and incubated for one hour. PMA was then added and the cells were incubated (50 ng/ml final conc) for a further 7 hours.

Positive controls (cells not exposed to plant extracts) and negative controls (unstimulated cells) were used. Each test was prepared in replicates of three.

Parthenolide was used as a control NF-kB inhibitor (results not shown).

Harvesting cells: The medium was removed and the cells washed with PBS and lysed by incubation with lysis buffer [1 M Tris-phosphate 6.25ml adjusted to pH 7.8 with NaH₂PO₄, 1 M DTT 500 μl, 0.5 M CDTA 1 ml, glycerol 25 ml, Triton X-100 (10 %) 2.5 ml, purified water to 50 ml, store at -20 °C] (50 μl per well) for 10 - 15 min at 4 °C. The suspensions were transferred to Eppendorf tubes and centrifuged (13000 rpm, 15 min, 4 °C), and the supernatent tested for luciferase activity.

Luciferase assay reagent

<table>
<thead>
<tr>
<th></th>
<th>Assay buffer</th>
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<tbody>
<tr>
<td>38 ml</td>
<td>Assay buffer</td>
</tr>
<tr>
<td>15.8 mg</td>
<td>CoA</td>
</tr>
<tr>
<td>10 mg</td>
<td>Luciferin</td>
</tr>
<tr>
<td>20.4 mg</td>
<td>ATP</td>
</tr>
<tr>
<td>purified water</td>
<td>to 76 ml</td>
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</tbody>
</table>

Assay buffer

<p>| | |</p>
<table>
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<tr>
<th></th>
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<tbody>
<tr>
<td>717 mg</td>
<td>Tricine</td>
</tr>
<tr>
<td>104 mg</td>
<td>Magnesium carbonate oxide pentahydrate</td>
</tr>
<tr>
<td>40 µl</td>
<td>EDTA (0.5 M) pH 8</td>
</tr>
<tr>
<td>1.03 g</td>
<td>DTT</td>
</tr>
<tr>
<td>purified water</td>
<td>to 100 ml</td>
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</tbody>
</table>

Results are shown in bar graphs as mean ± S.D.

11 Antibacterial assays

Antibacterial broth dilution minimum inhibitory concentration assay

*Staphylococcus aureus* strain XU212 (Tet(K) efflux system, tetracycline resistant) was cultured from clinical isolates from the Adnan hospital (Kuwait). Strain RN 4220 (Msr(A) efflux system, macrolide resistant) was provided by Dr. Jon Cove. Strain 1199-B (Nor(A) efflux system, norfloxacin resistant) was provided by Dr. Glenn W. Kaatz. Bacteria were subcultured on nutrient agar (Oxoid) 24 hours prior to assay.

Mueller-Hinton broth (MHB): (Oxoid) adjusted to contain 20 mg/ml and 10 mg/ml of Ca\(^{2+}\) and Mg\(^{2+}\) respectively.

Preparation of test compound solution: Test and antibiotic compounds were dissolved in DMSO and diluted out in MHB to give a final concentration of DMSO of 3.125 %.

Each test was performed in duplicate.

Preparation of inoculum: An inoculum density of 5 x 10\(^5\) colony forming units (cfu) of each test organism was prepared in normal saline (0.9 g/L). The McFarland...
Nephalo standard was used to obtain an inoculum suspension of density $5 \times 10^8$ cfu, which was diluted down to obtain $5 \times 10^5$ cfu.

Controls and standards: Assays using standard antibiotics (tetracycline, erythromycin and norfloxacin), DMSO, growth and sterile controls were run in parallel.

The assay is set up in a 96 well plate. 125 μl MHB is dispensed into 11 wells, and 125 μl of compound solution serially diluted two fold into 10 of the wells, before being deposited into well 12. 125 μl of inoculum is added to wells 1 - 11 and the plate incubated (36°C) for 18 hours. Minimum inhibitory concentration (MIC) is recorded as the lowest concentration at which no growth is observed. Well 12 is used for the sterile growth control, where no inoculum is added, well 11 for growth control, where the test compound is not added (Gibbons, Oluwatuyi, et al. 2003).

The addition of 20 μl of MTT solution (5 mg/ml in purified water, freshly prepared) is added to each well to aid visualisation, where the presence of bacteria can be clearly observed by the formation of dark blue formazan crystals.

12 Cytotoxicity assays

MTT reduction assay

MCF-7 breast cancer cells were seeded (1000 cells/well) in 96 well plates (medium: MEMα with Glutamax-1 supplemented with 5 % FCS - both from GibCo) and allowed to attach for 48 hours (at 5 % CO₂, 37 °C) before treatment.
Test compound solution (300 µg/ml) was prepared by dissolving in medium with 0.5 % v/v DMSO and allowed to solubilize overnight under refrigeration before filter sterilisation.

The cells were treated in replicates of 6 with the test compound at a series of different concentrations ranging from 1 - 100 µg/ml and incubated for 72 hours (final volume of 150 µl per well). Only the central wells were used, the wells along the edges of the plate were not used due to evaporation effects.

Blanks (wells containing no cells) and controls (untreated cells) were run in parallel. 20 µl of MTT solution (5 mg/ml in PBS) was added to each well and incubated for 3 hours. 100 µl of MTT solubilisation solution (500 ml H2O, 200 g SDS, 500 ml dimethylformamide, 20 ml glacial acetic acid, 10 ml 2M HCl) was added to each well and incubated overnight.

Optical density of each well was measured at 570 nm with a plate reader (Labsystems Multiskan Multisoft). The mean optical density obtained for the blanks was subtracted from the readings obtained, and cell viability in each well expressed as a percentage of the mean optical density of the controls. Cell viability was plotted against log concentration, and the IC50 obtained using linear regression. The results are given as the mean and standard deviations of IC50s obtained from 2-3 experiments.

Experiments for the isolates were repeated at least three times. ‘SigmaPlot’ software was used to obtain IC50s for each experiment.

MCF-7 cells were grown in T25 cell culture flasks, and were grown and split following standard protocol. Hank’s buffered salt solution (Gibco) was used for washing the cells. Cells were discarded after ten passages.
CHAPTER THREE: RESULTS

13  

OCHNA MACROCALYX

13.1 PHYTOCHEMISTRY

13.1.1 EXTRACTION OF BARK

50 g of the powdered bark was refluxed in 500 ml of 96 % ethanol for 30 min. The ethanol extract was filtered, and the remaining insoluble plant material refluxed twice more using 70 % ethanol. The ethanol extracts were combined, the ethanol removed using the rotary evaporator, and the remaining water removed by freeze drying, to obtain approximately 18 g of crude bark extract.

13.1.2 FRACTIONATION OF THE CRUDE EXTRACT

The crude bark extract was suspended in water, and partitioned first with hexane, and then with ethyl acetate. The majority of the extract went into the ethyl acetate, when dried the ethyl acetate extract was approximately 15 g. 26 mg was obtained for the hexane extract, and 1 g for the water extract. The ethyl acetate extract was fractionated using 300 g of Sephadex LH-20 in methanol to obtain 9 fractions (OM 1-9).

A second crude extract was obtained later on in the project, again via reflux with EtOH, but this time was fractionated with VLC without partitioning.
Fig. 10 TLC (silica) of *Ochna macrocalyx* extracts and fractions

Sephadex-LH20 fractions 'OM'1-9

Solvent system: DCM/MeOH/H$_2$O 80:20:2
C: crude ethanolic bark extract
E: ethyl acetate extract

VLC fractions of crude extract

Solvent system: DCM/MeOH/H$_2$O 90:10:1
13.1.3 ISOLATION AND IDENTIFICATION OF COMPOUNDS

TLC analysis of the crude extract using DC:MeOH:H₂O 80:20:2 showed the presence of two major orange and yellow compounds. UV showed the presence of a number of UV active compounds, and spraying showed the compounds up in strong orange, red, pink, yellow and brown colours, characteristic of flavanoidal type compounds (Fig. 10). During phytochemical investigations, all compounds that were isolated or partially isolated were flavanoidal or biflavonoidal, whose ¹H NMR spectra are all very characteristic and similar. NMR spectra were generally simple with few overlapping signals, with mostly strong characteristic aromatic peaks depicting several ring systems.

The compounds calodenin B, dihydrocalodenin B and ochnone isolated in this study were major constituents of the extract, whilst hexaspermone C and dehydroxyhexaspermone C (Fig. 12) were minor, and were all easily visualised. Calodenin B and dihydrocalodenin B are orange and yellow respectively, and are the main compounds responsible for the colouring of the bark. The chalcone dimers all had strong UV activity, under both short wave and long wave, where they presented as dark spots. Hexaspermone and dehydroxyhexaspermone C also had strong UV activity under short wave, but not under long wave UV light.

The presence of the isolated compounds is consistent with previously published work performed on other members of the Ochnaceae, which show that the family produces biflavonoids and related chalcones.
Fig. 11 *Ochna macrocalyx* isolates

Calodenin B

2,3-Dihydrocalodenin B

Ochnone

Cordigol

Isoflavanone subunit fragments from DHHC and HC

<table>
<thead>
<tr>
<th>R</th>
<th>m/z</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>568</td>
<td>Dehydroxyhexaspermone C</td>
</tr>
<tr>
<td>OH</td>
<td>584</td>
<td>Hexaspermone C</td>
</tr>
<tr>
<td>H</td>
<td>283</td>
<td>1b</td>
</tr>
<tr>
<td>OH</td>
<td>299</td>
<td>2b</td>
</tr>
</tbody>
</table>
Isolation scheme used for *Ochna macrocalyx*

- **Bark**
  - Refluxed 1x with 96% and 2x with 70% EtOH
  - **Crude extract**
    - Hexane extract
    - Partitioning
      - Aqueous extract
    - **Ethylacetate extract**
      - Sephadex-LH 20 fractionation in MeOH
        - 9 fractions OM1-9
          - Biotage flash 75i
          - PTLC and HPLC
            - DHCB
            - CB
            - Compound X
          - 9 fractions (5.1-9)
            - PTLC, HPLC
          - Biotage flash 40i
            - PTLC and HPLC
              - DHHC and HC
              - Ochnone and cordigol
            - 7 fractions (5.3.1-7)
**Fig 13** TLCs of *Ochna macrocalyx* fractions and compounds

Sephadex fractions OM5-8

Solvent system:
- DCM/MeOH/H\textsubscript{2}O 90:10:1 developed 4 times
- E: Ethyl acetate extract

Biotage flash fractions of OM5.3.1-7
solvent system: DCM/MeOH/H\textsubscript{2}O 90:10:1
13.1.3.1 Calodenin B

Calodenin B (135 mg) was isolated from fractions 7-9 (Fig. 13). Strong orange in colour, isolation was straightforward using preparative TLC (dichloromethane:methanol:H₂O 80:20:2, Rf 0.46). The compound is highly soluble in methanol, and less soluble in ethyl acetate and acetone.

FABMS gave a molecular weight of 524 ([M+H]⁺), consistent with C₃₀H₂₀O₉, a biflavonoid C₃₀ structure. Accurate mass measured: 525.116500, calculated mass of C₃₀H₂₁O₉: 525.118558.

Only a ¹H NMR spectrum in acetone-d₆ was required for identification (Table 9, Fig. 15). Mainly aromatic signals were present, two AA'BB' (δ 7.76, 7.00 and 7.65, 6.94), one ABX (δ 6.24 (dd, J = 2, 9 Hz), 6.39 (d, J = 2 Hz), 7.46 (d, J = 9 Hz), and a singlet (δ 6.27). Two strongly coupled doublets could be seen downfield (δ 7.94 (J = 15 Hz), 8.30 (J = 15 Hz)), indicative of trans coupled protons. One proton could also be seen far downstream at δ 14.33, indicative of a chelated hydroxy group.

Identification was by comparison with previously published spectral data. The orange colouring is due to its highly conjugated system, which also makes it relatively stable, contributing to its ease of isolation.
Table 8. References containing literature data on compounds isolated from *Ochna macrocalyx*.

<table>
<thead>
<tr>
<th>Isolated compound</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-dihydrocalodenin B</td>
<td>Drewes, Hudson, et al. 1987; Marston, Zagorski, et al. 1988</td>
</tr>
<tr>
<td>cordigol</td>
<td>Marston, Zagorski, et al. 1988</td>
</tr>
<tr>
<td>hexaspermone C</td>
<td>Moreira, Sobrinho, et al. 1994</td>
</tr>
</tbody>
</table>

13.1.3.2 Dihydro-calodenin B

Dihydrocalodenin B (DHCB) (320 mg) was isolated from fractions 6-7 (Fig. 13). Strong yellow in colour, isolation required multiple developments on PTLC using dichloromethane:methanol:H$_2$O 90:10:1, a further step using multiple development on analytical TLC using hexane:propan-2-ol 8:2, and final purification using RP-C18 semi-prep HPLC: isocratic, 55 % MeOH in H$_2$O, 5 ml/min, approximately 80 min. required for both compounds to elute. The compound is highly soluble in MeOH, but ethyl acetate was used preferentially, as DHCB has two chiral centres, one of which is next to a carbonyl group and therefore may enolise and isomerise in MeOH due to the proteolytic nature of the solvent.

FABMS gave a molecular weight of 526 (527 [M+H]$^+$), consistent with C$_{30}$H$_{22}$O$_9$. Accurate mass measured: 527.135900, calculated mass of C$_{30}$H$_{23}$O$_9$: 527.134208.
The $^1$H NMR spectrum in acetone-$d_6$ was similar to that of calodenin B (Table 9, Fig. 15), with two AA'BB' ($\delta$ 6.89, 7.53 and 6.94, 7.41), one ABX ($\delta$ 6.43 ($dd, J = 2.4$, 8.5 Hz), 6.40 ($d, J = 2.4$ Hz), 7.81 ($d, J = 8.5$ Hz), a singlet ($\delta$ 6.00), two trans coupled protons ($\delta$ 7.83 ($J = 15.6$ Hz), 8.05 ($J = 15.6$ Hz)), and two chelated hydroxyl groups ($\delta$ 12.02, 14.20). In addition, there is also the presence of two coupled doublets ($\delta$ 5.35, 6.10) with $J = 6.0$. Identification was through comparison with literature data. As the molecular formula of DHCB has two more protons than calodenin B, it could be deduced that DHCB only differs from calodenin B by the saturation of one of the two double bonds that are present in calodenin B. The trans coupled protons of the double bond at positions 2" and 3" are present in both spectra, therefore the two additional protons must be present at positions 2 and 3 on the furan ring in DHCB. Thus DHCB has a reduced conjugation system in comparison to calodenin B, giving it its lighter yellow colour, and also making it relatively less stable, enabling it to form isomers via enolisation. Whereas the isolation of calodenin B was straightforward, several isolation steps were required to obtain DHCB in a pure state. Also the molecular ion of DHCB could only be obtained via FAB (Drewes, Hudson, et al. 1987) or electron spray, which are softer ionisation methods in comparison with EI.
Table 9 1-D $^1$H NMR data (500 MHz, acetone-d$_6$) obtained for calodenin B and 2,3-dihydrocalodenin B, and comparison with literature data.

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<tr>
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<th>2,3-dihydrocalodenin B</th>
<th>Reference data**</th>
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</tr>
</tbody>
</table>

Chemical shift in ppm (integration, multiplicity, and coupling constant).

references:
* Messanga, Tih, et al. 1994
** Drewes, Hudson, et al. 1987
a data missing in reference.
Fig. 14 $^1$H NMR spectra for calodenin B and dihydrocalodenin B in acetone-d6 (400MHz)
Ochnone (180 mg), a white, colourless solid, was present as a major component in fraction 5, which was visible under short wave (254 nm) UV light, and showed as a dark spot under long wave UV light. Fraction 5 was further fractionated with Biotage flash 75i using a gradient system starting from 100% hexane and moving up to 100% ethyl acetate, resulting in 9 fractions (5.1-9). Fraction 5.3 was fractionated again with Biotage flash 40i, using isocratic DCM:MeOH:H₂O 86:13:1, obtaining another 7 fractions (5.3.1-5.3.7) (Fig. 12). Ochnone and cordigol were isolated from 5.3.5-7.

Using DCM:MeOH:H₂O 90:10:1 on PTLC, ochnone was isolated together with cordigol, both having very similar R_f values. Separation of the two compounds required multiple developments on analytical TLC plates using chloroform:butan-2-ol 8:2 (R_f ochnone: 0.5, orange when sprayed, R_f cordigol: 0.4 pink when sprayed, Fig. 13). Cordigol was present as a minor component in the NMR spectra used to elucidate ochnone. The quality and high resolution of the spectra, enabled the identification of cordigol. Only trace amounts of pure cordigol were obtained, not sufficient to enable biological testing.

The FABMS of ochnone gave a molecular ion at 528 (551 [M+Na])^+, consistent with C_{30}H_{24}O_9. The 'H NMR spectrum (Table 10, Fig. 16) showed mostly aromatic signals, with two AA'BB' (δ_H 6.61, 7.22 and 6.76, 7.69) and two ABX (δ_H 6.10, 6.32,
7.75 and 6.32, 6.38 and 7.35) ring systems. There were also four aliphatic protons ($\delta_H$ 4.93, 4.99, 5.37, 5.56) and two deshielded hydroxyl groups ($\delta_H$ 8.26 and 12.36) indicative of H-bonding to a carbonyl group. $^{13}$C-NMR data showed thirty carbons, most of which were in the aromatic region, 6 were oxygen bearing quaternary carbons and two were carbonyls ($\delta_C$ 197.7 and 203.7). $^1$H-$^1$H COSY, $^1$H-$^{13}$C HMQC and $^1$H-$^{13}$C HMBC enabled the assignment of the carbons and protons. Structure elucidation of ochnone was straightforward, the four ring system fragments were easily deduced, followed by the tetrohydrofuran fragment. Stereochemistry of the protons at positions 2-5 were deduced from $^1$H-$^1$H NOESY data (Fig. 15).

The $^1$H and $^{13}$C NMR spectra of cordigol (Table 11, Fig. 16) showed two AA'BB' ($\delta_H$ 6.83, 7.21 and 6.85, 7.29), one ABX ($\delta_H$ 5.95 ($dd$, $J = 2.4$, 8.9 Hz), 6.25 ($d$, $J = 2.4$ Hz), 6.29 ($d$, $J = 8.9$ Hz) and one meta coupled system ($\delta_H$ 5.98 ($d$, $J = 2.9$ Hz), 6.15 ($d$, $J = 2.9$ Hz)). Five aliphatic protons were present at $\delta_H$ 2.85 ($ddd$), 3.58 ($dd$), 4.81 ($d$), 5.14 ($d$), 5.17 ($d$), indicative of a CH-CH(CH)-CH-CH structure. One chelated hydroxyl group ($\delta_H$ 12.90) and one carbonyl group ($\delta_C$ 204.8) could also be seen. After elucidation of the structure it was found to be the same as that for cordigol, with data very similar to that previously published (Table 11) (Marston, Zagorski, et al. 1988).

![Figure 15 Selected NOE connectivities for ochnone](image-url)
Table 10

$^1$H (400MHz) and $^{13}$C NMR data of ochnone obtained by application of 2-D experiments COSY, HMQC and HMBC (acetone-d$_6$).

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<th>position</th>
<th>$\delta_C$</th>
<th>jmod</th>
<th>$\delta_H$</th>
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![Ochnone structural diagram](image-url)
Table 11  NMR data obtained for *cordigol* (400 MHz), and comparison with literature data.

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$^{a,b,c}$ signals may be interchanged.
Fig. 16 $^1$H NMR spectra for ochnone and cordigol in acetone-d$_6$ (400 MHz)
13.1.3.4 **Hexaspermone C and dehydroxyhexaspermone C**

Hexaspermone C (HC) (20 mg) and dehydroxyhexaspermone C (DHHC) (30 mg) (both white/colourless solids) were obtained from fraction 5.1, 5.2 and 5.3.1 (Fig. 12). More was also obtained from a second crude extract which was fractionated using VLC (Fig. 10). With very similar $R_f$ values, the two compounds were isolated together on TLC (multiple development) using chloroform ($R_f$ dehydroxyhexaspermone C: 0.06, $R_f$ hexaspermone C: 0.08, both red when sprayed), and then separated using semi-prep RP-C18 HPLC, isocratic, 55% acetonitrile in H$_2$O, 5 ml/min, 80 min. Analytical RP-C18 HPLC isocratic 55% acetonitrile in H$_2$O, 2 ml/min, $R_t$ DHHC: 16 min, $R_t$ HC: 22 min.

HC and DHHC are both isoflavone dimers, or bi-isoflavonoids. Both are composed of two isoflavone subunits joined together through a 2-2" link. HC was identified as a compound previously isolated from *Ouratea hexasperma* (Ochnaceae) by comparison of spectral data (Moreira, Sobrinho, et al. 1994). HC is an almost symmetrical compound, its two isoflavone subunits only differing at positions 4"" and 4'. The $^1$H NMR spectrum in CDCl$_3$ had a simplified appearance with many of the proton signals superimposed due to its high degree of symmetry. The spectrum showed two AA'BB' ring systems ($\delta_H$ 6.80, 7.33 and 6.87, 7.38), two
meta coupled systems ($\delta_H$ 6.13 (2H) and 6.15 (2H)), two chelated hydroxyls ($\delta_H$ 11.93 (2H)), and two of the methoxyl groups (position 7 and 7") at $\delta_H$ 3.76 with the third methoxyl at $\delta_H$ 3.80. Four aliphatic protons showed signals at $\delta_H$ 4.51 (m, 2H), 5.68 ($d, J = 10$ Hz) and 5.69 ($d, J = 10$ Hz). The $^{13}$C NMR spectrum showed the presence of two carbonyl groups at $\delta_C$ 198.2 (2C). FABMS gave a molecular ion of 584 (585 [M+H]$^+$), consistent with C$_{33}$H$_{28}$O$_{10}$. Accurate mass measured: 585.183000, calculated mass of C$_{33}$H$_{29}$O$_{10}$ : 585.176073. EIMS m/z 417, 300, 299, 286, 285, 252, 167, 121. UV $\lambda_{max}$ 220.4, 298.2 nm.

The $^{13}$C and $^1$H NMR spectra for DHHC was similar to HC. The spectra (in acetone-d$_6$) showed the presence of three methoxyl groups ($\delta_C$ 55.5, 56.2, 56.3, $\delta_H$ 3.77, 3.81, and 3.83), two carbonyl groups ($\delta_C$ 194.6 and 200.1), four aliphatic protons ($\delta_H$ 4.58, 4.67, 5.62 and 5.68), one chelated hydroxyl group (visible with CDCl$_3$) at $\delta_H$ 11.91 and four ring systems, two AA'BB' ($\delta_H$ 6.82, 7.39 and 6.89, 7.42), one ABX ($\delta_H$ 6.62, 6.71 and 7.58) and one meta coupled system ($\delta_H$ 6.12 and 6.17). FABMS of DHHC gave a molecular ion at 568 (569 [M+H]$^+$) consistent with C$_{33}$H$_{28}$O$_9$. Accurate mass measured: 569.181900, calculated mass of C$_{33}$H$_{29}$O$_9$ : 569.181158. EIMS m/z 417, 286, 285, 284, 283, 252, 167, 151, 135, 121, 107. UV $\lambda_{max}$ 218.1, 295.8 nm. $^1$H-$^1$H COSY and $^1$H-$^{13}$C HMQC spectra in acetone-d$_6$ were obtained, however $^1$H-$^{13}$C HMBC spectra could not be obtained as the compound later decomposed in the acetone-d$_6$.

Due to DHHC and HC differing only by an additional oxygen in their molecular formula, and the similarity of their $^1$H and $^{13}$C spectra, this led to the deduction that the two differ only by the absence of one OH group in DHHC. In addition only one
chelated hydroxyl group could be seen in the $^1$H NMR spectrum for DHHC. The position of this OH group was established using EIMS, where the compounds fragment into their isoflavanone subunits. The EIMS for DHHC showed two major signals at m/z 283 (1b) and 285, for HC the signals occurred at 285 and 299 (2b). The signal at m/z 285 consistent with C$_{16}$H$_{13}$O$_5$ corresponds to the isoflavanone fragment 1a/2a, which remains the same for both compounds. This then led to the deduction that the OH group is absent from position 5 in DHHC, giving rise to the ABX system observed in the $^1$H NMR spectrum. Although it may be possible for the methoxyl group to be in position 6 instead of 7, this was rejected on biosynthetic grounds as being highly unlikely.

Structures drawn show the isoflavanone subunits in their reduced forms, which correspond to the m/z of the signals seen in the EIMS. Assignments of the carbons and protons for DHHC (Table 12) were based on HMQC data and by similarity with HC.

The J-values between H3-H2, H2-H2", H2"-H3" for both compounds were 10.8, 8.0, and 10.8 Hz, respectively, consistent with three trans linkages, making the stereochemistry of the two isoflavanone subunits of both compounds the same.
In contrast to the assignments in (Moreira, Sobrinho, et al. 1994), $^1\text{H}$ signals at $\delta_\text{H} 4.51-4.67$ were assigned to positions 2 and 2" because of the multiplicity of the signals as two clear double doublets in particular in the spectra for DHHC (Table 12). $^1\text{H}$ signals at $\delta_\text{H} 5.6 - 5.7$ as doublets were thus assigned to positions 3 and 3", although HMQC showed them to be attached to carbons at $\delta_\text{C} 84.5 - 86.4$, which would seem further downfield than expected.
Table 12

$^1$H and $^{13}$C NMR (400 MHz) spectral data obtained for dehydroxy hexaspernone C (acetone-d$_6$) and hexaspernone C (CDCl$_3$) and comparison with literature data (200 MHz)* for hexaspernone C (CDCl$_3$). Chemical shifts ($\delta$) measured in ppm, coupling constants ($J$) in Hz.

<table>
<thead>
<tr>
<th>NMR data obtained</th>
<th>Literature data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydroxy hexaspernone C</td>
<td>Hexaspernone C</td>
</tr>
<tr>
<td>$\delta_C$</td>
<td>$\delta_H$ (m, J)</td>
</tr>
<tr>
<td>$\delta_C$</td>
<td>$\delta_H$ (m, J)</td>
</tr>
<tr>
<td>2</td>
<td>59.9$^a$</td>
</tr>
<tr>
<td>3</td>
<td>86.2$^b$</td>
</tr>
<tr>
<td>4</td>
<td>194.6$^c$</td>
</tr>
<tr>
<td>4a</td>
<td>109.2$^d$</td>
</tr>
<tr>
<td>5</td>
<td>131.7</td>
</tr>
<tr>
<td>6</td>
<td>111.6</td>
</tr>
<tr>
<td>7</td>
<td>166.7$^e$</td>
</tr>
<tr>
<td>8</td>
<td>105.7</td>
</tr>
<tr>
<td>8a</td>
<td>165.9</td>
</tr>
<tr>
<td>1'</td>
<td>122.5</td>
</tr>
<tr>
<td>2', 6'</td>
<td>129.2$^f$</td>
</tr>
<tr>
<td>3'/5'</td>
<td>114.6$^g$</td>
</tr>
<tr>
<td>4'</td>
<td>160.7$^h$</td>
</tr>
<tr>
<td>2''</td>
<td>60.4$^a$</td>
</tr>
<tr>
<td>3''</td>
<td>86.4$^b$</td>
</tr>
<tr>
<td>4''</td>
<td>200.1$^i$</td>
</tr>
<tr>
<td>4a''</td>
<td>109.3$^j$</td>
</tr>
<tr>
<td>5''</td>
<td>164.9</td>
</tr>
<tr>
<td>6''</td>
<td>97.3</td>
</tr>
<tr>
<td>7''</td>
<td>167.5$^k$</td>
</tr>
<tr>
<td>8''</td>
<td>99.4</td>
</tr>
<tr>
<td>8a''</td>
<td>166.0</td>
</tr>
<tr>
<td>1'''</td>
<td>132.7</td>
</tr>
<tr>
<td>2''/6''</td>
<td>129.6$^l$</td>
</tr>
<tr>
<td>3''/5''</td>
<td>116.0$^m$</td>
</tr>
<tr>
<td>4''</td>
<td>158.5$^n$</td>
</tr>
<tr>
<td>7-OMe</td>
<td>55.5</td>
</tr>
<tr>
<td>7'-OMe</td>
<td>56.2</td>
</tr>
<tr>
<td>4'-OMe</td>
<td>56.3</td>
</tr>
<tr>
<td>5''-OH</td>
<td>11.91 (s)**</td>
</tr>
<tr>
<td>5-OH</td>
<td>11.93 (s)</td>
</tr>
</tbody>
</table>

* Moreira, Sobrinho, et al. 1994

** visible with CDCl$_3$.

a, b, c, d, e, f, g, h, i, j signals may be interchangeable.

\[ R = \text{MeO} \quad \text{hexaspernone C} \]
\[ R = \text{H} \quad \text{dehydroxyhexaspernone C} \]
Fig. 17 $^1$H NMR spectra for hexaspermone C and dehydroxy-hexaspermone C in CDCl$_3$ (500 MHz)

Hexaspermone C

Dehydroxyhexaspermone C
13.1.3.5 **Unidentified compound (‘X’)\**

The TLC also showed the presence of a major component with a lower $R_f$ of 0.15 (DC:MeOH:H$_2$O 80:20:2), also with strong UV activity, which turns pink when sprayed (Fig. 13). Isolation on PTLC produced small quantities of compound, which initially under NMR looked very impure. The low yield was probably due to the polarity of the compound, which was reflected in the low $R_f$, suggesting that a lot of the compound was unrecoverable from silica. Analysis using RP-C18 HPLC showed the presence of one other compound. Although the two compounds showed as two distinct peaks on the analytical column and were fairly well separated, they were too close together for separation on the semi-prep column. In addition the peaks produced were broad and showed slight tailing. I was unable to find a solvent system that could purify the compound sufficiently using HPLC, so in the end purification was performed using analytical reverse-phase silica plates and scraping off the bands, but the bands were still close. Only 5 mg of the two compounds were obtained in the end, and even then the $^1$H NMR spectra were very complex, despite the purity of the compounds as visualised on TLC (Fig. 13) and HPLC (Fig. 18). No further NMR analysis was undertaken, as 5 mg was insufficient for full spectral experiments to be performed. The NMR spectra showed an abundance of overlapping signals in the aromatic region ($\delta$ 5.5-8.0) as well as between $\delta$ 4.0 and 5.5, which may indicate the presence of sugars. In addition a few signals consistent with chelated hydroxyl groups, which were another characteristic of the isolated biflavonoids, could be seen far down field at $\delta$ 12 - 13. The complexity of the spectra possibly suggests a tri- or tetra-flavonoid. Mass spectral data obtained using electron spray was inconclusive, in $^+$ve ion mode ([M+H]$^+$) the largest ion was seen at 633, but atleast 3 other major
peaks were present at 288, 375, and 537. The presence of the other peaks also suggests impurities, as fragmentation is unlikely with ES. A small number of tri/tetra-flavonoids have been isolated from other members of the Ochnaceae, with MWs ranging from 700-1000. The complexity of the $^1$H NMR spectra also indicates the presence of impurities, although this contrasts with the purity of the compound as seen on TLC and HPLC.
Fig. 18  $^1$H NMR spectrum for compound X in acetone-d$_6$ (500 MHz)

HPLC trace and UV spectrum

Analytical HPLC trace for X.
47 % MeOH in H$_2$O, 0.5 ml/min.
13.2 NF-κB results for Ochna macrocalyx

The crude ethanolic bark extract and subfractions (obtained by Sephadex separation) which later yielded the isolated compounds were tested and found to have inhibitory activity at 200 μg/ml using EMSA (Fig. 21). The crude and ethyl acetate extract were tested using the IL-6 luciferase reporter gene assay at 100 μg/ml, and reduced NF-κB activity to 42 % ± 8 % and 30 % ± 9 % (mean and S.D. of 9 replicates obtained from three experiments), respectively (Fig. 20). Some cytotoxicity in HeLa cells was observed in most of the extracts, which was more evident in CB and DHCB. CB and DHCB were observed to show cytotoxicity towards the HeLa cells at concentrations higher than 20 and 95 μM respectively. Both were tested at reduced concentrations where cytotoxicity was removed, but along with the other isolated compounds showed no NF-κB inhibitory effects in the IL-6 luciferase assay (Fig. 19).

Figure 19  NF-κB IL-6 luciferase results for Ochna macrocalyx compounds

* 100 % cytotoxicity
** some cytotoxicity observed
**Figure 20**  IL-6 Luciferase assay results for *Ochna macrocalyx* extracts

- Sephadex fractions and crude extracts (TNF-α, 200 µg/ml)
- Sephadex fractions OM6-8 (TNF-α, 50 and 10 µg/ml)

Results of luciferase assay for crude and ethyl acetate extract (PMA)
Fig. 21 Results of EMSA for *Ochna macrocalyx* fractions OM 5-9 (200 µg/ml)

<table>
<thead>
<tr>
<th>OM fractions</th>
<th>controls</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>origin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- unstimulated cells
+ cells stimulated with PMA

NF-κB – radiolabelled oligo complex
Radiolabelled oligo – excess protein complex
Unbound oligo
The preliminary screening assays used high concentrations of extracts of 200 µg/ml, and cytotoxicity was observed for the ‘active’ fractions at this concentration. Thus the inhibition seen could simply be due to toxic effects, which would explain why continued work in these fractions led to the isolation of compounds which did not have activity. However, other fractions and plant extracts were also tested at 200 µg/ml (Fig. 20), with no cytotoxicity nor NF-κB inhibitory activity recorded. Some extracts also had some toxicity with no significant NF-κB inhibitory effects.

One of the effects of inhibiting NF-κB is apoptosis, which is programmed cell death, as NF-κB is known to regulate the expression of anti-apoptotic genes. Determining whether the cells were dying from apoptosis or from toxic effects of the extract requires a different set of experiments, the facilities for which were not available on site.

Further bioassay guided fractionation was not possible at the time as the facilities were not readily available to us - the preliminary assays were performed at a different location. Hence only the main compounds whose isolation was straightforward from these fractions were obtained.

The screening procedure which is currently in place at the research centre tests crude plant extracts at 100 µg/ml using the luciferase assay, and ideally only extracts which reduce NF-κB activity down to less than 20 % are investigated further. In contrast the crude extract of Ochna macrocalyx at 100 µg/ml only reduced NF-κB activity to 40 %, its selection was based on screening using EMSA and testing at 200 µg/ml.

However this luciferase assay result is still significant, in comparison the preliminary data obtained for the crude ethanolic (‘total’) extract of Bupleurum fruticosum only reduced NF-κB activity to 60 %.
After the completion of the project and during the writing of this thesis, CB was re-tested using HeLa cells transfected with luciferase genes controlled by a different promoter construct containing glucocorticoid responsive elements ((GRE)2-2088-(kB)3-luc; (Hofmann & Schmitz 2002)). In this system (using TNF-α) CB was found to have activity at 20 μM with no cytotoxicity, reducing NF-κB activity to approximately 10 %, with an IC$_{50}$ of roughly 10 μM (Fig. 22). This result suggests that CB may have some NF-κB inhibitory activity, although more assays are required to obtain more accurate values, as the positive controls showed some variability. The other OM compounds were not tested in this system.

Figure 22  (GRE)2-20880(kB)3-luc NF-κB luciferase assay results for CB
13.3 Antibacterial results

Antibacterial activity against the three strains of multi-drug resistant *Staphylococcus aureus* was found for CB and DHCB, the minimum inhibitory concentrations (MIC) of which are presented in Table 13, alongside those for control antibiotics. HC and DHHC showed no activity at 512 µg/ml and ochnone showed an MIC of 128 µg/ml for the three strains. DHCB showed good activity, with an MIC of 8 µg/ml (15 µM) for the three strains.

CB showed good activity against one strain, with mild to moderate activity against two others. CB and DHCB are structurally similar, differing only in the presence of one double bond at position 2 and 3, and it would seem resulting in improved activity in DHCB in two of the mdr SA strains (RN 4220 and 1199-B). The strong antibacterial activity of DHCB found in this study may merit its further investigation.

The crude bark extract was also tested against a resistant strain of *Escherichia coli*, which is known to cause gastrointestinal problems especially diarrhoea, one of the traditional uses of the bark, but was found to have no activity against this organism. It may be worth seeing whether the extract has activity against standard wild type *E. coli* to see if the results support its traditional use.

### Table 13. Minimum inhibitory concentrations of test compounds

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>CB (µg/ml)</th>
<th>DHCB</th>
<th>Erythromycin</th>
<th>Tetracycline</th>
<th>Norfloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN4220</td>
<td>64</td>
<td>8</td>
<td>128</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>XU212</td>
<td>8</td>
<td>8</td>
<td>-</td>
<td>128</td>
<td>-</td>
</tr>
<tr>
<td>1199-B</td>
<td>16</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>32</td>
</tr>
</tbody>
</table>
13.4 Cytotoxicity assays

Cell proliferation

The main aim of the MTT assays was to assess the cytotoxicity profiles of compounds from *Ochna macrocalyx*. Some cell proliferation was also observed in OM6 and ochnone, although this was not examined in much detail and so the results are inconclusive. However, the results are worth including, as one of the traditional uses of *Ochna macrocalyx* reported was fertility problems, and a speculative link between the isolated compounds and phytoestrogens was also suggested in (Tang et al. 2003).

Preliminary MTT assays were performed on one of the crude fractions (fraction 6 'OM6') obtained from the original Sephadex LH-20 fractionation of the crude extract, from which DHCB as the major component was isolated from. A range of concentrations (obtained by two fold dilutions) were used ranging from 50 μg/ml down to 0.5 μg/ml.

The first preliminary assay using OM6 was performed using MDA-MB-231 oestrogen receptor negative breast cancer cells (Fig. 23). The assay was set up using two plates, so that cell viability could be measured at 72 hours and 120 hours after treatment. At 72 hours, it was observed that whilst at the lower concentrations below about 5 μg/ml, the cell viability was normal in relation to the controls, between 7 μg/ml and 25 μg/ml there appeared to be cell proliferation, where cell numbers seem to be almost 50 % more than the controls at 25 μg/ml. At 50 μg/ml there was total cell death, showing a very steep cytotoxicity curve. At 120 hours, significant cell proliferation, almost 50 % more cell numbers than the controls, could be seen at concentrations up to 25 μg/ml.
**Fig. 23** MTT assay results for 'OM6' using MDA-MB-231 cells

Assay was performed once, each concentration was performed in replicates of 6, and each point displayed above.

---

**Fig 24.** MTT assay results of 'OM6' using MCF-7 cells

Assay was performed four times, each concentration was performed in replicates of 6, all points displayed above.
However, it is not possible to properly quantify an exact percentage proliferation from these results, as the relationship between cell number and absorption measured by the spectrophotometer is not linear above a certain number of cells, which was exceeded in this assay. This assay was only performed once. Further assays to obtain cytotoxicity curves for OM6 used MCF-7 oestrogen receptor positive breast cancer cells. With the MCF-7 cells, slight cell proliferation could be seen (Fig. 24), but not as significantly in comparison to that seen with the MDA-MB-231 cells. As the aim of the assays was to measure toxicity, the observed proliferative activity was investigated no further, as we moved on to concentrate on cytotoxicity curves.

Slight proliferation could be seen in ochnone (Fig. 25) on MCF-7 cells, whose structure may be comparable to the phytoestrogen lignans. But the results are inconclusive, and thus any link speculative.

Cytotoxicity

CB showed cytotoxic activity against MCF-7 breast cancer cells, and DHCB showed moderate toxicity (Table 14). It is again interesting to see that although CB and DHCB are structurally similar, CB is significantly more toxic. Ochnone exhibited mild cytotoxicity, and also produced a particularly steep dose-response curve, similar to the one for OM6, reducing cell viability from 100% at 42 ± 7 μM to 10% at 78 ± 3 μM.

The steep dose-kill curve of ochnone and OM6 is notably unusual, although it is not possible to say what the cause of this is (Fig. 25). It does suggest that possibly a narrow therapeutic window may be an issue, and that care may be required in its use,
however it is difficult to relate the in vitro data to its use in traditional medicine. HC and DHHc did not show significant toxicity below 100 μM.

Table 14  Results of MTT assays, IC50s obtained.

<table>
<thead>
<tr>
<th>Extract</th>
<th>IC50 (mean and S.D. of 2-3 experiments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ochnone</td>
<td>56 ± 7 μM</td>
</tr>
<tr>
<td>CB</td>
<td>7 ± 0.5 μM</td>
</tr>
<tr>
<td>DHCB</td>
<td>35 ± 7 μM</td>
</tr>
<tr>
<td>crude extract</td>
<td>52 ± 10 μg/ml</td>
</tr>
</tbody>
</table>

Some isoflavonoids and lignans are referred to as phytoestrogens, with known oestrogenic effects (Ingram, Sanders, et al. 1997). Therefore the presence of compounds like calodenin B, ochnone, and other chalcone derivatives in Ochna macrocalyx may account for its use in gynaecological problems by the Washambaa, although this is speculative. Phytoestrogens are also thought to have anti-oestrogenic properties, which may be a possible mode of action for CB and DHCB in the cytotoxicity assay.

Figure 25  MTT assay results for ochnone, CB and DHCB

Cytotoxicity curves obtained for ochnone, calodenin B and dihydrocalodenin B against MCF-7 cells. Each point is shown as the mean ± S.D. obtained from three experiments.
14.1.1 Preliminary small scale crude extractions

The ground up plant material (20 g) was extracted three times at room temperature with petroleum ether, then again three times with ethyl acetate and finally three times with methanol (150 ml of solvent for 15 minutes each time).

A separate 'total' extract was also obtained, where another 20 g of the ground plant material was refluxed with 150 ml of 80 % ethanol in water for 30 min.

The four dried extracts obtained from each solvent were then included in the luciferase assay to test for NF-κB inhibitory activity. The leaves were also extracted separately from the stems and flowers (which were extracted together).

In the luciferase assays, using PMA as the inducer, NF-κB inhibitory activity was found in the leaves' petroleum ether extract, and the stem/flowers' pet. ether and EtOAc extracts, with cytotoxicity observed for all the active extracts. The preliminary stem/flower petroleum ether extract showed strong NF-κB inhibitory activity (with PMA as the stimulator) when tested at 50 μg/ml, which reduced activity to 12 %.

When tested at 100 μg/ml it showed toxicity, and at 25 μg/ml the activity was lost. Conversely, the initial stem/flower ethyl acetate extract when tested at 50 μg/ml did not show significant activity, only reducing activity to 60 %, with some toxicity observable (Fig. 26). However, the stem/flower ethyl acetate extract showed potent inhibitory activity when TNF-α was used as the stimulator, reducing activity to 9 %,
this time with no observable toxicity, and it was this result which led to the further investigation of the *Bupleurum* and the use of ethyl acetate in large scale extraction.

Additional biological data obtained from the other laboratories participating in the EU collaboration was also taken into consideration.

---

**Figure 26 Preliminary extraction of *Bupleurum fruticosum*.**

Protocol for the EU project used in screening plants

Aerial parts

- leaves
- Stems and flowers

- Reflux with 80% EtOH in 'Total' extract
- 'cold' extraction: petroleum ether → EtOAc → MeOH

- Pet. ether extract
- EtOAc extract
- MeOH extract

- 'cold' extraction: petroleum ether → EtOAc → MeOH

Stems and flowers

- Pet. ether extract
- EtOAc extract
- MeOH extract

Extracts showing inhibitory activity in the NF-κB luciferase assays

---

**NF-κB luciferase assay results for the stem/flowers extracts (PMA)**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>+ve controls</th>
<th>-ve controls</th>
<th>Pet. ether</th>
<th>EtOAc</th>
<th>MeOH</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μg/ml</td>
<td>15</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>50 μg/ml</td>
<td>10</td>
<td>5</td>
<td>15</td>
<td>10</td>
<td>5</td>
<td>20</td>
</tr>
</tbody>
</table>

n = 2-4  ** cytotoxic
There were initial concerns about the cytotoxicity of the extracts and fractions. But in several fractions it was seen that their cytotoxicity could be removed by reducing the concentration of the test extract, whilst still retaining significant activity.

14.1.2 Extraction of plant material

The ground stems and aerial parts (not leaves) of the shrub were exhaustively extracted at room temperature using ethyl acetate. About 300 g of plant material gave about 13 g of extract (approx. 4%).

14.1.3 Fractionation of the crude extract

The extract was dissolved in chloroform, and then partitioned with 10% methanol in water (Fig. 27). The chloroform extract was then fractionated using Sephadex LH-20 in chloroform. The original reason for using Sephadex LH-20 was to remove chlorophylls that were present, in the first main extraction the leaves were not removed due to a miscommunication of the preliminary results obtained. But then it was found that the extract separated well, with little loss of material. For crude fractionation about 150 g of Sephadex was used for about 2 g of extract, which was repeated several times. Methanol was used as a final wash. This gave seven fractions (BF1-7, Fig. 28). I did not continue working on the polar fraction (BF7) as it did not show activity in the luciferase assay. In the preliminary screening the methanol extract also did not show significant NF-κB inhibitory activity.
Figure 27  Isolation scheme used for *Bupleurum fruticosum*

Aerial parts

Exhaustive extraction with EtOAc at room temp

Crude extract

partitioning

$\text{CHCl}_3$ extract

Sephadex-LH 20 fractionation in $\text{CHCl}_3$

7 fractions

1 2 3 4 5 6 7

Sephadex-LH20 and PTLC

BFa, PP1, PP2, PP3, BFc

PTLC

BFb, d, e, f, g
**Fig. 28** TLC of *Bupleurum fruticosum* extract and Sephadex-LH20 fractions

E: ethyl acetate extract
Sephadex-LH20 fractions ‘BF’ 1-7

Solvent system: CHCl₃/MeOH 9:1

<table>
<thead>
<tr>
<th>E</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tbody>
<tr>
<td>PP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP2 + PP3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Solvent System:
- CHCl₃
- CHCl₃/MeOH 9:1
- Cyclohexane: EtOAc 8:2
- CHCl₃
The seven (BF1-7) fractions were tested in the luciferase assay at 100 μg/ml. BF1 had no activity (see section 14.2, Fig. 35). BF2 reduced NF-κB activity to about 10 % with no cytotoxicity. BF3-6 were toxic. BF7 reduced activity to about 30 %, with no toxicity. BF3-6 were retested at 50 μg/ml, and were found to reduce activity to almost zero, with BF3 and BF4 still showing toxicity. BF3-6 were retested at 25 μg/ml, but did not show significant activity (Fig. 35).

### 14.1.4 Isolation and identification of compounds

The TLC of the crude ethyl acetate extract in CHCl₃ was mostly colourless, and when sprayed showed a large number of compounds with mostly blue, purple, red and black colours, with some chlorophyll type compounds present (Fig 28). A number of them had UV activity under short wavelength. Use of CHCl₃ and cyclohexane:ethyl acetate as solvent systems were effective at producing good TLC traces, and the polarity of the solvents show the non-polar nature of the compounds present. More polar solvent systems used only moved all the compounds far up the plates, leaving behind only a few minor compounds seen as indistinct bands.

**Compounds isolated**

Three phenylpropanoid compounds were isolated and identified.

14.1.4.1 \((E)-3-(3,4\text{-dimethoxyphenyl})-2\text{-propen-1-yl} \ (Z)-2-[(Z)-2\text{-methyl-2-butenoxyloxy}methyl] \text{butenoate.} \ (PP1)\)

Colourless oil. PP1 is a major component (100 mg), present in fractions BF2-3. These were refractionated using flash with hexane : chloroform. Isolation on PTLC was straightforward using first CHCl₃, and then cyclohexane:ethyl acetate 8:2 for final
purification. The compound turns red when sprayed (Fig. 28), and has UV activity at 254 nm, but none at 365 nm.

EI and FABMS gave a molecular ion of 374 [M]^+, consistent with C_{21}H_{26}O_6. Accurate mass measured: 374.192800, calculated mass for C_{21}H_{26}O_6: 374.1729. EIMS m/z (relative intensity) 374 (30), 236 (50), 208 (25), 193 (50), 177 (100), 165 (35), 146 (60), 131 (30), 119 (30), 103 (35).

Identification was by comparison of the ^1H NMR spectral data (Table 15) with literature data (Massanet, Guerra, et al. 1997; Pistelli, Bilia, et al. 1995). The ^1H NMR showed the presence of two methoxyl groups (δ 3.87 and 3.91), three vinylic methyl groups (δ 1.86, 1.94, 2.10), two vinylic CH2O (δ 4.82, 4.85), four olefinic protons (δ 6.03 ddd, 6.17 dt, 6.45 dd, 6.61 d), and three aromatic protons (δ 6.82 d, 6.92 d, 6.94 s).

14.1.4.2 L-(3,4,5-trimethoxyphenyl)-2-propenyl 2-(2-methyl-2Z-
butenoyloxymethyl)-2Z-butenoate. (PP2)

Colourless oil. Another major component (50 mg), present in BF2-3. Isolation on PTLC required two steps, isolation using the solvent system 100 % CHCl_3, and then purification using cyclohexane:EtOAc 8:2. The compound turns grey when sprayed (R_f: 0.3 CHCl_3, Fig. 28), and also has UV activity at 254 nm but none at 365 nm.

FABMS gave a molecular ion of 404 [M]^+, consistent with C_{22}H_{28}O_7. Accurate mass measured: 404.1826, calculated mass for C_{22}H_{28}O_7: 404.1835. FABMS m/z (rel. int.) 404 (15), 318 (15), 223 (15), 208 (25), 207 (100), 181 (15), 176 (30), 133 (45), 83 (75).

Identification was by comparison of the ^1H NMR spectral data (Table 15) with literature data. The ^1H NMR (in CDCl_3) spectrum was very similar to that of PP1,
showing the presence of 28 protons. Signals for the diester portion of the side chain (positions 1-9) are almost identical for PP1 and PP2. Differences in the spectra for the two compounds are: only one vinylic CH$_2$O, one more methoxyl and one less aromatic proton present, and the presence of a -CH(OR)-CH=CH$_2$ group. Signals for three methoxyl groups could be seen, two of which are magnetically equivalent, at $\delta$ 3.80 (3H) and 3.85 (6H). Signals for three methyl groups came at $\delta$ 1.79 (s), 1.90 (d), 2.12 (d), one CH$_2$O group at $\delta$ 4.86, three olefinic protons at $\delta$ 5.96 (m), 6.03 (q), 6.48 (q), two geminally coupled protons belonging to an olefinic CH$_2$ at $\delta$ 5.24 (d, $J = 10$ Hz) and 5.33 (d, $J = 17$ Hz), and two magnetically equivalent aromatic protons at $\delta$ 6.59.

In (Massanet, Guerra, et al. 1997), the authors observed that this compound is less stable, stating that it spontaneously transforms into PP3 at room temperature.
Table 15. $^1$H NMR data obtained for PP1-3 (400 MHz, CDCl$_3$), and comparison with literature data.

<table>
<thead>
<tr>
<th></th>
<th>PP1</th>
<th>Literature*</th>
<th>PP2</th>
<th>Literature**</th>
<th>PP3</th>
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* Pistelli, Bilia, et al. 1995 (200 MHz)
** Massanet, Guerra, et al. 1997 (400 MHz)
14.1.4.3 3-(3,4-dimethoxyphenyl)-2E-propenyl2-2[2-methyl-2Z-
butenoyloxy)methyl]-2Z-butenoate (PP3)

Colourless oil. Grey when sprayed, and has the same \( R_f \) value as PP2 when 100 % 
CHCl\(_3\) is used. It was isolated together with PP2 on PTLC using CHCl\(_3\), and then 
separated on TLC using cyclohexane : ethyl acetate 8:2 (\( R_f :0.1 \)), it is a minor 
component (10 mg).

The \(^1\)H NMR spectrum (Table 15) for PP3 is very similar to PP1, differing only at the 
aromatic signals and the integration for the methoxyl signals. In the PP3 spectra, the 
total integration of the methoxyl signals was equivalent to 9 (\( \delta = 3.85 \) (3H), 3.87 
(6H)), corresponding to three OMe groups. In addition there was only one aromatic 
signal (\( \delta_H = 5.97 \)) the integration of which was equivalent to 2 protons, corresponding 
to the magnetically equivalent aromatic protons at positions 15 and 19. Identification 
was by comparison with literature data (Massanet, Guerra, et al. 1997).
This compound could not be obtained in a pure form, and was not included in 
biological testing.
Fig. 29  $^1$H NMR spectra for PP1 and PP3 in CDCl$_3$ (400 MHz)

PP1

PP3
Fig. 30  
$^1$H NMR spectra of PP2 and unknown 'blue' compound (BFc) in CDCl$_3$ (400 MHz)

PP2

BFc - 'blue' compound
14.1.4.4 Unidentified compounds

A number of fatty acids were present in the extract, which presented as compounds which tended to turn strong shades of blue, purple or red when sprayed (Fig. 28, 32) and were UV inactive. During the process of compound isolation several components were easily isolated (BFa, b, f, g), one of which precipitated out in large quantities in the presence of methanol, whose \(^1\)H NMR spectra (Fig. 33-4) were generally characterised by a complex mass of signals upfield at \(\delta 0.5-2.5\), and almost no other signals elsewhere, suggesting that they are fatty acids. However they have not been identified, their spectra were not analysed as they were poorly resolved. Being ubiquitous, fatty acids are not considered to be phytochemically interesting, nor of biological/pharmacological relevance. In the literature the main class of compounds isolated from the aerial parts of BF are phenylpropanoids, which themselves are synthesised from fatty acids.

The TLC of the crude ethyl acetate extract showed the presence of two major components (BFd and e) in BF4 (Fig. 28), which have very strong UV activity at 254 nm (none at 365 nm), and are colourless when sprayed. Impure samples containing these compounds were tested and found to have NF-\(\kappa\)B inhibitory activity, but attempts to purify and isolate them have been unsuccessful, as the compounds decompose rapidly even at -20°C. UV spectra were obtained when an HPLC analysis was attempted, which gave characteristic multi-peak spectra, suggesting the presence of several conjugates (Fig. 31). NMR spectra obtained showed signals which suggest a possible triterpenoidal structure, particularly with ‘BFd’ (Fig. 33).
Fig. 31  HPLC trace and UV spectra for ‘BFd’

Analytical HPLC trace for BFd (78 % MeOH in H₂O, 1 ml/min), showing the presence of two peaks, with almost identical UV spectra.
The TLC of the crude ethyl acetate extract also showed the presence of a number of blue bands under 365 nm (Fig. 32), which were also difficult to isolate, only obtainable in very small quantities whilst also still impure. Isolation of these components had been attempted with HPLC, but despite their fluorescence on TLC under long-wave UV, they were not detectable on the HPLC photo-diode array detector. A small amount of one of the compounds (‘BFc’) was accumulated via repeated TLC isolation, and the $^1$H NMR spectrum shows the clear presence of the same diester group which is present in PP1-PP3 (Fig. 30). Although the spectrum shows several methoxyl group signals at around 3.8 ppm, they lack the height which is a characteristic of methoxyl signals, indicating that these are part of a ‘minor’ component/impurity present. This plus the absence of the distinctive signals of aromatic protons down-field suggests that this compound does not have an aromatic group, and is therefore not a phenylpropanoid. The propanyl side chain portion (C11-C13), which in PP1-3 produced signals between 4.8 and 6.5 ppm, also seems to be absent here. In addition, the signal at 4.8 ppm which correlates with the methyl group (C6) adjacent to the oxygen/ester group is a clear singlet, suggesting the presence of only one such group. Possibly this compound is simply the diester side chain ([2-methyl-2-butenyloxymethyl]-2-butenoate) found in PP1-PP3, although as the spectra shows impurities, it is inconclusive. Unfortunately, as isolation and purification of the compound was difficult, and due to time constraints, no more compound was obtained. I had attempted to repurify the compound again using TLC with the same solvent systems as described before, but recovery was poor.
TLC (solvent system CHCl₃) showing the progressive fractionation of the crude *Bupleurum fruticosum* extract. Using Sephadex-LH20 in CHCl₃, components isolated (BFa-g) shown.
Fig. 33  $^1$H NMR (250 MHz) spectra for BFa, b and d (CDCl$_3$).

BFa

BFb

BFd
Fig. 34  $^1$H NMR (250 MHz) spectra for BFe, f and g (CDCl$_3$)
14.2 NF-κB results for *Bupleurum fruticosum*

Toxicity was observed for many of the fractions obtained from the plant extract when tested at 100 μg/ml, although at lower concentrations the toxicity was removed. Fractions which were toxic at 100 μg/ml were retested at 50 μg/ml, which reduced the toxicity but in some fractions did not remove it completely. NF-κB inhibitory activity was observed for most of the fractions and subfractions obtained from the ethyl acetate extract, although the results were treated with caution as they may be due to the toxic effects observed (Fig. 35).

For the preliminary screening assay, the plant material was extracted first with petroleum ether before being extracted with ethyl acetate. In the full phytochemical investigation which followed, the plant material was extracted with ethyl acetate only, and the compounds which would have been removed by the petroleum ether in the preliminary extraction would most likely now also be present in the ethyl acetate extraction.

Bioassay guided fractionations were performed, although most of the fractions obtained showed activity. PP1 and PP2 were two of the main components present and were the most straightforward to isolate. Tests showed that PP1 and PP2 had activity down to 50 μM, against PMA and TNF-α. Dose-response curves were obtained (Fig. 36), and PP1 and 2 show very similar concentration-activity relationships. Both show maximal activity at 50 μM, and become ineffective at 10 μM, with an IC₅₀ of approximately 30 μM.
Figure 35  Results of luciferase assay for *Bupleurum fruticosum* extracts BF1-7

PMA was used as the inducer. All assays were performed once, with at least 6 replicates for the controls, and 2-3 replicates for the extracts. Error bars show mean ± S.D.

100 μg/ml: BF3-6 were cytotoxic and produced no readings.

50 μg/ml: Some cytotoxicity in BF3 and 4.
Sometimes NF-κB activity readings for PP1 and PP2 went below that for the negative control at 50 μM. It may be that the compounds are still exerting some cytotoxic effects even at this concentration which are not visible in this short time period. Another possibility is that the compounds may have a direct inhibitory effect on the luciferase activity itself, and so may not have any actual intrinsic NF-κB inhibitory activity of its own. Further assays can be performed to establish whether this is the case or not, such as by using EMSA.

As neither PP1 nor PP2 show NF-κB inhibitory activity below 10 μM, the compounds are not considered to be of clinical importance, which would require compounds to show activity at concentrations of less than 10 μM. However the activity they show is still significant, and may support the traditional use of *Bupleurum fruticosum* as an anti-inflammatory agent.
Results of one assay shown, each concentration performed in triplicate. Each point shown as mean ± S.D.
15.1 Discussion of phytochemical results

In this study most of the species' main compounds, as seen on TLC, have been isolated from the bark of *Ochna macrocalyx* (OM). Their isolation was aided by their UV activity and their strong colouration with vanillin and sulphuric acid. Of the six compounds identified, five have been previously reported in related species. The sixth compound, dehydroxyhexaspermone C (DHHC), is a new natural product - a derivative of the known isoflavanone dimer hexaspermone C (HC). Most of the compounds isolated were abundant enough to enable their isolation in relatively large quantities, in which a high percentage yield of crude extract also helped.

The OM compounds are unusual and interesting, and provided different phytochemical challenges in their isolation and elucidation. The results of this phytochemical study also reinforce the relationship of OM with other members of its family, and it was interesting to see that plant species which are botanically related also have a similar phytochemistry.

Although ochnone has been previously isolated (Hostettmann & Marston 2001), its spectral data was not published, so was published for the first time in (Tang et al. 2003). Cordigol was first reported in (Marston et al. 1988) as allegedly being isolated from *Cordia goetzei*, which was found later to be *Brackenridgea zanguebarica* (personal comment March 2002, Ioset and Hostettmann). Calodenin B (CB),
dihydroxycalodenin B (DHCB) and cordigone (a closely related analogue of ochnone) have also been isolated from *Brackenridgea zanguebarica*, a small tree from Zambia with bright yellow root bark (Drewes, Hudson, et al. 1984; Drewes, Hudson, et al. 1987), possibly suggesting that this species is closely related to *Ochna macrocalyx*. CB has also been isolated from *Ochna calodendron* Gilg. et Mildbr. (Ochnaceae), a medicinal tree with yellowish stem bark from Cameroon, from which its trivial name is derived (Messanga, Tih, et al. 1994). HC was previously isolated from the stem bark of *Ouratea hexasperma* (St. Hil.) Baill (Ochnaceae), a Brazilian medicinal plant (Moreira, Sobrinho, et al. 1994).

The main biflavonoids isolated, CB, DHCB, ochnone, and the minor component cordigol, are chalcone dimers, composed of two trihydroxy chalcone units. The Ochnaceae chalcone dimers appear to arise from initial dimerisation followed by further elaboration, such as cyclisation. In CB and DHCB, one chalcone unit is linked by its two central aliphatic carbons to the phenyl group of the second unit, via one C-C bond and one O-bridge forming a benzofuran structure. In ochnone and cordigol, the two chalcone units are both joined together through their central aliphatic carbons, also via one C-C bond and one O-bridge, forming a central furan ring structure. The way in which these chalcones are dimerised seems to be unique to the Ochnaceae.

The minor components isolated, DHHC and HC are isoflavanone dimers, and possess methoxyl groups. The dimerisation of the isoflavanone subunits is classical, where the subunits are linked via a single bond at the same position on both units making the dimer almost symmetrical, although it would appear that such compounds are also unusual.
Examples of other biflavonoid and related compounds isolated from the Ochnaceae

Calodenin B, which is completely conjugated, is relatively stable, which may have contributed to the absence of CB isomers with similar $R_f$ values, making its isolation by far the simplest out of all the other compounds. In (Hostettmann & Marston 2001) the authors even described their isolation of CB from *Brackenridgea zanguebarica* by recrystallisation. Its isolation was also aided by its strong orange colour and UV activity.

In contrast, dihydrocalodenin B is only partially conjugated, and has a strong yellow colour. It is the main principle responsible for the distinctive colouring of the *Ochna macrocalyx* bark, and was also the most abundant in terms of quantity isolated. Its purification was relatively difficult, and it was clear that there are several other closely related DHCB isomers were present with very similar $R_f$ values. Attempts made to isolate these other compounds only obtained trace amounts which were not clean under NMR analysis. Some of these compounds may have been formed or derived from DHCB. One of the protons present on the furan ring is situated next to a carbonyl group, so it is possible for the compound to enolise (Fig. 37). A proton may move back to the original position on the furan ring, but in the opposite
stereochemistry, so what was a trans bond may change to a cis bond. This could occur during isolation procedures, where the use of proteolytic solvents such as methanol and acetone may encourage proton migration. This tautomerism could also occur in cordigol and ochnone.

Figure 37  Enolisation of a carbonyl group next to a CH group

I had not established the stereochemistry of the two DHCB furan ring protons. Its isolation was first reported in Drewes et al. who published two papers on their results (Drewes, Hudson, et al. 1984; Drewes, Hudson, et al. 1987). They do not detail how they decided on the stereochemistry of DHCB, only mentioning a trans configuration when labelling it in the structure diagram and in the list of isolates under the experimental section.

The trans-dihydrofuran configuration may have been decided from the coupling constants of the two protons, which they reported to be 5.8 Hz. Typically trans protons have much larger coupling constants, such as 15-17 Hz, and a value like 6 Hz would be typical of cis configuration. Here trans protons can give a low J value, as on the benzofuran they would still be close to each other on the diaxial plane due to contortion of the furan by the aromatic ring. More evidence may be provided in a NOESY spectra, although correlation between directly coupled protons is shown regardless of stereochemistry. Possible configuration may be deduced according to
whether or not the two protons show the same correlations, perhaps with protons on
the adjoining phenyl or benzoyl group. More evidence may be obtained using X-ray
crystallography, which was not performed in this study.

Ochnone and cordigol are analogues, with the same molecular weight and formula. It
is possible that cordigol is derived from ochnone, as the two compounds show the
same substitution patterns on their phenyl and benzoyl groups, although their relative
stereochemistry is not identical. The additional 6 member ring present in cordigol
could be formed by a carbonyl group joining with the neighbouring phenyl ring to
form an O-bridge. A derivative of cordigol has been isolated from *Lophira lanceolata*,
lophirone H, which has one less hydroxyl group on the benzopyran ring.

Ochnone also has three other closely related analogues, cordigone, isolated from
*Brackenridgea zanguerebarica*, lophirone F and G from *Lophira lanceolata* (Fig. 38).
The three analogues are enantiomers, with the same planer structure, which only
differs from the planar structure of ochnone in the position of one hydroxyl group.
Unlike ochnone, the planar structure of the enantiomers is symmetrical, with two
dihydroxybenzoyl groups and two hydroxyphenyl groups.
Previous isolation of ochnone was only mentioned in a review on counter current chromatography in preparative isolation (Hostettmann & Marston 2001), published in the Journal of Liquid Chromatography and Related Technology. The review, which was found through the Chemical Abstracts database, only shows the structure of the compound, and does not have any details on its isolation nor its structure elucidation. In the review, one reference was given which may have had the NMR details on the compound, in the proceedings of the first IOCD-symposium, published by the University of Zimbabwe. As a copy could not be obtained through the British Library, a direct comparison of NMR data was not possible. The structure elucidation of ochnone in this study was performed through interpretation of 1-D and 2-D NMR data. As the review does not detail stereochemistry, it is also possible that they may have isolated an enantiomer of ochnone.

When the 2-D NMR spectra for ochnone was obtained there was still a small amount of cordigol present, but the contrast between the two compounds was such that it was
fairly straightforward to distinguish which peaks belonged to which compound. The spectra obtained was of such good quality that it enabled the structure elucidation of both compounds, even though there would have only been trace amounts of cordigol present, perhaps 1-2 mg, to 10 mg of ochnone. Thus even though cordigol had not actually been isolated, the quality of the NMR spectra still enabled its identification. The spectra were obtained at Strathclyde University by Prof. A. I. Gray, an experienced NMR expert, whose help may have contributed to the quality of the spectra.

I was not so fortunate with the unknown compound X, of which there was approximately 4-5 mg, although the amount in the final acetone-d$_6$ solution would have been less, as the compound was not completely soluble in the solvent. Although two attempts were made to obtain a $^{13}$C spectrum using a 400 MHz and a 500 MHz magnet, where with the 500 MHz the carbon experiment was run for 12 hours, almost no signals could be obtained.

Whereas the chalcone dimers where isolated because they were major components present in NF-κB bioactive fractions, the isoflavanone dimers were isolated more for phytochemical interest. They were present in the original Sephadex ‘fraction 5’ which showed some activity in the luciferase assay. Their presence was noticed after a second bark extraction, which was fractionated using VLC. On TLC the biisoflavonoids were clearly visible under short-wave UV as a band with a high $R_f$ value which turned red when sprayed. The polarity of the biisoflavonones was very different from the chalcone dimers, readily dissolving in chloroform, although they also dissolved in polar solvents such as methanol.

In terms of the amount of time spent in developing an isolation method, isolation of the biisoflavonones was quick and straightforward. This may have partly been due to
phytochemical experience as they were the last compounds which were isolated from *Ochna macrocalyx*. Their purification on RP-C18 HPLC was straightforward, where they separate well. There was also no problem with getting them to dissolve in the mobile phase (55% ACN in H₂O), where they seem to become oily drops, forming an emulsion which passed through a 0.45 μm filter easily. Thus, even though they are not as abundant as the other compounds, they were easily isolated.

One unfortunate mistake I made was in putting dehydroxyhexaspermone C (DHHC) in acetone-d₆ when submitting it for the full set of NMR experiments, as this was undoubtedly what led to its subsequent decomposition. The decision to use acetone-d₆ was due to the fact that the ¹H spectrum obtained gave a better separation of the signals than the ¹H spectrum obtained using CDCl₃. Acetone-d₆ had also been used for the chalcone dimers with good results, where although they dissolved better in methanol, the signals obtained in acetone-d₆ were again better defined.

At the time when the spectra for DHHC and hexaspermone C (HC) were obtained, the NMR facilities at the School of Pharmacy were not able to perform 2-D experiments, and the samples were sent to the Queen Mary and Westfield College in Mile End. If the NMR facilities had been on site as they are now, DHHC would have been resubmitted in CDCl₃ for another set of experiments.

Although HMBC and NOESY spectra were obtained for DHHC, they were unuseable, and the challenge became whether it was possible to deduce the structure of DHHC without HMBC. The identification of HC and the subsequent comparison of their NMR and EI mass spectra made this possible.

The full set of NMR experiments for HC were obtained in CDCl₃, where unlike DHHC no decomposition had occurred. I had attempted structure elucidation of HC
from the NMR spectra, and had deduced another ochnone like substituted
tetrahydrofuran derivative, with an O-bridge between
the two benzoyl groups (a).
This was due to the spectra showing two AA’BB’ and
two meta coupled ring systems, as well as four
aliphatic signals, the positions and multiplicities of
which were very similar to those of the furan ring
protons in ochnone. The O-bridge was then brought in as the only way in which such
a system could also have two meta coupled ring systems which correlated with its
molecular formula. All the other NMR data supported this arrangement and possibly I
would have continued to believe that this was the correct structure if a search in the
Dictionary of Natural Products had not produced hexaspermone C, reported in
(Moreira, Sobrinho, et al. 1994). The structure in which I had deduced is also
illustrated in this paper, and the authors go into much detail on the fragmentation
patterns obtained in the EIMS. And as the main base peak for HC was at m/z 299, the
hypothetical compound was rejected as not having the structural conditions to
produce such a fragment.
This observation which was almost pivotal in deciding on the structure of HC, led to
my reviewing of the EIMS obtained for DHHC. The EIMS for the compounds are
notable in which they show two distinct signals, alongside other less prominent
signals. In HC, two signals stand out at 299 and 285 (Fig. 39), as detailed in the paper
(Moreira, Sobrinho, et al. 1994), corresponding to two isoflavanone subunits.
Fig 39  EIMS and fragmentation pattern for hexaspermone C

Source: Moreira, Sobrinho, et al. 1994
Fig 40  EIMS and fragmentation pattern for dehydroxyhexaspermonone C
It was clear that the isoflavanone subunit which had given the signal at m/z 299 in HC (2b) must have changed in DHHC, where it is absent, replaced by signals at 283 and 285. The proposed structure for DHHC is also supported by the fragmentation patterns in the EIMS (Fig. 40). Unfortunately, the EIMS for these compounds were not obtained on site and the spectra were not processed well, with the main signals going off the top of the scale so relative intensities could not be determined.

The DHHC structure is also supported by the $^1$H spectrum. The $^1$H spectra of both bisoflavonoid compounds are almost the same except what was a meta coupled ring system in HC was now an ABX system in DHHC. Therefore the ABX system can only be on one of the two 'A' rings present in the DHHC biflavonoid. And it could not be on the other subunit (1a/2a), which would give fragment of m/z 269 (285 - 16), with the signal at m/z 299 remaining the same.

Two other variations which may give the same proton signals are also possible. One is where the
methoxyl group on the ABX system is at position 6, instead of 7 (b), which is biosynthetically unlikely. The other (c) is if the methoxyl group on the remaining meta coupled ring system has switched places with the hydroxyl group on the phenyl ring, however this is not supported by the EIMS, which would have displayed a different fragmentation pattern.

Although the two bi-isoflavanones showed no bioactivity in our assays, they provided an interesting and valuable phytochemical exercise. In this case, my natural assumption was to deduce another substituted tetrahydrofuran derivative structure for HC based on my previous experience with the elucidation of ochnone. It was only after reviewing the paper by Moreira et al (Moreira, Sobrinho, et al. 1994) which showed that structure elucidation from NMR data may produce more than one possible structure, in which case MS interpretation becomes important. The paper also reported the isolation of two other closely related isoflavanone dimers from Ouratea hexasperma, hexaspermone A and B, which are almost identical to HC, but with one other additional methylolation of one of the hydroxyl groups. DHHC differs in the absence of one hydroxyl or methoxyl group from position 5, a departure from the 5, 7 hydroxylation pattern typically found in flavonoids. As the biosynthesis of the ‘A’ ring in flavonoids involves the linking of three malonyl CoA units, this typically
results in two hydroxyl groups in positions 5 and 7. In this respect DHHC QWalso
differs from the OM chalcone dimers, which all have chalcone subunits hydroxylated
at the 5 and 7 positions. It may be more logical to assume that there would be more
HC than DHHC present, although this was not the case, and the prevalence of DHHC
over HC may also be evidence that it is not an artifact. It is perhaps more unusual to
see flavonoids which do not have a hydroxyl group in position 5, although there are
many examples which occur in nature, such as liquiritigenin, daidzein and butin.

One possible concern is in the deterioration of compounds in the extraction of the
bark via ethanol reflux. However, refluxing with ethanol is a very common extraction
procedure, and is probably the most appropriate method for extracting bark material,
which may otherwise be difficult to penetrate. In addition some of the Washambaan
healers, in the preparation of the bark as a medicinal remedy, boiled the bark in milk.
If there is deterioration of the compounds in the boiling process, this may still not be
detrimental, as the compounds originally present may even break down or change into
bioactive components.

At the beginning of the project only silica was used in compound isolation, until
almost the second year of the project when HPLC equipment became available in the
Centre and use of RP-C18 was included. Silica was suitable for the isolation of
calodenin B and ochnone, although much time was spent finding a suitable solvent
system to separate ochnone from cordigol. RP-C18 was required for the purification
of DHCB, DHHC and HC. Method development in the isolation of the Ochna
compounds may have been quicker and more successful with higher yields if RP-C18
had been used earlier. The compounds are phenolic and polar, dissolving easily in
methanol, and so the use of RP-C18 may be particularly suitable. It is likely that
*Ochna* compounds ‘stick’ to the silica, due to strong interaction or reaction with the
polar SiOH groups, lowering their yields. This could be clearly seen with the coloured
compounds during desorption.

Use of RP-C18 HPLC may have been useful with the unknown compound X whose
presence was clearly visible on TLCs of the extracts, but whose isolation was very
difficult on silica, with extremely low yields obtained. On silica TLC, compound X
had the lowest $R_f$ value out of the other compounds, so it is likely that recovery was
poor due to binding of compound X to silica. Better results may have been obtained
using only RP-C18 and if more material were available to work with.

The original fractionation of the extract was performed using Sephadex LH-20,
although using silica gel (in flash and VLC) also produced satisfactory results. With
Sephadex, some fractionation of the extract was obtained, particularly in separating
CB from DHCB, but fraction OM5 still contained a large number of different
compounds. The extract was not completely soluble in methanol, and its migration
down the Sephadex column was slow, with a significant amount of residual material
remaining on the column despite large amounts of elution with methanol. A large
quantity of Sephadex LH-20 was also required in order to fractionate 15 g of the
extract.

In contrast the use of VLC and flash was much faster, and convenient to use. The loss
of polar compounds due to binding to silica also seems reduced, probably due to the
speed at which the solvents and the fractions move through the column.
15.2 Discussion of biochemical results

The investigation of the bioactivity of the medicinal plant made the study more comprehensive, and data obtained provided some insight into its traditional uses. I found performing the bioassays challenging, and had all the problems that are typically faced with obtaining biological data. Common general errors occurred, including the making up of the test extracts, contamination, pipetting and calculation errors. There were problems with solubility, and concerns of stability of the compounds. These errors could be minimised with care, experience and repetition of assays, but sometimes unexplained variations still seemed to occur. The compounds were all tested in vitro, very different to an in vivo situation, where compounds are likely to undergo various metabolic changes, if they are absorbed at all. And although they may not show activity in this study, there are many other possible targets in which they may be acting on to produce the medicinal effect in which they are traditionally employed. Thus the results presented are at best only an indication of the capabilities of the compounds. The amount of test compounds available was also limited. The quantities quoted in the results sections are theoretical, based on calculations of percentage yields obtained. Thus although the amount of some of the test compounds which could theoretically be obtained was high, in practice obtaining enough compound for testing in some cases required repeated, complicated and labour intensive isolation procedures.
15.2.1 NF-κB

It was the NF-κB inhibition found in an EMSA by the crude ethanolic bark extract of *Ochna macrocalyx* which led to this phytochemical and biological study being performed.

The NF-κB results obtained for the crude extract and fractions may be partly explained by cytotoxic effects, where reduced cell numbers will reduce NF-κB readings. However, cytotoxicity seen in screening does not necessarily mean extracts are not worth further investigation. With further fractionation, cytotoxic components may be separated from bioactive components, and cytotoxic effects may in itself be an indication of NF-κB inhibitory activity. As there is strong evidence that NF-κB is important in the inhibition of apoptosis, it is possible that the suppression of NF-κB activity would lead to apoptotic effects being observed. And active compounds which are cytotoxic at higher concentrations may become less so when diluted, whilst still retaining bioactivity.

It is also possible there are synergistic or additive effects between compounds in the extract, and on their own the compounds show no activity (Williamson 2001).

For the first 18 months of the study on *Ochna macrocalyx*, the assays were performed at different sites, as NF-κB testing facilities were not in place at the centre. Thus, unfortunately a proper bioassay guided fractionation was not performed, and only the original Sephadex LH-20 fractions from the crude bark extract were assayed for activity, which led to the further investigation of fractions 5-8. Only the main
components of these fractions were isolated. As these compounds have now been shown to have no relevant NF-κB inhibitory activity, it perhaps may have been better in retrospect to have fractionated these fractions first. Further testing of these subfractions may then have produced more successful results, perhaps leading to the concentration of effort in the isolation of more minor components.

Information obtained from literature research may support possible NF-κB inhibitory activity in OM. The structure of theoflavin-3,3-digallate is not unlike that for the tetraflavonoids found in the Ochnaceae, specifically in Lophira alata and L. lanceolata. Theoflavin digallate is a component of black tea (Thea sinensis), which was found to have NF-κB inhibitory activity in an EMSA at 30 μM. It is highly polyphenolic, and some other common features that it has with some of the Ochnaceae compounds include benzopyran units, and tetrahydroxy benzoyl units.

In a search for inhibitors of tumour promotion in cancer prevention, one research group performed extensive research on Lophira alata (Murakami, Ohigashi, et al. 1991; Murakami, Tanaka, et al. 1992; Murakami, Tanaka, et al. 1992), and its ability to inhibit Epstein Barr Virus-Early Antigen (EBV-EA) induction. EBV is known to be activated in vitro by tumour promotors such as PMA and teleocidin B-4, which induce the viral early antigen (EA). EBV is also an NF-κB activator, capable of inducing persistent NF-κB activation, which has also been linked to tumour promotion. Their research led to the isolation of the bi- and tetraflavonoids lophirone A, lophirachalcone, alatachalcone and azobechalcone A as EBV-EA induction inhibitors (Fig 41). Although these results do not necessarily mean there is a definite connection with our work with NF-κB, it would be interesting to see if these compounds are also able to inhibit NF-κB induction. Interestingly, they tested
lophirone F, which is structurally similar to ochnone, and only found marginal activity. There are several other Ochnaceae compounds closely related to azobechalcone A, including CB and DHCB, lophirone C and its derivatives, isolated from *Lophira lanceolata, Ochna calodendron* and *Ochna afzelii*.

*L. alata* is described as being widely exploited for commercial and medicinal uses, where it is used to cure several diseases, which includes its use as an analgesic. Other biological studies have also found it to have antibacterial and anti-inflammatory activity.

One of the traditional uses of *Ochna macrocalyx* is dysmenorrhoea and stomach pains, and the extract may be used for this purpose due to anti-inflammatory/analgesic properties.
Fig. 41  Compounds from *Lophira alata* (Ochnaceae) with anti Epstein-Barr Virus Early Antigen (EBV-EA) Induction activity.

Lophirone F

Lophirone A

Lophirachalcone

Alatachalcone

Azobechalcone A

Compounds from *Lophira alata*
Anti EBV-EA induction activity  
% inhibition

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lophirone F</td>
<td>18 ± 5</td>
</tr>
<tr>
<td>Lophirone A</td>
<td>45 ± 12</td>
</tr>
<tr>
<td>Lophirachalcone</td>
<td>64 ± 5</td>
</tr>
<tr>
<td>Alatachalcone</td>
<td>72 ± 8</td>
</tr>
<tr>
<td>Azobechalcone A</td>
<td>83 ± 12</td>
</tr>
</tbody>
</table>

References:
Murakami, Ohigashi et al. 1991
Murakami, Tanaka et al. Phytochemistry 1992
15.2.2 Cytotoxicity

Cytoxicity assays were performed because of the observed cytotoxicity of the *Ochna macrocalyx* fractions at 200 µg/ml in the NF-κB assays performed at the beginning of the project. The assays were performed with breast cancer cells, and from this a possible link between the OM compounds and phytoestrogens was also speculated.

Five of the OM compounds were tested in the MTT assays using MCF-7 breast cancer cells. Only the two chalcone dimers CB and DHCB showed cytotoxic effects below 50 µM. In the bark preparation administered as a herbal remedy, they would be present in even lower concentrations. However it is not possible to determine whether the extract is harmful or not from these results.

As different cell lines have varying sensitivities, the MTT assays perhaps should have been performed with HeLa in order to relate them to the observations reported during the NF-κB assays. However, as it was concluded that the compounds did not show inhibitory activity in the NF-κB assays, further investigation was not considered essential.

The main difficulty I had in these assays was the solubility of the test compounds. The compounds were not readily soluble in the medium, although the chalcone dimers dissolved easily in DMSO. Even though a small amount of DMSO was added to facilitate their solubilisation into the medium, any affect it may have had looked marginal, and as DMSO itself has toxic effects, its concentration in the test solutions could not be increased. To aid solubilisation the test solutions were sonicated, but not
heated due to concerns on the stability of the compounds, which was also the reason why they were stored overnight in the fridge before being filter sterilised.

Variations in cytotoxicity were also seen between different test solution batches of the same compound. This was first noticed with the OM extract fraction 'OM6', and to try to minimise this I made all the stock solutions up to the same concentration (300 µg/ml). In retrospect the test solutions should have all been made up to the same protocol, perhaps incorporating a set sonication time and solubilisation period. Several batch solutions should probably be made up as well, to account for variation in the test solution concentrations, although this would be difficult if the quantities of test compound available is limited.

It was clear that the compounds were not completely soluble in the medium, and therefore the actual final concentrations of the test solutions would have been less than the intended concentrations. And the compounds which showed more potent cytotoxic effects in the assays may have only done so because they were more soluble than the others. This may have been the case with HC and DHHC, which are relatively non-polar in comparison to the other compounds, and in the assays showed virtually no cytotoxic effects at 100 µg/ml.

With respect to a possible link between the OM compounds and phytoestrogens, no definite conclusions can be drawn from the results obtained in this study. However a link may be hypothesised, as there are some similarities between known phytoestrogens and the OM compounds. For instance there are some relatable structural characteristics between chalcone dimers and the lignan phytoestrogens, such as between ochnone and enterolactone. The isoflavonoid phytoestrogens can be more directly related to the OM isoflavanone dimers (Fig. 42). It is also possible that
there are other related compounds in the bark with oestrogenic activity, perhaps more closely related in structure to the phytoestrogens than those isolated so far.

Some of the literature on other *Ochna* species may also support to this theory. *Ochna afzelii*, from which lophirone A and four isoflavonoids have been isolated from its leaves, is used to treat (amongst other medical uses) female sterility and menstrual complaints (Pegnyemb, Tih, et al. 2001). Closely related isoflavonoids have also been isolated from *Ochna obtusata*, which is also used to treat menstrual complaints (Rao et al. 1989, Nia et al. 1992). All the isoflavonoids isolated from *O. afzelii* and *O. obtusata* have methylenedioxy groups.

Figure 42  Lophirone A, isoflavones and phytoestrogens

Examples of phytoestrogens

- enterolactone
- secoisolariciresinol
- enterodiol
- daidzein
- genistein
15.2.3 Antibacterial

Although the MICs obtained may not be low enough to be of interest at a clinical level, CB and DHCB have shown undoubted anti-mdr SA activity. Out of all three bioactivity assays performed on the OM compounds, the results obtained for the antimicrobial activity may be the most promising in terms of useful activity. The results obtained from the antibacterial assays repeated several times also showed good reproducibility. In the interpretation of the antibacterial results, they also relate better to the traditional uses of other similar species which have the same compounds.

The chalcone dimers have also been found to have antifungal activity against the spores of the plant pathogenic Cladosporium cucumerinum, a pathogenic plant fungus by Marston et al. in 1988 who worked on Brackenridgea zanguebarica. However activity against this strain is no longer considered to be as good an indicator of promising bioactivity as other fungal strains which are more currently in use.

Unlike the MTT assays, the minimum inhibitory concentration method does not produce a dose-kill curve, which would perhaps have provided a better representation of the activity range of the test compounds. As the concentrations are made up through two fold dilution, there are large gaps between the test concentrations at the concentrated end of the range. And so although the MIC of a compound is recorded at the well in which there is no growth observed, the actual value may lie between the recorded MIC, and that of the next well at half the concentration. However, compounds are only of interest if they show activity at low concentrations, and on the
lower range of concentrations tested, the range of test concentrations are much closer together.

There are no obvious connections between this antimicrobial activity and the traditional use of OM, although it can be speculated that these compounds perform a protective role in the bark of the plant.

In the list of use-reports collected for OM, the use of OM against diarrhoea and/or bloody diarrhoea, gastric ulcer and anaemia, as well as 'chango' and 'ngiri' was recorded. There may be a possible link with its use in treating diarrhoea, although I had also tested CB and DHCB against a resistant strain of *E. coli*, which is known to cause diarrhoea, and neither of them showed any activity. The other OM compounds and the extract were not tested in this system. Amoebic and bacillary dysentery are caused by infection by the protozoan *Entamoeba histolytica* and the *Shigella* bacteria respectively, which can be treated with antibiotics including metronidazole, ciprofloxacin and trimethoprim. As amoebic dysentery causes intestinal ulceration, indigestion, loss of weight and anaemia, the symptoms described in the OM use-reports may possibly be linked with dysentery. However OM collected a relatively low number of use-reports, suggesting that it may not be as relevant as the other medicinal plants used in the same category. Medicinal uses for *Ochna obtusata* and *Ochna afzelii* include treatment against dysentery, phytochemical studies on the two species have isolated related isoflavones from the bark and leaves, respectively. Although no common isolates have as yet been found in both species with those from *O. macrocalyx*, lophirone C and its derivatives, isolated from *Ochna afzelii*, are structurally related to CB and DHCB.
However, anti-diarrhoeal activity may not be due to anti-bacterial activity. Diarrhoea remedies in Western medicine use anti-spasmodics such as loperamide and morphine. Preparations containing magnesium trisilicate, bismuth, or kaolin are also used, although it is not entirely clear how they work. Kaolin is thought to act by ‘adsorbing toxins’.

When ingested the *Ochna* compounds may not show the antibacterial activity which they displayed in the MIC assays. But these results do suggest that topical application of the extract for external use may be of medicinal value, perhaps in bacterial or fungal skin infections. However the cytotoxic effects of the extracts seen in the NF-κB assays and in the MTT assays may not allow this, except perhaps at a suitably low concentration. Cytotoxicity assays using skin keratocytes may give a better indication of suitability for topical use.

Interestingly, the powdered dry stem bark of *Ochna calodendron* is said to have antimicrobial properties (Messanga, Pegnyemb et al. 1998). As well as CB, the derivative calodenin A and two analogues lophirone C and K have been isolated from *O. calodendron*. However, according to the ethnobotanical study, the Washambaa did not use OM for skin infections. As it seems unlikely that it would not have at some point been experimentally used for this purpose, it may have been found to be ineffective, or perhaps showed some adverse effect. [Calodenin A and Lophirone K](#)
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16  BUPLEURUM FRUTICOSUM

16.1 Discussion of phytochemical results

Two Bupleurum fruticosum phenylpropanoids PP1 and PP2 were isolated in a pure form and identified in this study. A third related phenylpropanoid PP3 was also identified, and the diester compound which is found as a subunit in PP1-3 may also have been isolated. There were many other components present in the extract, some of which were isolated but not identified, although most of them also appeared to be fatty acids from their NMR spectra. The different classes of compounds encountered in this phytochemical investigation during attempted identification included phenylpropanoids, fatty acids, and possibly triterpenoids.

The BF phenylpropanoids are characterised by having extended sidechains, typically made up of the same unusual diester moiety ([2-methyl-2-butenyloxymethyl]-2-butenoate). These phenylpropanoids are characteristic for this species, PP1, PP2 and several other related compounds have been described in the previous publications on BF phytochemistry. As the majority of the phytochemical studies on other Bupleurum species have only been on the roots, it is not known whether others may also have the same compounds present in their aerial parts.

Phenylpropanoids are defined as having a phenyl group attached to a propane sidechain, and are not in themselves particularly remarkable. Thus calling PP1-3 phenylpropanoids as well may be a slight misnomer. ‘Ordinary’ phenylpropanoids have also been isolated from BF in previous studies.
From previously published papers BF seems to be a locally well known aromatic shrub, used in folk medicine, with one report of its use as a spice as well (Manunta et al. 1992). Additional and more recent interest in BF may also be due to its more famous related species including *B. falcatum*, which is an important ingredient used in Japanese and Chinese medicine with well established bioactivity. The phytochemical information on BF looked extensive, so it seemed likely that all the main components in the extract had already been isolated.

I had become used to working with the relatively polar compounds in OM, and had to adjust to the non-polar compounds of BF. As ethyl acetate had been used in the main extraction, it had not been immediately apparent to me that the BF compounds would have very different properties from the OM compounds. It was only after readjusting the polarity of the solvent system to reduce the $R_f$ values of the BF components on TLC that I found using chloroform alone produced very good TLC traces of the extracts. Using this system also had the main advantage of convenience, in comparison to the triple solvent system used in OM (DCM:MEOH:H$_2$O) which needed to be made up fresh before being used, as the composition of the solvent mixture changed easily due to the different rates of evaporation of the different solvents. The second solvent system I also used for the BF extracts was cyclohexane and ethyl acetate. Various other solvent systems were tried, but this one produced good TLC results and was also convenient to use, without the problems of toxicity associated with using chloroform.

Sephadex LH-20 worked well with the BF extract, providing good fractionation results. As the BF extract was almost completely soluble in chloroform, Sephadex-
LH-20 seemed a good option to use for fractionation, and recovery of compounds from Sephadex, if not complete, is typically better than silica. Chloroform worked well with Sephadex LH-20 despite problems with floating, where packing and eluting the column required practice. The fractionation procedure was also fast, the majority of the extract moved quickly through the column. This may also have been due to the relative low density of the Sephadex packing in chloroform, in comparison to using methanol which produces higher density packing.

Isolation of the two main BF phenylpropanoids which were present in relatively large quantities was straightforward on TLC. Both the chloroform system and the cyclohexane:EtOAc system were important in purifying PP2. When chloroform was used, PP2 had the same Rf value as several other components, including PP3, which separate with cyclohexane:EtOAc. Conversely, PP1 and PP2 do not separate well in cyclohexane:EtOAc. Even PP1, when isolated with chloroform alone is almost clean, still requires a second TLC step using cyclohexane:EtOAc to remove minor components which were still present. This stepwise technique was very effective in the obtaining of the two compounds in a pure state. PP2 also showed some signs of instability, even though it was readily purified.

Although isolation of some of the compounds was straightforward, many were more problematic. Apart from problems with instability which were encountered, very low yields of compounds were obtained from silica TLC isolation. It is possible that these compounds may simply be present in small amounts, although they did appear to be much more abundant on TLC. As an alternative HPLC was tried in compound isolation as well, even though the compounds are non-polar, there was some
promising separation shown with some of the fractions. PP2 was actually first isolated using HPLC, although it still required further purifying. However, I stopped using HPLC after I noticed that some of the fractions tended to form a white precipitate in methanol, in particular with the unknown BF component (‘BFd’) which had shown promising NF-κB activity. I had been attempting to purify the compound on HPLC and on dissolving it in methanol, the solution became cloudy. After filtering, more precipitate formed, and the amount of the original compound left remaining was reduced. Thus the use of methanol was avoided, and although the stability of the compounds in acetonitrile was not established, as a precaution no further work was performed with HPLC. TLCs were also performed with RP-C18 plates on extracts using polar solvent systems, and did not show useful separation.

PP3 was isolated during repeated TLC isolation procedures performed in the obtaining and purification of PP2. On a single plate its presence is very minor, thus accumulation of PP3 was labour intensive, over numerous isolation procedures. The final product obtained (15 mg) was still not completely pure, but still gave a relatively clean NMR trace, which was almost identical to that of PP1 with the exception of an additional methoxyl group present. Unfortunately, a clean mass spectrum was not obtained using electron spray (ES), which produced several signals without a clear molecular ion present. Previous good mass spectral results were obtained using FAB and EI, but due to unfortunate circumstances these on site facilities at the School of Pharmacy became unavailable. As the poor results obtained on ES could have been due to the sample being impure, I attempted to further purify PP3 on TLC using cyclohexane:EtOAc and then again with chloroform. Despite this, the final product obtained was still not pure even on TLC, possibly some of the compound had
decomposed or changed. However the $^1$H NMR spectrum is unambiguous and correlates well with published NMR data.

TLC of the extract showed the presence of numerous other components which produced vivid colours with vanillin and sulphuric acid, the isolation of which were not difficult, the majority of which appeared to be fatty acids. These compounds did not show UV activity on TLC, and turned red or blue when sprayed, which are typical characteristics for fatty acids. In addition their $^1$H NMR spectra also showed characteristic signalling patterns typical for fatty acids, and no further steps were taken to identify them. Interestingly one of them when tested in the NF-κB luciferase showed good activity, which could mean that for the first time a fatty acid has been found to have activity. Conversely, this activity may show that it is unlikely that this compound is a fatty acid after all, and the NMR data should be reviewed further.

After PP1 and PP2, the next major component on TLC showed strong UV activity under short-wave UV, and also turned blue-black when sprayed (BFd and e). After isolation on TLC it was found that the band was made up of one or more components, which when tested showed potent NF-κB inhibitory activity, and $^1$H NMR spectra obtained suggested that these could be triterpenoid type compounds. Unfortunately isolation and purification of these compounds was difficult as they were found to be very unstable, decomposing quickly even when stored dry under refrigeration. Numerous unsuccessful attempts were made to purify them on TLC with various solvent systems. HPLC analysis was also attempted as mentioned before, but then it was seen that the components decomposed quickly in methanol, and further use of RP-C18 HPLC was thought to be inappropriate.
The TLC plate also showed the presence of bands which fluoresced blue under long-wave UV, which although were distinctive were also difficult to isolate, only obtainable in very small quantities whilst also still impure. A small amount of one of these 'blue' compounds was accumulated via repeated TLC isolation, which was collected at the same time as PP3 and was equally labour intensive. The NMR spectrum shows the presence of the same diester group which is present in PP1-PP3. Possibly this compound is simply the diester side chain ([2-methyl-2-butenyloxymethyl]-2-butenoate) found in PP1-PP3, although as the spectra shows the presence of impurities, it is difficult to be conclusive. Finding the presence of such a compound, is perhaps not surprising, likely to be synthesised separately in the plant before being joined to a phenylpropanoid unit to form the characteristic BF phenylpropanoids. It may have also been formed from the breakdown of one of the BF phenylpropanoids, perhaps during fractionation or isolation procedures. It is possible such a compound has not been successfully isolated from BF before, previous phytochemical papers have not reported isolating a compound like it. Fatty acids have not been mentioned in the published studies, although this may be due to their ubiquitous nature. However the presence of the free diester side chain is worth mentioning, as its presence would contribute to the hypothesis of how the BF phenylpropanoids are synthesised.

The yield of the crude extract obtained from the plant material was low, approximately 4 %, which may be usual for a cold solvent extraction. As I experienced problems with low yields in the isolation of the BF compounds, having more crude extract may have helped. In one of the papers (Massanet et al. 1997)
which successfully isolated several compounds from the aerial parts of BF, their plant material had been extracted with dichloromethane in a Soxhlet apparatus, obtaining an initial yield of 1.5%. They further extracted it with ethanol, giving a second yield of 8%. In our study, Soxhlet apparatus was not used, due to concerns on the stability of the compounds in the extract. In addition, a crude ethanolic extract had been obtained by reflux as part of the preliminary screening protocol, which was tested but found to have no NF-κB inhibitory activity. For the main plant extraction, it may have been worth further extracting the same plant material using Soxhlet as a second step to see if more extract could be obtained. This second extract could be kept separate from the first, and TLC or HPLC used to determine if the extracts are different. As the preliminary screening also showed that the hexane extract of BF had bioactivity, hexane, or maybe chloroform, could also be included in the extraction procedure.

16.2 Discussion of NF-κB biochemical results

Both PP1 and PP2 showed good inhibitory activity at 50 μM in the luciferase assay, the results of which also showed good reproducibility, although to be clinically useful requires activity at concentrations less than 10 μM. They are currently being further investigated in additional biochemical assays. As PP1 and PP2 are similar and both show activity, is it likely that other closely related compounds present, including PP3, would also show activity. Closely related BF phenylpropanoids have been reported in previous studies, thus the combined bioactivity of the various compounds may produce an additive effect.
There are likely to be other components in BF which have activity but unfortunately could not be obtained pure. These include the unknown, possibly triterpenoidal compounds, or the possible fatty acid discussed in the previous section. The presence of compounds such as erythrodiol and spinasterol may also account for some of the activity seen in the assays.

In the preliminary screening, the plant material was extracted successively with petroleum ether, ethyl acetate and finally with methanol. Both the petroleum ether and ethyl acetate extracts showed activity, although the main extraction performed for the study was performed with ethyl acetate alone. A TLC of the hexane extract showed that its composition was very similar to that of fractions BF1-2, and contains both PP1 and PP2. In a previous study (Pistelli et al. 1995) PP1 had also been isolated from the leaves of BF. The presence of PP1, which appears as a large red band when sprayed with vanillin and sulphuric acid can be seen on the TLC of a petroleum ether extract of the leaves, and the same leaf extract also showed activity in the luciferase assay.

The luciferase assay NF-κB testing facilities were in place at the Centre when this study on BF commenced, and this, in comparison to the previous study on OM enabled a much more comprehensive bioassay guided fractionation to be performed. However, almost all the fractions and subfractions obtained from the ethyl acetate extract showed activity when tested in the NF-κB assays, which suggests it is likely there may be several other bioactive components still remaining which have not been isolated in this study. Cytotoxicity was also observed in most fractions, which may also account for the results seen in the bioassays.

PP1-3 bear some similarity to the vanilloid capsaicinoids present in the Capsicum species, which have an aromatic ring with a hydroxyl and methoxyl group, with a
long aliphatic side chain. They also showed a similar level of activity, at about 50 μM. There is also some similarity with curcumin, which also has a methoxylphenyl group connected to long straight chain unit with two carbonyl groups, although it differs with an additional aromatic group.

The results of the NF-κB assays complements previous biological data performed on BF. The results also seem to support the traditional use of BF as an anti-inflammatory agent, although the cytotoxicity of the extract seen in the assays would seem to suggest that its use would not be appropriate. The papers do not detail which parts of the plant are traditionally used in this way, possibly only the leaves or the flowers are used, which may be less toxic than the stems. Traditional methods used in preparing and administering the plant material may also somehow reduce the toxicity of the extract. In addition, cytotoxicity seen in vitro for one particular cell type may not necessarily translate to toxic effects in vivo.

It may have been worth performing MTT assays on these compounds, as even at 50 μM there were some signs of cytotoxicity. Such signs included visual observation of slight cytotoxicity, as well as occasional negative luciferase activity readings, which were less than those obtained for the negative controls. Although the observed cytotoxicity of the compounds at 50 μM was slight and borderline, this may possibly be the reason for the results given in the assay. In the assays the cells are incubated with the cells for 7 hours, and it is likely that if incubated for longer, more pronounced cytotoxic effects would appear. The compounds also quickly lose the NF-κB activity once the concentrations are reduced from 50 μM.
More biological testing is being performed on the compounds by the other laboratories in the EU collaboration, the results of which may provide a clearer picture on their pharmacological activity.
APPENDIXES

1. FABMS for calodenin B and dihydrocalodenin B
2. FABMS for ochnone and ESMS for compound X
3. $^{13}$C NMR spectrum for ochnone with cordigol contaminant
4. $^1$H NMR spectra for ochnone with cordigol contaminant
5. $^1$H NMR spectrum for cordigol (δ 2.5-4) showing protons at positions 3 and 4 and NOE spectrum for ochnone with cordigol contaminant
6. COSY and HMQC spectrum for ochnone with cordigol contaminant
7. HMBC spectrum for ochnone with cordigol contaminant
8. EIMS for dehydroxyhexaspermone C
9. EIMS for hexaspermone C
10. FABMS for DHHC and HC
11. $^{13}$C NMR spectra for HC (CDCl$_3$) and DHHC (acetone-d$_6$)
12. $^1$H NMR spectrum for DHHC (acetone-d$_6$)
13. HMQC and COSY spectra for DHHC (acetone-d$_6$)
14. HMQC and HMBC spectra for HC
15. UV spectra and HPLC trace for HC and DHHC
16. FABMS for PP1 and PP2, EIMS for PP2
$^{13}$C NMR (acetone-d6) spectrum for ochnone (with cordigol contaminant)
$^1$H NMR spectrum for ochnone (acetone-d6) (with cordigol contaminant)
$^1$H NMR (acetone-d6) spectrum for cordigol ($\delta$ 2.5 - 4) showing protons at positions 3 and 4

NOE spectrum for ochnone (acetone-d6) (with cordigol contaminant)
'\textsuperscript{1}H-'\textsuperscript{1}H COSY spectrum for ochnone (acetone-d6) (with cordigol contaminant)

HMQC spectrum for ochnone (acetone-d6) (with cordigol contaminant)
OMSA 834 in Acetone-d6 SimonG
HMBC: long range H-x-C-C-C couplings

HMBC (acetone-d6) for caffeine
(with corrigal comments)
EIMS for DHHC
$^1$H NMR (acetone-d$_6$) spectrum for DHHC
OM55A in d6-acetone;  $^1$H- $^1$H COSY spectrum for DHHC (acetone-d6)
UV spectrum and analytical HPLC trace
(55% ACN in H₂O, 2ml/min)
for HC

UV spectrum and analytical HPLC trace
(55% ACN in H₂O, 2ml/min)
for DHHC
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