Immunomodulatory Effects of *Zingiber officinale* Roscoe var. *rubrum* (Halia Bara) on Inflammatory Responses Relevant to Psoriasis

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DECLARATION

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ABSTRACT

Psoriasis is a chronic autoimmune skin disease characterised by hyperplasia of epidermal keratinocytes and the accumulation of activated immune cells at sites of the disease. The disease is associated with aberrant activation of phagocytes, T-lymphocytes and the production of pro-inflammatory cytokines and chemokines. This thesis examines the therapeutic efficacy and mechanisms of action of the ginger species Halia Bara, or *Zingiber officinale* Roscoe var. *rubrum* (ZOR), on key immunopathogenic mechanisms relevant to psoriasis.

In-depth experiments first determined the effect of a ZOR extract in chloroform (HB02) and its fractions on nitric oxide (NO) and prostaglandin E$_2$ (PGE$_2$) production. The results of these experiments showed that HB02 and its fractions efficiently inhibited NO and PGE$_2$ production by activated macrophages. Extensive fractionation and characterisation experiments succeeded in identifying two compounds 6-shogaol (6S) and 1-dehydro-6-gingerdione (GD) with potent inhibitory effects on NO and PGE$_2$. These effects were comparable to dexamethasone and indomethacin.

Studies on the effects of HB02, its fractions and compounds showed inhibitory effects on the level of mRNA for iNOS, TNFα, IL-12p40 and IL-23p19 in pre-treatment experiments of macrophages. Studies of cell migration showed that the fractions and compounds from ZOR inhibited the migration of polymorphonuclear neutrophils (PMNs) through human vascular endothelial cells (HUVEC) by influencing CD11b expression and CD62L shedding. Further studies showed that the ZOR samples also inhibited the activation of
CD8$^+$ cytotoxic T-lymphocytes and reduced CD25 and CD69 expression. Furthermore, an *in vitro* model of epidermal inflammation showed that ZOR directly inhibits keratinocyte proliferation and the production of IL-20 and IL-8, both key psoriasis-promoting cytokines.

The studies reported in this thesis provide experimental evidence for potent anti-inflammatory properties of ZOR and for potential mechanisms of action in ameliorating psoriasis.
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<td>DEPT</td>
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CHAPTER 1

General Introduction
1 Psoriasis: Immunological aspects

1.1 The Immune system

The immune system is highly evolved to protect one from infectious organisms. It is broadly organised into two compartments or arms, innate and adaptive, each consisting of cells, receptors and proteins that cooperate to deal with infectious pathogens and cancer. These arms of the immune system provide effective immunity during infections and is then followed by lasting immunity against the specific pathogen (immunological memory). An acute inflammatory response is a feature of the immune response following an infection and this aids in the recruitment of various effector components of that result in the killing of the infectious agent (bacteria, viruses or parasites) while facilitating wound repair. However, persistent and prolonged chronic immune responses and inflammation are associated with the development of immune-mediated chronic diseases, such as rheumatoid arthritis (RA), Crohn’s disease and psoriasis.

The innate immune system constitutes the first line of defence against a wide range of microorganisms during infections, providing immediate protection without requiring prior exposure. Components of the innate immune system express conserved receptors that recognise and interact with pathogen-associated molecular patterns (PAMPs). Such conserved receptors include toll-like receptors (TLR), mannose receptors and scavenger receptors. PAMPs are small molecules unique to a group of related microorganisms such as bacteria and virus, and not expressed by human cells. PAMPS are recognised on invading microorganisms by conserved receptors on innate immune cells and are then engulfed and destroyed at the site of infection by phagocytic cells such
as macrophages, monocytes, and polymorphonuclear leukocytes (PMN). In addition to recognition and destruction of infectious pathogens, cells of the innate immune system help to prime the adaptive immune system by processing and presenting pathogens to T- and B- lymphocytes at sites of infection and also in secondary lymphoid organs. Upon receptor binding with PAMPS, phagocytes react by releasing chemical mediators, known as cytokines and chemokines that collectively lead to inflammation. Other than the release of cytokines/chemokines, innate immune cells also help combat infectious pathogens by releasing inflammatory mediators such as nitric oxide (NO) and prostanoids.

Cytokines are small cell-signalling molecules that are protein, peptides or glycoproteins, grouped into lymphokine, chemokines or interleukins depending on the originating cell and on their functions. They are produced by various cell types, particularly immune cells and cells of the nervous system in response to inflammation, infection and stress. Cytokines display redundancy in their functions. They are also multifunctional, involved in intercellular signalling, in systemic immune-modulation and in embryogenesis. Cytokines can act in both autocrine and paracrine manner and activate cascades of biological responses upon binding to their receptors. Cytokines can induce different biological responses depending on the cell type, activation status of the cell and previous or simultaneous exposure to other cytokines. The different and, at times, paradoxical, biological effects of cytokines can be important in the pathogenesis of chronic diseases such as psoriasis as will be discussed in this thesis.
Another key molecule relevant to this study that plays important roles in immunity, inflammation and immune regulation is nitric oxide (NO). NO is a short-lived free radical with a range of physiological and pathological effects including vascular homeostasis, neurotransmission and immunity (1). NO is produced from L-arginine by nitric oxide synthase (NOS). There are three isoforms of NOS: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). eNOS and nNOS are constitutively-produced and Ca2+/calmodulin-dependent. These two isoforms only synthesize small amounts of NO, triggered by various agonists (2). iNOS, however, is calcium-independent and is expressed in response to certain stimuli such as endotoxins, lipopolysaccharide (LPS) and cytokines.

In macrophages, iNOS is the key regulator of NO production which, in low concentrations, provides for host protection against bacterial and viral infections. NO eliminates pathogens by direct killing of the organism and/or weakening the mechanisms, used by the pathogen to avoid immune recognition. Prolonged inflammation, however, leads to the production of high levels of NO which, in turn, leads to inflammation-associated cellular and tissue damage (2) as observed in chronic autoimmune disease. Nevertheless, there are also studies showing that NO could act as a negative regulator of inflammation, as shown by its ability to down-regulate the development of Th17 T-cells and, thus, potentially modulate autoimmune mechanisms in diseases such as RA and psoriasis (3). Interestingly, NO also plays pivotal roles in immunity independent of its pro-or anti-inflammatory effects.
In addition to cytokines and NO, arachidonic acid metabolites are also produced during inflammation. In response to a variety of nonspecific stimuli, phospholipase A\textsubscript{2} induces the production of arachidonic acid from membrane phospholipids which are further catalysed by cyclooxygenases 1 or 2 (COX-1/2) leading to the production of prostanoids such as prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), PGF\textsubscript{2α}, PGD\textsubscript{2}, PGI\textsubscript{2} and thromboxane (Tx) A\textsubscript{2}. Numerous studies have shown prostaglandins play key roles in pathophysiology involving complex interactions with many cell types including innate and adaptive immune cells (4-7)

Among the prostaglandins, PGE\textsubscript{2} is the most abundant in the body. The function and mechanisms of PGE\textsubscript{2} actions in host defences and in modulating adaptive immune responses have acquired particular interest and are widely studied. Apart from its role in driving inflammation (5, 8) and as a tumour inducer (9), a number of studies have suggested that it has an anti-inflammatory role (9), or even immune suppressor similar to non-steroidal anti-inflammatory drugs (NSAID) (4) and as an anti-cancer agent (10, 11). During bacterial infections, monocytes and macrophages produce PGE\textsubscript{2} and interleukin 12 (IL-12). Bacterial LPS and IL-1β stimulate COX-2 transcription via the nuclear factor κB (NF-κB) transcription factor leading to inflammatory cascades that ultimately promote T-cell responses (12). Eventually, the balance between PGE\textsubscript{2} and IL-12 production determines the type of T-cell response, whether T helper type 1 (Th1) or Th2. Subsequently, monocyte/macrophage-derived PGE\textsubscript{2} results in the activation and maturation of immature DCs. Activated/mature DCs then migrate to lymph nodes to serve as antigen-presenting cells (APCs) for naive T-cells (Th0 cells). Mature DCs produce chemotactic cytokines (chemokines)
stimulating Th1 or Th2 cell migration to sites of inflammation and leading to adaptive immune responses against pathogens.

Studies of the role of PGE$_2$ in adaptive immunity have revealed that exposure of DCs to PGE$_2$ leads to DC maturation with suppressed ability to secrete IL-12, thus, lowering their Th1-promoting capacity and promoting Th2 immunity (7, 12). This proposition that PGE$_2$ promotes Th2 immunity is supported by in vivo studies in BALB/c mice which showed Th2-dominant immune responses that were PGE$_2$-dependent (8, 13). More recently, it was observed that PGE$_2$ activates the Th17 pathway, increases IL-17 production while inhibiting IFN-γ production (14). The effect of PGE$_2$ on DCs and Th cells was speculated to occur through three potential modes of signalling (Figure 1.1). These are: 1) that PGE$_2$ acts through the EP-4 receptor on DC to produce IL-23 production which, in turn, promotes Th17 cell differentiation; 2) PGE$_2$ acts directly through the EP2 and EP4 receptors on naive T-cells leading to Th17 expansion by activating cAMP-PKA pathway; and 3) IL-12-induces Th1 differentiation through activating the phosphoinositide-3-kinase (PI3K) (8). Paradoxically, similar to NO, PGE$_2$ is a ubiquitous metabolite and could act as a pro- or an anti-inflammatory mediator depending on the micro environment. Hence, understanding the mechanisms involving PGE$_2$ release and actions could be beneficial in designing a new anti-inflammatory agent.
Figure 1.1: The diverse effects of PGE\textsubscript{2} on DC and Th1 and Th17 cells. PGE\textsubscript{2} produced during inflammation can: a) interact with EP-4 to stimulate DC to produce IL-23 via cyclic AMP (cAMP)-the EPAC pathway; b) interact with EP2 and EP4 receptors to facilitate activation of cAMP-PKA pathway that leads to IL-23-induced Th17 differentiation; c) interact with EP2 and EP4 to facilitate IL-12-induced Th1 differentiation through the activation of phosphoinositdie-3-kinase (PI3K). Adapted from Chizzolini et al., 2009 (8).

The presence of cytokines, free radicals and prostanoids further triggers the up-regulation of other pro-inflammatory cytokines and chemokines at sites of inflammation and, thus, increase the expression of many cellular adhesion molecules including selectins and integrins. The production of cytokines induces dilation of vascular smooth muscles and increases permeability of blood vessel which increases blood flow and outflow of fluid throughout the endothelium of blood vessel. These cytokines induce adhesion and migration of PMNs through the endothelium of the blood vessels to the site of infection inducing inflammation, which in turn causes pain.
Pathogens and other infectious agents invade and replicate in two intracellular compartments of the host cells; in the cytosol or in vesicles. During infection, peptides from antigen fragments derived from pathogens bind to major histocompatibility complex (MHC) proteins which present antigen peptides on the cell surface to activate antigen-specific T-cells. MHC class I (MHC-I) molecules deliver peptides from antigens produced into the cytosolic compartments to the surface creating peptide: MHC-I complexes are then recognised by cytotoxic CD8\(^+\) T-cells. CD8\(^+\) T-cells act immediately to eliminate cells infected with viruses and cytosolic bacteria. In contrast, extracellular pathogens that are endocytosed by immune cells and their products are processed in the vesicular compartment are presented within the context of MHC class II. These complexes are then recognised by helper CD4\(^+\) T-cells. This T-cell population is further classified into two functional subsets; Th1 cells which promote cellular immunity including the activation of macrophages to kill pathogens, and Th2 cells which primarily function by activating B cells to produce antibodies against pathogens (12).

Lymphocytes play pivotal roles in immune surveillance and upon stimulation by foreign antigens counteract danger by mounting specific immune responses and initiating appropriate effector mechanisms. Naive T-cells circulate between blood and secondary lymphoid tissues where they differentiate into effector Th1 or Th2 cells after encountering antigen presented by APCs in secondary lymphoid organs. Naive T-cells activated by IFN-\(\gamma\) develop and expand into Th1 cells while those activated by IL-4 expand into the Th2 cell lineage. Th1 cells produce IFN-\(\gamma\) and TNF\(\alpha\), and mediate immune responses against intracellular bacteria, viruses and tumour cells. Th2 cells mostly produce IL-4, IL-5 and
IL-13, and stimulate humoral responses against extracellular parasites. Another subset of Th cells, Th17 cells, which secrete IL-17, are involved in host defences and provide immunity to extracellular bacteria and fungi (15).

1.2 Immune responses within the skin

Skin is the largest organ in the body. It consists of the epidermis and the dermis. The main cell types in the epidermis are keratinocytes, melanocytes, Merkel cells, Langerhans cells and innate immune including macrophages and leukocytes. The epidermis can be further subdivided into the stratum-corneum, -lucidum, -granulosum, -spinosum and -basale layers (Figure 1.2a). Skin cell divisions occur in the basal layer (stratum basale) and then proliferate, differentiate and mature into specialised cells such as keratinocytes and melanocytes as it approached the epidermal outmost layer, the stratum-corneum. The dermis is the layer of skin beneath the epidermis and consists of connective tissue that protects the body from environmental elements including stresses and strains. The skin serves as an element of host defence, or barrier to the entry of foreign substances including infectious pathogens as it is equipped with a network of immune cells and resident cutaneous cells referred to as “skin-associated lymphoid tissue (SALT)” or “skin immune system” (16). SALT acts as a peripheral lymphoid organ that provides instantaneous immune protection against pathogens and environmental changes in skin (16, 17).
Langerhans cells (LCs) are dendritic cells (DCs) of the epidermis. They are normally present in the stratum spinosum and lymph nodes. Studies have identified distinctions between LCs and dermal DCs in terms of their roles in mediating immune responses in skin-draining lymph nodes. Thus, whereas LCs have prominent roles in the induction of T-cell responses, such as cell activation, dermal DCs induce humoral responses (18). As mentioned earlier, resting DCs have an essential ‘house-keeping’ role in maintaining tissue homeostasis. However, in an environment of chronic inflammation such as in autoimmune disease involving the skin, such as psoriasis, DCs mature into inflammatory cells that sustain and exacerbate the inflammatory response.

Keratinocytes, the largest cell component of the epidermis, play an integral part in skin-associated immune responses. Thus, keratinocytes can function as APCs, produce innate immune mediators, and subsequently stimulate skin homing and local activation of immune cells. Many types of TLRs are expressed by keratinocytes that have been activated in response to microbial stimuli. Engagement of TLRs induces keratinocytes to release pro-inflammatory cytokines including TNFα, IL-1α, IL-6 and IL-18, chemokines such as IL-8 and chemokine ligand 20 (CCL20) and antimicrobial peptides such as human β-defensin (HBD) -2, HBD-3 and Cathelicidin (or LL37 peptide) (19).
Numerous studies have suggested that crosstalks between immune cells and keratinocytes act as the central mechanism that maintains skin immune homeostasis. However, altered crosstalk due to defects in the regulation of multiple genes could initiate, sustain and exacerbate skin inflammation as seen in skin disease including psoriasis, atopic dermatitis and skin allergy. Autoimmune diseases result from a hyperactive immune system attacking normal tissues as if they were foreign organisms. This results in a chronic process and often leads to disruption of immune homeostasis and complete destruction of the target tissue. Figure 1.2 shows the difference of skin morphology in (b) normal and (c) psoriatic skin. In psoriatic skin, there is increased keratinocyte proliferation, disrupted terminal differentiation and excessive infiltration of immune cells. In contrast with normal skin, psoriatic
lesions are characterised by elongated epidermal rete and thickened skin which exhibit the characteristics of disorganized cornified layer (stratum corneum) at the skin surface (Figure 1.2c).

1.3 The pathology of psoriasis

Psoriasis is an autoimmune disease mediated by aberrant T lymphocyte and B lymphocyte responses. The aetiology of psoriasis is unknown but there is good evidence that susceptibility to the disease involves the interaction between genetic and environmental factors (20-23). Clinically, the disease is characterised by the appearance of red scaling plaques that range from a few lesions to total coverage of the skin. The disease is non-contagious and while symptoms can be managed by medical intervention there is, at present, no cure. Six clinical sub-types of psoriasis have been clinically distinguishable and these range from mild, severe and chronic plaques. These are plaque, guttate, pustular, inverse, erythrodermic and psoriatic arthritis (Figure 1.3a-f). The most common type of psoriasis is the plaque-type psoriasis (Figure 1.3a). Psoriatic lesions are characterised either as localised, or generalised, plaques asymmetrically distributed in the vicinity of the elbows, knees, ears, umbilicus, gluteal cleft, joints, nails and scalp. The localised plaque-type psoriasis is the least severe type that can be treated with topical therapy whereas the generalised plaque-type is the most severe and needs oral medications, ultraviolet light treatment and the provision of medical care as outpatients or inpatients (24).
Guttate psoriasis is characterized by the presence of small, scattered papules that develop after an acute upper respiratory tract infection (Figure 1.3b). Pustular psoriasis is characterised by the appearance of blisters of non-infectious pus on the skin (Figure 1.3d). This subtype may be triggered by medication, infection, stress or exposure to certain chemicals. Most severe psoriatic patients experience smooth red patches in the folds of the skin, near the genitals, or armpits and this type is also known as inverse psoriasis (Figure 1.3e). More severe types can be seen on patients with erythrodemic psoriasis which is characterised by widespread reddening and scaling of the skin (Figure 1.3f). The occurrence of this type of psoriasis may be due to severe sunburn, or a prolonged period of increased activity of psoriasis which is poorly controlled. Lately, severe psoriasis was found to be associated with the occurrence of arthritis and this type is referred to as psoriatic arthritis (Figure 1.3c).
Figure 1.3: Clinical sub-types of psoriasis. Psoriasis are categorised clinically into: a) Plaque; b) Guttate; c) Psoriatic arthritis; d) Pustular; e) Inverse and f) Erythrodermic psoriasis. Among these, occurrence of plaque psoriasis is the most prevalent. Adapted from https://www.psoriasis.org with consent.
Lesion formation is caused by excessive growth and aberrant differentiation of keratinocytes where the normal differentiation process has been altered (Figure 1.2c). The granular layer in the psoriatic skin is reduced or absent. The abnormal differentiation process caused by the failure of the corneocytes to stack normally causes scaling and consequential break of the cutaneous skin (25). Altered differentiation of keratinocytes is equivalent to the “regenerative maturation”, a cell differentiation process momentarily expressed during wound repair (25).

Other features of the disease include visible redness of lesions. This is caused by marked dilation of blood vessels due to the adherence of lymphocytes, monocytes and PMNs to endothelial cells. Endothelial cells in psoriatic lesions are activated and have characteristics of the high endothelial venules present in lymph nodes. During the inflammatory process, the COX pathway is activated and this causes the release of PGE₂ which promotes the dilation of capillaries and, thus, facilitates a chemoattractant environment for PMN. Such an environment favours the migration of intermixed T-cells and dendritic cells (DCs) to the lesion whereby the lesion functions as an organized lymphoid tissue. Leukocytes transmigrate to the reactive blood vessel while resident leukocytes expand and become dense infiltrates. Studies using immunostaining detected the presence of PMNs within small foci in the stratum corneum and significant amount of mononuclear infiltrates in the epidermis, as well as in the dermis region and in the hyperplastic blood vessels between the epidermal rete within the psoriatic skin (25). These immune cells are the source of the pro-inflammatory factors that induce the formation of psoriatic lesions.
The pathogenic mechanism that underpins psoriasis basically involves the interaction between resident skin cells and an altered immune system that mediates a combination of innate and adaptive effector immunity. In a normal healthy skin, keratinocytes mediate the trafficking of immune cells. In susceptible individuals, however, the trafficking of immune cells, especially leukocytes, is higher and trafficking cells are activated.

1.4 Immunopathogenic mechanisms in psoriasis

Potential inflammatory pathways likely to be involved in psoriasis pathogenesis have been extensively studied. Increased interest in this area of research has been driven by the increasing number of patients diagnosed with the disease. Multistage immunopathological pathways have also been suggested to be involved in the induction of asymptomatic skin lesions that lead, eventually, to psoriatic plaques. According to these scenarios immunopathological processes that underpin psoriasis pathology could be triggered by extrinsic signals (pathogen-associated signals), or by intrinsic signals, such as by heat-shock proteins, HIV-1 infection or by medications. This leads to a sensitisation phase whereby naive T-cells evolve into effector, or memory T-cells under the influence of APCs including DCs in secondary lymphoid organs (Figure 1.4). This stage is followed by an asymptomatic phase which can vary in duration. The next phase is the effector phase, which involves the infiltration and activation of T-cells and other immune cells. These events are then followed by visible skin alteration during which keratinocytes respond to the activation of immune cells. The condition is, thus, an overshoot of the skin regeneration process. Currently, patients are treated during this stage of the disease. Even
though patients do recover from skin lesions, the chances of reoccurrence are still high whereby the disease veers from the silent to the effector phase under the influence of intrinsic or extrinsic factors.

**Figure 1.4: The phases of psoriasis pathogenesis.** a) Sensitisation phase involves the induction of effector and memory T-cell as a result of interaction with activated DC in secondary lymphatic organ; b) The silent phase is an asymptomatic stage of psoriasis; c) The effector phase involves the interaction between infiltrating immune cells and keratinocytes leading to an overshot of skin re-generation. Adapted and modified from Sabat et al., 2007(26).

### 1.4.1 The role of T-cells in the pathogenesis of psoriasis.

There are several lines of evidence supporting the hypothesis that psoriasis is mediated mainly by T-cells. Psoriatic patients treated with T-cell targeting drugs such as Ciclosporin (27, 28), or given allogeneic bone marrow transplantation from normal individuals show clinical improvement (29). The majority of studies implicate pathogenic roles for CD8$^+$ and CD4$^+$ T-cells as well as for Th1-type cytokines in stimulating keratinocyte proliferation and plaque formation (30).
CD4\(^+\) T-cells normally reside at the dermis layer whereas CD8\(^+\) T-cells, which when activated upregulate CD25, CD69 and HLA-DR expression, are found in the epidermis at twice the number of CD4\(^+\) T-cells. The differentiation of T-cells at plaque sites appears to be polarized towards Th1 cells, based on their ability to produce IFN\(\gamma\) and TNF\(\alpha\) (31).

Evidence implicating Th1- and Th17-induced inflammatory pathways in the pathogenesis of psoriasis is demonstrated by a linear relationship between proximal inducer cytokines, such as IL-23, IL-12, IFN-\(\gamma\), TNF\(\alpha\) and activation of many IFN-responsive genes through signal transducer and activator of transcription (STAT) -1 and/or -3 (25) (Figure 1.5). Induction of the cascades leads ultimately to the production of keratinocyte-derived cytokines such as ECGF, VEGF and PDGF as well as IL-1\(\alpha\), IL-7, IL-19, IL-20 and IL-22 which play parts in keratinocytes proliferation.

Th1 cells are activated by APCs that produce IL-12. In contrast, differentiation of naive T-cells to Th17 cells is promoted by the presence of IL-23. The increased proportion of Th17 in psoriasis has been associated with single nucleotide polymorphisms (SNPs) in the \(il-12b\) gene which encodes for IL-12/IL23p40, the subunit of IL-12 and IL-23, as well as in the \(il-23r\) gene. Studies of cytokine expression in psoriatic skins have identified high levels of IL-12/IL-23p40 and IL-23p19 and an abundance of Th17 cells (32). Understanding the IL-12/IL-23p40 subunit is regulated has led to the establishment and application of Ustekinumab, an IL-12/IL-23 inhibitor for the treatment of psoriasis (33-35).
Figure 1.5: Involvement of Th/Tc1 and Th17 in the pathogenesis of psoriasis. Interactions between Inflammatory DCs (IDCs) such as TIP-DCs (TNFα and iNOS positive DCs), plasmacytoid DCs (pDCs) and T-cells promotes Th1 and Th17 differentiation and inflammation mediated by these cells subsets. This inflammation involves the production of chemokines, cytokines including IL-23, IL-12, IL-17, IL-22, IL-20, TNFα and IFN-γ. These cytokines, in turn, enhance the production of innate defence products upon keratinocyte activation Adapted and modified from Lowes et al., 2007 (25).

The infiltration of Th17 cell leads to the production of IL-17 and IL-22. IL-22 is a critical cytokine in the establishment of an inflammatory environment that promotes autoreactive T-cell activation. Production of IL-17 is also facilitated by IL-22. Thus, Ma et al. (2008) reported that blockade of IL-22 resulted in a decrease in the number of Th17 cells, but, surprisingly, led to an increase in IFN-γ (Th1 cytokine) production (36). This finding indicates that IL-22 regulates both Th17 and Th1 cells.
Although Th1 and Th17 cells are highlighted as key players in the pathogenesis of psoriasis, CD8\(^{+}\) cells or cytotoxic cell (Tc), also play important roles in initiating and sustaining the inflammatory process in psoriasis. CD8\(^{+}\) cells are known to be involved in innate and adaptive immune response (37). CD8\(^{+}\) cells can be subdivided into two subpopulations, termed Tc1 and Tc2 in accordance with the Th1 and Th2 T-cell paradigm. Tc1 cells are characterised by the production of IFN\(\gamma\), TNF\(\beta\) and IL-2 whilst Tc2 cells produce IL-4 and IL5. According to studies by Ovigne et al. (2001), CD8\(^{+}\) T-cells of the Tc1 subtype found in patients with chronic psoriasis plaques produce higher levels of IFN\(\gamma\) compared with matched healthy controls (30). IL-4 and IL-10, however, were either found at low levels, or not detected following stimulation with PMA. Studies by Torres-Alvares et al. (2007) revealed that CD8\(^{+}\) T-cells expressing CD69 were consistently found in psoriatic plaques after treatment with methotrexate while other inflammatory cells were reduced in number (38). CD69 is an activation inducer molecule involved in activation, differentiation and proliferation of T-cells. It is also persistently expressed on CD8\(^{+}\) infiltrating cells in RA (39). The persistence of CD8\(^{+}\)CD69\(^{+}\) T-cells in psoriatic plaques was thought to contribute to the reactivation and recurrence of psoriasis after cessation of treatment (38). Most recently, the pathogenesis of psoriasis was associated with another T-cell subset, Th22 cells. The notion that expansion of Th1 and Th17 cells are indirectly influenced by IL-22 suggested that IL-22 could play a specific role in psoriasis. The link between Th17 and Th22 is that both are activated by IL-23, denoting IL-23 as the possible key cytokine in psoriasis pathogenesis. However, there is no evidence that Th22 is a Th-17 subtype in that Th22 cells only produce IL-22 but not IL-17 (32). Nevertheless, Th17 cells produce both IL-17 and IL-22. Furthermore, in innate immune responses, Th22
cells are stimulated by Langerhans cells (LC) whilst Th17 cells are activated by CD11\(^+\) dermal DCs \((40)\). IL-22 has been shown to demonstrate additive or synergistic pathogenic effects with IL-17 and with other cytokines \((41)\). IL-22 itself, however, does not have profound effects in psoriasis. IL-22 and IL-17 have been shown to synergistically induce the secretion of anti-microbial peptides in the keratinocytes \((42, 43)\). The presence of IL-22, however, has limited effects on the expression of chemokines such as CXCL1 and CXCL3 when compared with IL-17.

In epidermal keratinocytes, IL-22 induces IL-20, an upstream inflammatory product in the epidermis of psoriatic skins, which specifically cause phenotypic skin changes including hyperproliferation and aberrant differentiation. Although IL-22 has been shown to induce hyperproliferation and altered differentiation in epidermal keratinocytes in vitro that resemble acanthosis, its role in the manifestation of psoriatic lesions might be magnified by presence of IL-20 \((41)\). Nevertheless, detailed studies on the role of the Th22 pathway in psoriasis might be an interesting area to be explored in the future.

### 1.4.2 Role of dendritic cells and macrophages in psoriasis.

Myeloid DCs \((CD11c^+)\), which are abundant in the dermal layer, are known as TNF\(\alpha\) and inducible nitric oxide synthase (iNOS)-producing DCs, or TIP-DC. In addition to producing high levels of TNF\(\alpha\) and iNOS, TIP-DCs produce IL-23, IL-12 and IL-20 which activate T-cells and upregulate gene transcription in keratinocytes through activation of STAT-3 (Figure 1.5). TIP-DCs are found in large numbers in psoriatic lesions and, in some cases, outnumber T-cells in
psoriatic plaques (31). Cytokines produced by TIP-DCs play critical roles in initiating Th1 and Th17 cell differentiation and proliferation.

DCs and macrophages also produce NO in response to mediators of inflammation (44). NO has been shown to potently-regulate keratinocytes growth and differentiation that are implicated in psoriasis and studies have shown that this could be influenced by NO synthase gene polymorphism (45). More recently, upregulation of NO has been noted even at the mesenchymal stage of skin development as high immune expression of iNOS in a comparative study carried out in psoriatic and atopic dermatitis patients and normal individuals (46). This preliminary study proposed that psoriasis has an early onset that could develop as early as the mesenchymal stage. Mesenchymal cells, thus, can produce angiogenic and pro-inflammatory mediators which contribute to the pathophysiology of psoriasis.

Some DCs mediate direct effector immune functions and have significant effects on the inflammatory process. These DCs are known as “inflammatory” DCs (IDCs). IDCs include plasmacytoid and myeloid DCs (pDCs and mDCs, respectively), which are responsible for bridging the innate and adaptive immune systems.

1.4.3 Crosstalk between DCs, T-cells and keratinocytes in psoriasis.

Overall, interactive cellular responses in psoriatic lesions appear to be due to an imbalance between the activation of both innate and acquired immune systems and factors produced by keratinocytes that directly influence T-cells and DCs.
PMNs, mDCs and CD11c⁺ mDCs are effector innate immune cells that are stimulated by chemokines produced by keratinocytes in the epidermis. Consequently, immune cell-derived cytokines produced through this interaction activate the transcription of genes encoding pro-inflammatory proteins and those that promote keratinocyte proliferation. Guttman-Yassky et al. (2007) proposed two models for the pathogenesis of psoriasis based on numerous reports of therapeutic approaches, histological and immunological studies (Figure 1.6) (31). The first model is based on the presence of primary growth defects in keratinocytes which initiate regenerative activation by various immune-activating cytokines and chemokines. This model is built on passive immunity in which cytokines and chemokines play significant roles in activating and recruiting leukocytes. The second model focuses on the role of activated leukocytes as the initiator of the inflammatory process by infiltrating focal skin regions and inducing epidermal hyperplasia. Following activation, keratinocytes produce further immune mediators which exacerbate the inflammatory response. These two models support a link between innate and adaptive immunity in initiating the pathogenesis of psoriasis (25).
Figure 1.6: Proposed models for the immunopathogenesis of psoriasis. Two types of immunity could underpin the immunopathogenesis of psoriasis: a) passive immunity in which leukocytes are activated by cytokines and chemokines produced by keratinocytes (KC), and b) active immunity in which DCs stimulate a conventional T-cell mediated immune response. (Adapted and modified from Guttman-Yassky et al., 2007)(31).

2 Factors that predispose to the development of psoriasis.

2.1 Genetic susceptibility factors

The development of psoriasis has been associated with a number of genetic loci including genes in the MHC-I locus (10% penetrance) known as Psoriasis Susceptibility 1 (PSORS1) locus. The PSORS1 includes genes for the human leukocyte antigen (HLA) Cw6 (HLA-Cw6). Several other psoriasis susceptibility loci have been identified (47). These include PSORS2 on 17q, PSORS3 on 4q,
PSORS4 on 1cen-q21, PSORS5 on 3q21, PSORS6 on 19p, PSORS7 on 1p, PSORS8 on 16q and PSORS9 on 4q31.

The available evidence indicates that HLA-Cw6 contributes to the onset of psoriasis and severity of lesions. Studies on HLA association with psoriasis indicate that psoriatic patients with HLA-Cw6 have an early age of disease onset compared with psoriatic patients with other types of HLA-C (48). Most Cw6-positive patients have severe symptoms such as observed in patients with guttate psoriasis. Indeed, Gudjonsson et al. (2002) observed that the clinical features of guttate psoriasis in patients depended on the HLA-Cw6 gene (48). Patients with the HLA-Cw6 allele experience more severe plaques and higher incidences of the Koebner phenomenon. The Koebner phenomenon is a condition of a worsening psoriasis that occurs along the site of injury. Gudjonsson and colleagues concluded that early age onset of psoriasis is associated with homozygosity for HLA-Cw*0602 (49). Nair et al. (2006)(50) confirmed that HLA-Cw6 is the PSORS1 risk allele and Azfar et al. (2008)(51) suggested that HLA-Cw6 was a key factor in pathogenicity of psoriasis, but may not be the only risk allele within the PSOR1.

Other susceptible loci contributing to the genetic predisposition to psoriasis are genes expressed by keratinocytes, LCE3B (late cornified envelope 3B) and LCE3C1 (late cornified envelope 3C1), which are involved in skin barrier functions and host defence. Environmental triggers, along with LCE3B/ LCE3C1 gene defects, alter the skin barrier functions and contribute to the formation of self-DNA/RNA and microbicidal cathelicidin (LL37) complexes (Figure 1.7). A recent study proposed that this scenario could initiate immune responses and
inflammatory cascades in psoriasis (19). The self RNA/DNA LL37 complexes stimulate maturation of mDCs and the production of IFN-α by pDC. Mature DCs induce the production of pro-inflammatory mediators that lead to differentiation and expansion of Th0 into Th1 cells (IL-12), Th17 (IL-23, IL-6 and TGF-β1) and Th22 (TNFα and IL-6). Presence of Th1 and Th17-related cytokines stimulate CCL20 production by keratinocytes, which subsequently promote migration of CCR6-expressing DCs and T-cells in the psoriatic skins. Th17-related cytokines induce DCs and keratinocytes to produce IL-20 which eventually promotes keratinocyte hyperproliferation. Activated keratinocytes further enhance DC activation and local inflammation by releasing inflammatory cytokines namely IL-1β, IL-6 and TNFα. Besides that, psoriasis is also associated with excess IL-23 production and activation of the NF-κB pathway (52). Thus, the PSORS1 locus includes HLA-C, three IL-23-related genes (IL-23A, IL-23R and IL-12B), two genes that encode proteins which act downstream of signaling triggered by TNFα binding to its receptors that, in turn, activate the NF-κB pathway (TNIP1, TNFAIP3) and il-4 and il-13 genes, which encode IL-4 and 13, respectively. These two cytokines are key factors in promoting Th2-mediated responses.

In vitro studies by Reich and colleagues (53) suggested that the early onset of psoriasis might be due to gene polymorphism associated with altered cytokine production. The inflammatory process in psoriasis is characterised by over-expression of TNFα and IL-1β and down regulation of anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist (IL-1RA). This indicates that psoriasis is associated with a cytokine imbalance.
Figure 1.7: Environmental triggers and genetic defects affecting skin barriers. These factors have been proposed to initiate the activation and maturation of DCs. The activated DCs then produce cytokines that stimulate differentiation of effector T-cells, Th1, Th17 and Th22 cells that promote keratinocytes hyper-proliferation and increase migration of inflammatory cells to the skin which, in turn, exacerbate inflammation and promote epidermal hyperplasia. Adapted and modified from Monteleone et al., 2011(19).
2.2 Environmental and non-environmental factors that contribute to the pathogenesis of psoriasis.

Although psoriasis is strongly associated with genetic factors not everyone with the known susceptibility alleles will have the disease nor do all patients have plaques or severe disease. Thus, psoriasis and psoriatic flares are also influenced by a number of environmental factors such as skin injury, stress, smoking and alcohol consumption.

Skin injury could lead to the formation of psoriasis plaques in susceptible individuals due to the Koebner phenomenon. The Koebner phenomenon is depicted by the appearance of psoriatic lesions in uninvolved skin of psoriatic patients due to trauma and injury (54). The appearance of psoriatic skin lesions in susceptible individuals might occur 2-6 weeks after skin injury. Such skin injury could be caused by abrasion, sunburn, viral rashes, drug-induced rashes or irritation due to clothes upon skin rubbing.

Weather changes could also contribute to the development of psoriasis. The exposure to different weathers could also determine severity. Generally, psoriatic lesions are milder in warmer than cooler seasons. Psychological wellbeing could also be a factor in susceptibility to psoriasis. Stress has been identified as one of the factors that influence the development and prognosis of psoriasis. Stress could trigger, or worsen, conditions of psoriatic plaques. Individuals with stressful lifestyles or character are more prone to severe psoriasis compared with individuals with a relaxed character. However, the evidence also indicates that individuals with severe psoriasis experience more
stress and have lower quality of life (QoL) (55) due to their physical appearance and other disease side effects. However, the mechanism by which stress increases severity of psoriasis is not well understood.

Bacterial and viral infections can also lead to the development of psoriasis. Guttate psoriasis in children, for instance, could be caused by streptococcal infection, tooth abscesses, cellulites and impetigo (56). In addition, co-administration of incompatible drugs are reported to induce, or worsen psoriasis flares (57). For example, angiotensin-converting enzyme inhibitors, beta-adrenergic blockers, lithium and anti-malarial agents and hydroxychloroquine (Plaquenil) have all been reported to be exacerbating factors for psoriasis (58).

According to an Italian case study, smoking, or nicotine exposure and alcohol consumption are factors that could be linked to the initiation of psoriasis (22). Interestingly, the occurrence of pustular lesions is strongly associated with smoking habits, in a dose-dependent manner. Nicotine as well as the other 400 substances identified in cigarettes could lead to oxidative damage in the body which then causes the activation of phagocytic cells. Phagocytic cells produce reactive oxygen species and chemokines. Other studies have also shown that the nicotinic cholinocytes stimulate Ca^{12} influx and accelerates keratinocyte proliferation (58). Furthermore, heavy and chronic alcohol consumption worsens the existing inflammatory conditions (59), which contribute to the imbalance between oxidants and antioxidants in psoriatic patients (60). This, in turn, leads to the up-regulation of TNFα converting enzyme (TACE) and increases soluble TNFα receptor type 1 (sTNF-R1) (60). TACE is a metalloproteinase-disintegrin
which releases TNFα and its receptors and generates the soluble and mature form of the molecules. TNF-R1 binds to TNFα and stabilizes the molecule, thus, triggering associated inflammatory cascades.

Overall, based on the available knowledge of the aetiology and immune mechanisms prevalent in psoriasis, specific gene or drug therapies could be highly desirable to disrupt the vicious cycles and re-programme the immune system to maintain homeostasis.

3 Treatment of psoriasis

Early treatments for psoriasis were typically serendipitous observation and no specific mechanism of disease was targeted. These treatments included arsenic (Fowler’s solution) and ammoniated mercury. The overall aims of current treatments are to control the initiation and progression of the disease by reducing the volume of lesions and percentage of body surface areas involved, promote remission, minimise side effects and improve quality of life (67). Topical therapy is the most commonly used treatment in mild-severe forms of psoriasis. The most common topical therapies for mild conditions are corticosteroids, calcipotriene (Dovonex), coal tar products, tazarotene (tazaroc), anthralin (Anthra-Derm) and keratolytic agents. Topical therapies used for localised and generalised plaque-type psoriasis are prescribed to patients with moderate to severe psoriasis who are resistant to the above-cited topical therapies. These patients are generally treated with systemic therapies such as intra-lesional corticosteroid injections, phototherapy and systemic therapy with oral retinoid, acitretin, methotrexate, Ciclosporin and biologic agents. However,
the appropriate therapeutic approach for each patient varies depending on the patient's physiological, immunological and psychological response (61). Based on clinicians' points of view, combination, rotational or sequential therapies are more efficacious than administration of monotherapy which has shown to be less effective in addition to potentially causing more side effects (61). Among systemic agents used, methotrexate (MTX) and Ciclosporin are most frequently prescribed individually or in combination with other treatments with appropriate dosages and monitoring. A study revealed that both drugs were equally effective and tolerable in patients with moderate-severe-psoriasis (62).

3.1 Methotrexate.

Methotrexate (MTX) is the drug of choice for treating psoriasis despite the systemic side effects it can cause. Administration of this drug needs careful monitoring because it can cause hepatotoxicity, gastrointestinal, malaise, headache, reactivation of phototoxic reactions, ulcerative stomatitis, myelosuppression, anemia, pneumonitis, pulmonary fibrosis, induction of lymphomas and teratogenicity (61). The mechanism of MTX action in psoriasis involves blocking DNA synthesis in hyper proliferating epidermal cells, inhibiting T- and B-lymphocyte responses and interrupting cytokine production (61).

In a 16-week randomized and, controlled trial to investigate the efficacy of MTX monotherapy in patients (N=85) with moderate-to-severe plaque psoriasis, 60% (26/43) of MTX-treated patients achieved Psoriasis Area and Severity Index 75 (PASI 75) and 40% (17/43) achieved PASI90, compared with 71% (P=0.29) and 33% (P=0.55) with Ciclosporin-treated patients, respectively. PASI is an
estimate of the severity of psoriatic lesions (redness, scaling and thickness) and the area affected into a single score in the range 0 (no disease) to 72 (maximal disease). Among patients studies by Barker and colleagues (2011), twelve discontinued MTX because of elevated liver enzymes (63). Thus, despite its effectiveness, MTX should be used with caution because of its systemic side effects.

### 3.2 Ciclosporin

Ciclosporin is a cyclic non-ribosomal peptide of 11 amino acids that contains a single D-amino acid, which is rarely encountered in mammal biochemistry. It was isolated from the soil fungus *Tolypocladium inflatum Gams* when Borel and colleagues were searching for novel antifungal agents in 1970 (64). In 1979 it was observed to demonstrate anti-psoriatic activity (64, 65).

Ciclosporin is an immunosuppressant drug widely used in post-allogeneic organ transplantation to reduce the risk of organ rejection and acts by suppressing the activity of patients’ immune system. It has been used in organ transplants, treatment of psoriasis, severe atopic dermatitis, pyoderma gangrenosum, chronic autoimmune urticaria and, infrequently, in RA and related diseases. Ciclosporin acts by specifically targeting Th cells and, indirectly, by affecting T-suppressor cells or cytotoxic cells by inhibiting IL-2, an important cytokine for T-cell activation (65). Ciclosporin forms a complex with cyclophilin and inhibits the activity of calcineurin and, thus, indirectly inhibits phosphorylation of nuclear factor of activated T-cells (NFAT) transcription factor (28, 65). Consequently,
NFAT is not transported to the nucleus for the initiation of IL-2 gene transcription. In addition, Ciclosporin downregulates keratinocytes hyperproliferation (66), histamine release by skin mast-cells (67) and expression of cellular adhesion molecules in dermal capillary endothelium (68, 69).

According to a 2008 National Psoriasis Foundation Consensus Conference (27), administration of Ciclosporin with appropriate monitoring significantly-reduced chances of side effects. Continuous use of Ciclosporin, depending on treatment duration and dose, is commonly associated with the development of hypertension and renal impairment (70). These side effects are reversible by discontinuation of the drug (70). Based on many studies, low doses of 2.5-5.0 mg/kg/day of Ciclosporin is safe and highly effective in the treatment of the majority of psoriatic patients (28, 71). More importantly, to-date, Ciclosporin is shown to be effective in controlling psoriatic crises and, treating psoriatic conditions which were unresponsive to other treatments and managing psoriasis using a rotational approach with other medications.

3.3 Biological therapies

The administration of topical and systemic agents temporarily suppresses psoriasis and limits the appearance of psoriatic plaques, thus, continual application is needed. Systemic agents, such as MTX and Ciclosporin cause side effects such as hepatotoxicity, nephrotoxicity and bone marrow suppression (72). In the USA, a majority of dermatologists only prescribe systemic agents for complex cases of psoriasis due to adverse side effects. In
recent years, however, a new class of therapeutics called biological therapies has been developed. Biological therapies, or “Biologics” as they are also known, are generated in living organisms. They include recombinant proteins and engineered antibodies that target specific molecules such as inflammatory mediators (e.g. TNFα). Biologics function by either stimulating the immune system to act against the diseases, or by neutralizing and inhibiting key molecules that drive diseases.

One school of thought is that future treatments for psoriasis should focus on four potential targets. These are T-cell trafficking, T-cell activation, inhibiting pro-inflammatory cytokines and counteroffensive strategies (73). Mechanisms of T-cell trafficking and activation have gained extensive attention through studies of the adhesion and migration of T-cells and IDCs to sites of inflammation. These studies were followed by the design of several inhibitors targeting T-cells using recombinant technology. Counteroffensive strategies involve enhancing the number and function of regulatory T-cell subsets (Treg cells) in order to counteract the effect of disease-promoting T-cells. Most recently, approaches focussed on more specific and selective targeting have taken centre stage in treating psoriatic patients by using inhibitors of IL-12/IL-23 (Ustekinumab), to minimise side effects associated with broad immunosuppressive agents.

Many factors should be considered before the application of biologics for treating patients due to concern that such therapies might induce long term immunosuppression, thus, promoting infections and cancer. To date, three
approaches have been widely used and proven to be effective in psoriasis. These include inhibition of T-cell receptor binding to antigenic peptides, blockade of TNFα and most recently inhibitors of IL-12/IL-23 (Ustekinumab).

### 3.3.1 Inhibiting T-cell receptor binding.

Therapies based on targeting T-cells, or therapies targeting T-cell receptor binding were the first to be used. These involve the application of the T-cell agents Alefacept (Amevive®) and Efalizumab (Raptiva®). The mechanism by which these agents act involve blockade of T-cell receptor (TCR) signaling following TCR engagement. Alefacept is a fusion protein consisting of the extracellular domain of CD58 (or LFA-3) and the FC region of human IgG1. The fusion protein binds to the membrane co-stimulatory molecule CD2. This biologic is designed to block the activation of T-cells and DCs and suppress the production of pro-inflammatory cytokines. The mechanism of action of Alefacept involves enhancing apoptosis of T-cells, by killing NK cells and by blocking T-cell proliferation by preventing binding to APCs. Binding of the fusion protein to the CD2 molecule favours NK cell binding and this causes T-cell killing. The fusion protein is designed also to inhibit APC engagement of T-cells.

Efalizumab is also designed to modulate T-cell adhesion and co-stimulation. It is composed of a humanized murine monoclonal antibody (mAb) with specificity for LFA1 (CD11a). LFA1 is a heterodimer glycoprotein which binds intracellular adhesion molecules 1-3 (ICAM1-3) during T-cell circulation and trafficking, antigen presentation by dendritic cell and T-cell co-stimulation (74). Efalizumab inhibits T-cell migration from blood vessels into the skin by inhibiting the binding
of T-cells with cells expressing ICAM1 which functions to mediate T-cell transmigration (75).

Both biologics, alefacept and efalizumab have been shown to effectively reduce psoriatic lesions in clinical trials (75). Intravenous efalizumab (at \( \geq 0.3 \text{ mg/kg per week} \)) was shown to lead to significant clinical and histologic improvement in psoriasis, in association with sustained serum efalizumab levels and T-cell CD11a saturation and down-regulation (76). Alefacept, given intravenously, significantly reduced psoriatic lesions by 75% (PASI 75), after two weeks of administration, with 71% of the responders maintained at least 50% improvement within 12 weeks follow-up (77).

3.3.2 TNF\(\alpha\) antagonists

Currently, there are three biologic TNF\(\alpha\) inhibitors in clinical use. These are: Etanercept, Infliximab and Adalimumab. These inhibitors have been administered to patients with psoriasis and psoriatic arthritis (PsA). Etanercept is a recombinant receptor of human TNF\(\alpha\) with a backbone of IgG1-FC that binds TNF\(\alpha\) and lymphotoxin \(\alpha\) (LT\(\alpha\)). Studies carried out by Van Lingen and colleagues (2008) demonstrated significant reduction in PASI after 12 weeks of treatment with Etanercept (78). Such clinical benefits were not achieved with the administration of Efalizumab. Efalizumab, however, induced a significant reduction in the number of T-cells in the dermis and epidermis and lymphocytes in peripheral blood (78). Therefore, it seems that the reduction in the number of T-cells might not be sufficient for a clinical response to therapy.
Infliximab (Remicade) is a chimeric human-murine mAb against TNFα that binds both the soluble and receptor-bound forms of TNFα. It is administered to patients with Crohn’s disease, rheumatoid arthritis, psoriasis, ankylosing spondylitis and psoriatic arthritis. Adalimumab (Humira) is the first fully-human recombinant Ab that blocks TNFα. It specifically binds TNFα and blocks its interaction with the p55 and p75 surface TNFα receptors.

Numerous studies have been carried out to determine the efficacy and side effects of these anti-TNFα biologics. There have been cases where patients with primary diagnoses of RA, PsA, ankylosing spondylitis, Crohn’s disease and other diagnosis who were treated with anti-TNF biologics with no history of cutaneous inflammatory disease, but then developed cutaneous eruptions such as psoriasis, pustular folliculitis, lupus-like syndromes and others (79, 80). The mechanism that caused such adverse side effects, however, is unclear. Nonetheless, the cutaneous eruption could be resolved by discontinuation of the anti-TNFα agent and by switching to other anti-TNFα treatment, or by steroid/topical treatment (79, 80).

Recent studies on the effect of biologics in patients with moderate to severe psoriasis showed that Infliximab was 93% more effective than placebo (93% probability), followed by a mAb to IL-12 and IL-23 (Ustekinumab) at 90mg (81% probability). The 3rd most-effective biologic in psoriasis is Ustekinumab 45mg with 79% probability (81). This latter study covered a meta-analysis of randomised controlled trials for all biologic treatments currently available in Europe. Efficacy studies of infliximab in comparison with MTX have shown that
Infliximab was well tolerated and more efficacious than MTX in naive patients as well as patients who were unresponsive towards MTX (63).

Overall, these studies have shown that although anti-TNFα biologics have been proven to be efficacious, there are side effects. Different individuals appear to react differently towards each biologic, therefore, monitoring is mandatory.

3.3.3 IL-12/IL-23 antagonist, Ustekinumab.

Recently, extensive studies were carried out to explore the role of IL-12 and IL-23 in the pathogenesis of psoriasis by using a fully human IL-12/IL-23 mAb, Ustekinumab. This mAb functions by blocking the activity of IL-12 and IL-23 that share the same p40 subunit (Figure 1.8). The mAb binds to p40 and neutralizes the activity of the two cytokines by blocking their interaction with the cell surface receptor, IL-12β1 which forms part of the IL-12R and IL-23R complexes (34). The administration of Ustekinumab was shown to be effective with prolonged efficacy after one dose, or four weekly doses (31).

Ustekinumab is the first IL-12/IL-23 inhibitor. It was produced using human immunoglobulin (Ig)-expressing transgenic mice and the mAb consists of human Ig heavy- and light-chain variable-and constant region genes (34). The transgenic mice produced human antibodies after antigen challenge and the mAb established by using the hybridoma technology as used in the generation of murine mAbs. The advantage of Ustekinumab is that it has a half life comparable to endogenous IgG1 as well as lower immunogenicity. Therefore, the effect of Ustekinumab is sustained and frequency of dose administration
reduced. This was demonstrated in studies conducted on a large number of patients with moderate to severe psoriasis (n=2,899), showing that, in some individuals, a single intravenous (i.v) dose of Ustekinumab led to rapid and significant clinical response that persists for 16-24 weeks (34). This finding was supported by a recent clinical study carried out by Reich et al.(2012) which showed that Ustekinumab was nearly as effective as Infliximab in the treatment of psoriasis (81).

**Figure 1.8: Mechanism of action of Ustekinumab**. Ustekinumab functions by binding the p40 subunit of IL-12 and IL-23 and blocks binding of the heterodimeric cytokines to IL-12β1 of both IL-12R and IL-23R. This binding inhibits intracellular signaling by IL-12 and IL-23 and suppresses activation and cytokine production. Adapted and modified from Yielding et al., 2011(34).
3.4 Traditional herbal therapies.

Unlike modern medicines, traditional medicines focus mainly on the systemic effects of herbs and organic substances to treat skin disorders. In Asia, the oldest medicinal therapies include traditional Chinese medicine and traditional Indian medicine.

3.4.1 Anti-psoriatic treatment in Traditional Chinese Medicine (TCM).

In TCM, the severity of psoriasis is classified into three stages namely blood heat, blood dryness and blood stasis (82). Blood Heat refers to reddened skin, erosion, pustule, scorching, itching and pain, accompanied possibly by constipation and dark urine, as well as heat sensation and thirst. This is followed by blood dryness which is characterised by skin damage (xerosis, rhagades, squamae, atrophy trichoxerosis and trichomadesis), normally accompanied by severe itching. Further, blood stasis is manifested by petechia, ecchymosis, violet red or dark red spots, pigmented spots, thickened and hardened skin, verrucous vegetations. Based on these symptoms, two types of treatments are available that are internal and external treatments. Internal treatment involves the use of herbal prescriptions (phytotherapy) whilst external treatment focuses on physiotherapy including acupuncture and cupping techniques.

Many studies have been carried out to define mechanisms involved in use of TCM herbs on psoriasis. Indigo naturalis, one herbal treatment in TCM used in psoriasis has gained interest for its immunologic and dermatologic effects (83, 84). Studies showed that Indigo naturalis and its active compound, indirubin,
inhibit keratinocyte hyperproliferation resulting in decreased levels of the proliferating cell nuclear antigen (PCNA) and stimulate their differentiation with an increase in involucrin (a differentiation protein) level at both mRNA and protein levels (84).

Despite these findings, comparative studies of the effect of MTX vs. TCM medicinal herbs (a standardised herb formulation known as wen-tong-hua-yu) in psoriatic patients showed that TCM medicinal herbs were less efficacious than MTX. Although with less adverse effect compared with MTX, TCM medicinal herbs did not achieve PASI 75 which was achieved by MTX with 63% of patients achieving PASI 75 (85). It was proposed that this might be due to inefficient delivery methods (i.e. use in the form of decoction instead of capsule) of the active ingredients of TCM to sites of inflammation, dosage or the composition of the active ingredients not been effective for the treatment (85). Therefore, it was suggested that further detailed studies to explore the potential of TCM and assess the side effects of the medications should be carried out. These studies should also aim to eliminate the potential of liver dysfunction, cardiomyopathy and hepatoxicty as reported by some studies (86, 87).

3.4.2 Anti-psoriatic treatment in Ayurveda.

Ayurveda in traditional Indian Medicine has evolved before the emergence of modern medicine. The Ayurveda materia medica has described more than 1500 herbs and 10,000 formulations for health, well-being and treatment for diseases (88). Moreover, it has documented over 5000 signs and symptoms of diseases. Ayurveda has also standardised prescriptions for the treatment of skin disorders
including psoriasis. Herbal plants used by Ayurveda in the treatment of psoriasis that have been studied include *Centella asiatica* (89), *Caesalpinia bonduc* (L.) Roxb. (90) and *Argemone mexicana* (91). Extract of *Caesalpinia bonduc* (L.) Roxb has been shown to have anti-proliferative effects on a human keratinocytes cell line (HaCaT), inhibited lipooxygenese and promoted significant orthokeratosis (a normal configuration of stratum corneum) in mouse tail test (90). In vivo mouse tail parakeratosis is used as a hallmark of psoriatic skin. *Argemone mexicana*, the plant ingredient in Desoris, botanical drug product, has been found to effectively modulate cellular functions in psoriasis and has been the subject of phase 3 clinical trial (90).

### 3.4.3 Anti-psoriatic agents in traditional medicine in Malaysia.

In Malaysia, traditional Malay medicines have diverse uses in applied medicine by combining medicine with religion and spiritual beliefs. Vast numbers and diverse types of herbs are used to treat skin diseases by topical applications and by oral consumption. *Centella asiatica* has been used for the treatment of psoriasis, eczema and ulceration in TCM and Ayurveda. This herb has also been used in Malaysian traditional medicine for the same conditions. Studies have shown that the constituents of the plant, triterpenoid glycosides displayed potency equal to the commercially-available anti-psoriatic agent, dithranol (89, 92). To date, however, very few reliable scientific studies have been carried out to understand the effect of Malaysian herbal traditional medicines on the modulation of immunopathogenesis in psoriasis.
3.5 Commercially-available plant-derived anti-psoriatic inhibitors

Prior to the emergence of synthetic drugs and biologics, phytotherapeutic agents, or drugs originated from plants have been used in the treatment of psoriasis (Table 1.1). These drugs were discovered serendipitously and normally applied topically in combination with treatment such as ultra-violet (UV) light. Anthralin and psoralen have been shown to have excellent efficacy in regulating keratinocyte hyperproliferation.

Anthralin originally known as cignolin, was derived from chrysarobin a component from the bark of Goa tree or *Andira araroba* which is a native plant of the Amazon. Nonetheless, Anthralin that is available today is synthetically-produced and is known as Ditranol. Anthralin is used as a topical agent which induces clearance of psoriatic lesions and remission from disease. Anthralin has been shown to inhibit the release of pro-inflammatory mediators and possess anti-proliferative effects on keratinocytes (83). While displaying both anti-proliferative (93) and anti-inflammatory effects on psoriasis (94), it also induces inflammation on normal skin (95). The inflammation could be controlled by including free-radical scavenger and anti-oxidant as part of the treatment. Clinical studies on psoriatic patients indicated that anthralin is more potent than calcipotriol (83) and similarly effective compared with topical corticosteroid (0.05% diflorasone diacetate ointment) and traditional treatment which requires overnight application (95). Obviously, this indicates that potent drugs against psoriasis could be discovered in herbal plants serendipitously.
Psoralen was isolated from Bishop’s weed (*Ammi majus*) and has been used topically to treat psoriasis. The main active component is 8-methoxypsoralen (8-MOP). There were numerous clinical studies that highlighted the potency of psoralen in combination with UVA irradiation, generically known as PUVA. It was found that the anti-psoriatic effect of PUVA therapy was due to its ability to impair T-cell functions. However, the precise mechanism of action involved in its clinical effectiveness in the clearance of psoriatic lesions is still not well understood. PUVA treatment in transgenic mice (k5.hTGFb1) which exhibited close phenotypic resemblance to human psoriasis had shown that PUVA successfully cleared the lesion by suppressing IL-23/Th17 and Th1 pathways while activating Th2 cells and by inducing Treg cells and CTLA4 signaling (96).

According to Commission E of the German Federal Drugs Administration, the quality of clinical studies on these drugs was classified as LOE A. Level of evidence (LOE), according to the UK National Health Service is categorised in range of A to D as depicted in Table 2 of the Appendices. This indicates that these drugs had undergone the highest level of assessment with randomised, controlled clinical and cohort studies, which validate the effectiveness of these drugs in the treatment of psoriasis.
Table 1.1: Plants and their active compounds with dermatological effects. The effectiveness of the compounds is classified according to the former Commission E at the German Federal Drugs Administration based on the quality of the clinical studies known as LOE. Reference: Reuter et al. JDDG (2010) band 8 (83).

<table>
<thead>
<tr>
<th>Plants</th>
<th>Active compound</th>
<th>Levels of evidence (LOE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Araroba tree (Andira araroba)</td>
<td>Anthralin</td>
<td>A</td>
</tr>
<tr>
<td>Bishop’s weed (Ammi majus)</td>
<td>Psoralen</td>
<td>A</td>
</tr>
<tr>
<td>Indigo naturalis (Baphicacanthus cusia)</td>
<td>Indirubin</td>
<td>A</td>
</tr>
<tr>
<td>Cayenne pepper (Capsicum frutescens)</td>
<td>Capsaicin</td>
<td>A</td>
</tr>
<tr>
<td>White willow (Salix alba)</td>
<td>Salicylic acid</td>
<td>D</td>
</tr>
</tbody>
</table>
4 Hypothesis

Psoriasis is a complex and multifactorial disease that is influenced by genetics and environmental factors, the combination of which could vary among different individuals. Studies on its aetiology, pathogenesis, and immunological abnormalities have resulted in the development of a range of therapies including natural and synthetic drugs and biologics. Despite integrated scientific and clinical findings and postulations, key mechanisms and inflammatory pathways involved in pathogenesis remain debated. Hence, more insights into disease mechanisms, genetic roles, epigenetics, and metabolomics are required to develop new and better treatments.

Therefore, by gaining further knowledge of the roles of T- and B-cells, particularly Th17 cells, in the disease process, using naturally-derived compound/s, more targeted therapies could be identified and developed. Although, natural products and plant-derived constituents have not been the subject of recent drug discoveries, their potential should not be ignored since important drugs such as Ciclosporin and dithranol were derived from natural product/resources. Plant-derived active compositions and extracts have been shown to possess multiple functions in disease therapy. In many, so-called traditional medicinal systems such as Ayurveda and Traditional Chinese Medicine, nutrition and herbal medication are a normal part of prevention and healthcare.
With these notions in mind, it is hypothesised that Halia Bara or *Z. officinale* Roscoe var. *rubrum* (ZOR) may have therapeutic potential in regulating inflammatory events related to psoriasis. Therefore, this study will identify the key mechanism of action of this ginger species against inflammatory responses relevant to psoriasis.
5 Aims and Rationales

The key aims of this study were to evaluate the potential of active constituents from *Z. officinale* Roscoe var. *rubrum* (ZOR) or Halia Bara as anti-psoriatic agents. Specifically, the aims and rationales for this study were;

1. To investigate if ZOR active constituents possess inhibitory effects against nitric oxide and prostaglandins produced by activated macrophages. This provides a line of evidence wherein these plant-derived constituents may act at the early phases of the inflammatory process mediated by the innate immune system.

2. To identify if ZOR-derived active constituents possess inhibitory effects against cytokines involved in the induction of Th1 and Th17 cells. These cytokines would include TNFα and IL-12/IL-23 produced by activated macrophages.

3. To examine the effect of ZOR samples on leukocyte migration and T-cell activation. Thus, the effect of ZOR on the expression of adhesion molecules involved in immune cell activation and recruitment to site of inflammation to be defined.

4. To identify ZOR samples suppressive effects on keratinocyte proliferation, differentiation and chemokines production. This would define the effect of ZOR active compounds on key responses by which keratinocytes contribute to pathogenesis.
All of these aims, it is hoped, would determine if ZOR has the potential to regulate inflammatory pathways involved in psoriasis. For all of these objectives to be achieved, the study could raise the possibility that ZOR, or more precisely compounds derived from it, could be therapeutically-beneficial in psoriasis and, possibly, other inflammatory conditions. This study was, therefore set up to address if ZOR and active compound could modulate the key mechanisms known to drive inflammation involved in psoriasis and identify functional compounds of therapeutic value.
CHAPTER 2

Identification of anti-inflammatory fractions and compounds in *Zingiber officinale* Roscoe var. *rubrum* (Halial Bara)
1 Introduction

1.1 *Zingiber officinale* Roscoe var. *rubrum* (ZOR), ginger variant of South East Asia

Ginger (*Zingiber officinale*) is a versatile plant which has been used as a spice and herbal medicine for around 3000 years in India and China (97). Ginger is thought to have originated from the Indo-Malayan region (South-east Asia). It was disseminated from India throughout Asia, Africa, Mediterranean and Pacific Islands through trading activity since the 1st century (98).

Ginger belongs to the genus *Zingiber* of the *Zingiberaceae* family that is part of the order Zingiberales and tribe Zingibereae (99). Worldwide, there are estimated to be about 1,200 species of *Zingiberaceae* belonging to 18 genera. Among these, approximately 160 species from 18 genera are found in Peninsular Malaysia and, therefore, comprises at least 20% of the world taxa and 40% of the world genera of *Zingiberaceae* (100).

Early studies by Ridley (1915), demonstrated that there are four types of ginger in Malaysia namely: Halia Betel (true ginger), Halia Bara, Halia Padi and Halia Udang. Ridley also described the red variety of ginger, *Zingiber officinale* var. *rubra* (or *rubrum*), which has a reddish rhizome skin (101), and closely resembles the ginger sp. of interest, *Z.officinale* Roscoe var. *rubrum*, also known as Halia Bara. Therefore, this suggests that the ginger variant of interest, Halia Bara (ZOR), is most probably local to Malaysia and its neighbouring countries in South-east Asia.
Halia Bara (ZOR) can be distinguished genetically and phenotypically from other ginger varieties and variants. Phenotypically, Halia Bara is slightly different from the common culinary ginger whereby its rhizomes are red, smaller, more pungent and have pinkish rhizomes in its cross section (Figure 2.1(a)(b)) (102). The base of its leaf shoot is red and it has a reddish petiole when young, as well as scarlet red lip blotched with cream (100). Authentication of Halia Bara and other local varieties and variants in Peninsular Malaysia was carried out by utilising DNA fingerprinting (100). According to DNA fingerprinting of a local variety of the species in Peninsular Malaysia, *Z. officinale* Roscoe var. *officinale* (common ginger), *Z. officinale* Roscoe var. *rubrum* (Halia Padi) and *Z. officinale* Roscoe var. *rubrum* (Halia Bara) are slightly differentiated by their DNA profiles at RAPD (Random Amplified Polymorphic DNA) Operon primers; OPA1, OPA8 and OPA20 (Figure 2.2) (100). RAPD involves the use of a single random primer in a polymerase chain reaction which results in the amplification of several distinctive DNA products. It is a useful approach to identify variation within plant varieties (103). The DNA profiles indicated that the varieties were differentiated by the presence of polymorphic banding patterns shown as ‘X’ in Figure 2.2 (OPA1: X=1500bp, OPA8: X=2000BP, OPA20: X1=1350bp, X2=900bp, X3=800bp, X4=550bp). Halia Bara (ZOR) can be differentiated from the common ginger (halia) and halia padi by the presence X3 band of OPA20 (Figure 2.2).
Figure 2.1: Phenotype of Halia Bara or *Z. officinale* Roscoe var. *rubrum* (ZOR). It is as indicated by (a) a photograph of the whole plant of and (b) its pink reddish rhizome and the rhizome cross section.
Figure 2.2: DNA profiles of three ginger varieties. DNA fingerprinting demonstrating slight variations in DNA between *Z. officinale* (common ginger, H) and its varieties; *Z. officinale* var. *rubrum* (Halia Bara, HB) and *Z. officinale* var. *rubrum* (halia padi, HP) (100) Halia Bara and halia padi can be differentiated by band X3 and X4 of OPA20. These bands are not observed in halia DNA profile. (OPA1: X=1500bp. OPA8: X=2000BP, OPA20: X1=1350bp, X2=900bp, X3=800bp, X4=550bp). Adapted from Ibrahim et al. 2007 (100).

Ethnobotanical studies of Zingiberaceae sp. in Peninsular Malaysia by Ibrahim et al. (2007) found that, traditionally, many medicinal ginger plants are used for female-related ailments, for example to treat post-partum and post-natal symptoms after childbirth (100). The Zingiberaceae sp have been effective to improve blood circulation and encourage contraction of the uterus during post-partum period (104). The treatments are in the form of tonics, herbal extracts, decoctions (herbal extract prepared by boiling) and ointment, whereby they might consist of a single ginger sp. or a herbal mixtures consisting of several Zingiberaceae sp. In Malay traditional medicine, *Z. officinale* Roscoe var. *rubrum* (Halia Bara) was used to treat aching joints, being applied topically as a
mixture of rhizome juice and vinegar, or drank as a rhizome juice \((100)\). Thus far, the anti-arthritic effect of this ginger variant has not been determined scientifically though it was reported in several studies on \(Z.\ officinale\) Roscoe sp. \((105, 106)\).

To-date, few studies have been carried out on this particular ginger variant in terms of the chemical composition of the rhizomes, apart from a single study that indicates that an essential oil from the rhizomes consisted mainly of geranial \((28.43\%)\), neral \((14.20\%)\) and geranyl acetate \((8.77\%)\) \((107)\). The essential oil of the common ginger, however, consists mainly of zingiberene \((16.7\%)\), \((E,E)\)-\(\alpha\)-farnesene \((13.10\%)\) and geranial \((7.60\%)\). Thus, geranial seems to be a compound present in the essential oil of both ginger species. Therefore, it could be deduced that closely related ginger variants might share one or two common compounds. Nonetheless, there are a very limited number of studies carried out on the composition of the oleoresin (a mixture of extracted essential oil and resin) of Halia Bara and its potential biological effects.

### 1.2 Chemical components of ginger in general

The rhizome of ginger consists mainly of volatile (constituting the essential oils) and non-volatile compounds \((101)\), that appear to contribute to its healing properties \((108)\). More than 66 compounds have been identified in the volatile oil which are mainly sesquiterpene hydrocarbons that include zingiberene, curcumene and farnesene, \(\beta\)-sesquiphelandrene, bisabolene, 1,8-cineole, linalool, borneol, nerol, geraniol, camphen, limonene, myrcene, \(\beta\)-phellandrene,
α-pinene, citronellol, geranial, neral and others (109). These constituents of essential oil contribute to the distinct aroma and taste of ginger. The essential oil is normally used in small amounts by the pharmaceutical, cosmetic and perfumery industries. As well as its use as a fragrance, the essential oil possesses therapeutic effects (97). A high yield of essential oil is obtained by steam distillation and supercritical carbon dioxide extraction. It is also detected as a product in solvent extraction (97, 110).

The major characteristic of ginger is the pungent odour which is due to its oleoresin content. The oleoresin mainly consists of non-volatile pungent compounds, which are classified as phenols such as gingerols, shogaols, paradols, zingerone and related compounds, and resin. The oleoresin is a dark brown viscous liquid which contains 20-25% volatile oil, 25-30% pungency stimuli and non-flavour compounds; fats, waxes and carbohydrates (111). Some trace elements of the usual organic and inorganic compounds found in food, as well as a high content of vitamin C, manganese and iron were also identified in ginger (97, 110).

Biochemically, these active compounds are produced through the phenylpropanoid pathway that mainly controls the production of gingerols, the major pungent compounds in ginger (111). Hence, this pathway indirectly also becomes the limiting factor for the generation of other related metabolites. Studies indicate that gingerol and shogaol are the major compounds that contribute to the pungent smell in both fresh and dried ginger (111). Gingerols are mainly observed in the oleoresin of fresh ginger with yield of 20%-33% of the oleoresin (97). In dried ginger, gingerols are converted to shogaols.
Shogaols are found in increasing amounts during the drying process of ginger as a result of dehydration of gingerols. Therefore, shogaols are the dehydrated products of gingerols and, thus, are more chemically and thermally stable than gingerol (112). Studies by Connell (1970) indicated that gingerols are biosynthesized from dihydroferrulic acid via condensation with mevalonic acid and hexanoic acid to give the intermediate gingerdione. Among the gingerols, 6-gingerol is the highest content. In the production of 6-gingerol, Macleod & Whiting proposed that a diketone, 6-dehydrogingerdione, is the initial product which undergoes two reduction steps which are C=O and C=C reduction that lead to the conversion into 6-gingerol (113). In this biosynthesis reaction, 6-dehydrogingerol is the intermediate product (Figure 2.3). Upon dehydration, the 6-gingerol are converted to 6-shogaol and when undergoes hydrogenation, 6-shogaol is converted into 6-paradol upon storage. Hence, this demonstrates that the presence of the ginger compounds in the pathway are inter-related and varied according to the biochemical environment such as the presence of enzymes, pH etc (97). The presence and biological activity of these compounds differ depending on the state of the rhizome whether it is in a fresh, dried or roasted condition, the cultivar, storage, processing condition (114) and methods of extract preparation (115).
Figure 2.3: Synthesis of gingerol-related compounds. The biosynthesis pathway of 6-gingerol, with 6-dehydrogingerdione as the initial product and 6-dehydrogingerol and 6-gingerdione as the intermediate products. $\text{Ar} = \text{C}_7\text{H}_7\text{O}_2$ and $\text{R} = \text{C}_5\text{H}_{11}$. Adapted and modified from Macleod et al 1979 (113).
1.3 Bioactivity-structure relationship of known ginger constituents

Over the centuries, ginger has been used to cure colds, inflammation, gastrointestinal discomfort, rheumatic disorders, neuralgia and motion sickness \((112)\). Despite the efficacy of ginger in treating these symptoms, the exact mechanisms of ginger-containing-herbal preparations remain unclear. Nonetheless, it is still used in traditional medicines such as in Traditional Chinese Medicine, Ayurveda and others. The challenge in understanding the mechanistic properties of traditional medicines is that such traditional medicines normally contain several types of herbs with each herb containing several potentially therapeutically effective compounds.

To-date, many studies have been carried out to understand the bioactivity-structure relationship (SAR) of ginger’s constituents. Several bioactive compounds have been identified in ginger, known as phenylpropanoids such as gingerols, and gingerol-related compounds; shogaols, gingerdiones and paradols. Among these compounds, the bioactivity of the gingerols, 6-gingerol in particular, has been widely studied. It has been shown, for example, that 6-gingerol possesses antioxidant, anti-inflammatory, anti-microbial, anti-thrombotic, antispasmodic, anti-tumour and anti-arthritic properties \((96, 97)\). Recently, it was shown that the dehydrated form of 6-gingerol, 6-shogaol, was a more potent antioxidant and anti-inflammatory agent than gingerols due to its ‘drug-like’ structure \((116)\). Among the gingerols, 6-gingerol was the least potent while 10-gingerol was the most potent antioxidant agent. It was proposed that the length of the alkyl side chain determines the potency of these compounds. The longer the carbon side chain the more potent the compound. This was
evidenced by the interaction of phenylpropanoid with the human serotonin 5-HT$_{1A}$ receptor for anti-anxiolytic effects, whereby the binding affinity of compounds is correlated with the length of the aliphatic chain. Phenylpropanoids with a longer chain such as 10-gingerol, 10-shogaol and 1-dehydro-10-gingerdione were shown to have the strongest affinity among the tested phenypropanoid (117).

The presence of an α,β-unsaturated ketone moiety within the carbon chain of the alkyl group of gingerol and gingerol-related compound was speculated to contribute to its potency. A study was carried out by Kimura and colleagues (2005) to gain insight into the possible interaction of these compounds with endogenous target ligands such as prostaglandin F$_{2\alpha}$, which is involved in the modulation of eicosanoid responses in mouse mesenteric veins, in a study related to mechanisms in diabetes mellitus. Mesenteric veins are involved in controlling blood flow from the liver to the digestive organs such as the intestines. This subsequently regulates blood glucose levels in the circulation. Kimura and colleagues (2005) showed that 6-gingerol and hexahydrocurcumin potentiated PGF$_{2\alpha}$-induced contraction of mice mesenteric veins whilst 6-shogaol and 6-gingerdione inhibited PGF$_{2\alpha}$ (112).

In contrast, no potentiation of PGF$_{2\alpha}$-induced contraction of mice mesenteric veins by 6-dehydrogingerdione or 6-gingerdiacetate was observed. Interestingly, however, 6-gingerdione in aqueous solution demonstrated increasing potentiating effects on PGF$_{2\alpha}$ production. In aqueous solution, 6-gingerdione with a α,β-unsaturated diketone moiety at C-3 and C-5, is stabilised into an enolic form, employing both carbonyl and hydroxyl (112). This
is believed to be due to conversion of the keto to the enol-OH, as a result of the activation by the carbonyl group of the α,β-unsaturated ketone moiety, which favours enolisation. Therefore, the effect of the hydroxyl group that mimics PGF$_{2α}$ is evidence that part of the chemical structure of gingerol and related compounds in ginger resemble prostanoids (112) (Figure 2.3).

Equal potentiation effects of 6-gingerol in normal and diabetic states suggest that it could be involved in the activation of COX-1. 6-shogaol and other gingerol-related compounds have been shown to exhibit inhibitory effects on COX-2 on the basis of three structural features. These are; 1) lipophilicity of the alkyl side chain, 2) substitution pattern of hydroxyl and carbonyl groups on the side chain and, 3) substitution pattern of hydroxyl and methoxy groups on the aromatic moiety (118).

The above cited data, therefore, indicate that the effect of ginger constituents on prostaglandins could be due to the close resemblance between the structure of prostaglandins such as PGF, PGE$_2$ and PGH and the compounds present in ginger such as gingerols, shogoals, hexahydrocurcumin, gingeracetate and gingerdiones (112) (Figure 2.4). It was also highlighted that only compounds that contain the hydroxyl group at C-5 in the side chain produced potentiation effects on PGF$_{2α}$-induced contraction in mice mesenteric vein (Figure 2.4(a)(b)). Kimura and colleagues (2005) concluded that the different effects of gingerol and shogaol on potentiation of PGF$_{2α}$-induced contraction in mice mesenteric vein suggested that a combination of both compounds could have synergistic effects against diabetes and other inflammatory conditions (112).
The findings cited above, therefore, suggest that ginger phenylpropanoids have ‘drug-like’ effects, possibly similar to those by NSAIDs such as aspirin, ibuprofen and indomethacin. The ‘drug-like’ structure of phenylpropanoids as stated by Dugasani and colleagues (2010) is in accordance with Lipinski’s rule of five (Ro5). According to Lipinski, a ‘drug-like’ compound is potentially orally active when it follows four simple physicochemical parameters, 1) molecular weight ≤500; 2) lipophilicity, expressed by log P≤5 (P= calculated octanol/water partition coefficient); 3) H-bond donors ≤5 (calculated as the sum of OHs and NHs within the structure) and, 4) H-bond acceptor ≤10 (calculated as the sum of Ns and Os within the structure). These characteristics are based on 90% of orally-active drugs that have achieved phase II clinical trials. Log P is useful in predicting the distribution of drugs within the body whereby high log P values indicate hydrophobic drugs which are predominantly distributed into the hydrophobic compartments such as lipid bilayers while the preferred, hydrophilic drugs with low log P, are distributed into hydrophilic compartment such as blood serum. Therefore, a lower log P value may lead to better drug-delivery. To qualify as a drug, a compound must reach the target site, interact with a molecular target and preferably be structurally similar to the native ligand, in this case, catecholamines, prostanoids, indoles and quaternary amines.
Figure 2.4: Structure-activity interaction of the phenylpropanoid (gingerol-related compounds) and prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$). Structure comparison between six ginger phenylpropanoids (a-g) with (h) PGF$_{2\alpha}$. It was proposed that the presence of hydroxyl group on the side chain of the phenylpropanoids mimics PGF$_{2\alpha}$. Adapted and modified from Kimura et al. 2005 (112).
In summary, ginger contains potential ‘drugs’ or therapeutically active compounds that will be characterised and discussed in terms of their effect on modulating inflammatory mediators related to psoriasis later in the following chapters.

2 Objectives

This chapter focuses on the fractionation of *Zingiber officinale* Roscoe var *rubrum* (ZOR) or Halia Bara extract, isolation and chemical characterisation of compounds with therapeutic effects. Prior to the start of this study, 20 extracts from 12 types of plants from Malaysia were screened for their inhibitory effects on the production of NO and PGE$_2$ as well as changes in the expression of iNOS and TNF$\alpha$ by PCR (detailed in Chapter 3 and 4). Amongst the plant extracts tested, ZOR was identified with the most efficacious activity against NO and PGE$_2$ production by macrophages, thus, it was selected for further characterisation both chemically and biologically. The bioactive compounds were isolated using conventional isolation methods, such as vacuum liquid chromatography (VLC), solid phase extraction (SPE) and preparative thin layer chromatography (Prep-TLC) and characterised via nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry, infra red (IR) and ultraviolet (UV) spectrometry.
3 Methods

3.1 Preparation of plant extracts and profiling

Preparation of plant extracts were carried out in two stages. Phase I involved preparation of extracts from a number of plants of known therapeutic effects (Table 1 of the Appendices). These plants were labeled as indicated in Table 2.1. Phase II involved modifying the original extraction protocols to obtain a ZOR extract for detailed assessment.

3.2 Sample preparation

Twelve varieties of plants were collected for the initial screening (Table 2.1). These plants were collected from three states in Malaysia; Selangor, Negeri Sembilan and Pahang. Based on the biological potency in initial biological assays (will be discussed in chapter 3), a ginger species, *Zingiber officinale* Roscoe var *rubrum* (Halia Bara) (ZOR) was selected for further detailed studies. Rhizomes from ZOR were collected from Pahang, Malaysia and specimens deposited at Kew Gardens Herbarium, Kew, and U.K (voucher: Nordin 1). It was classified as *Zingiber officinale* sp. native to Peninsular Malaysia according to the morphology of the leaves and petiole. It is known as variant *rubrum* due to the purplish rhizome and pungent odour (99). It was identified as Halia Bara due to its pungent smell and red-skin rhizome (Figure 2.1). The rhizomes were washed, sliced and dried for 48 hours at 40±5°C. The dried rhizome were then grounded into powder and stored at 4°C until used.
Table 2.1: Selected plants with known therapeutic effects. These plants were collected from Malaysia and extracted using sonication (SN) and shaking (SK) methods described in 3.3. Their general use in traditional medicine are summarised in Table 1 of the Appendices.

<table>
<thead>
<tr>
<th>Plants</th>
<th>Coding</th>
<th>Extraction method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves of <em>Mangifera indica</em></td>
<td>MP</td>
<td>SK, SN</td>
</tr>
<tr>
<td>Rhizomes of <em>Ipomoea batatas</em></td>
<td>UB</td>
<td>SK, SN</td>
</tr>
<tr>
<td>Leaves of <em>Crinum asiaticum</em></td>
<td>TS</td>
<td>SK</td>
</tr>
<tr>
<td>Leaves of <em>Pandanus amaryllifolius</em></td>
<td>DP</td>
<td>SK, SN</td>
</tr>
<tr>
<td>Rhizomes of <em>Zingiber officinale</em> Roscoe var. rubrum (Halia Bara)</td>
<td>HB</td>
<td>SK</td>
</tr>
<tr>
<td>Leaves of <em>Artocarpus heterophyllus</em></td>
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<td>SK, SN</td>
</tr>
<tr>
<td>Leaves of <em>Piper sarmentosum</em></td>
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<td>SK, SN</td>
</tr>
<tr>
<td>Leaves of <em>Morinda citrifolia</em></td>
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</tr>
<tr>
<td>Roots of <em>Tinospora crispa</em></td>
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<td>SK</td>
</tr>
<tr>
<td>Seeds of <em>Archidendron bubalinum</em></td>
<td>KD</td>
<td>SK, SN</td>
</tr>
<tr>
<td>Rhizomes of <em>Marantha arundinacea</em></td>
<td>UBM</td>
<td>SK</td>
</tr>
</tbody>
</table>
3.3 Extraction phase I: Preliminary studies

The first stage involved preparation of extracts from twelve varieties of the plants in Table 2.1. The extractions were carried out using 80% ethanol by two different methods.

**Method 1:** Dried ground plants (30g) were extracted in 100 mL 80% ethanol. The extraction was carried out on a shaker at 40°C for 24 hours. The extracts were filtered and then concentrated using a rotary evaporator at 72 Pa at <40°C. The samples were re-dissolved in 80% ethanol for bioassays.

**Method 2:** Dried ground plants (30g) were extracted in 100 mL 80% ethanol. The extraction was carried out by sonicating the samples at 50°C for 30 minutes. The extracts were filtered and the remaining solid fractions sonicated twice more. Filtered extracts were combined and concentrated in a rotary evaporator at 72 Pa at <40°C. The samples were re-dissolved in 80% ethanol for bioactivity studies.

3.4 Extraction phase II: *Zingiber officinale* Roscoe var. *rubrum* (ZOR) *(Halia Bara)*

Phase II involved an optimised extraction protocol for ZOR. Active components in new batch of ZOR powder (499g) were extracted with 3 L chloroform for 30 minutes using sonication and then maceration for 5 days at room temperature. The extract was collected, filtered and evaporated using a rotary evaporator at 200 Pa at 45°C. The powder residue was then re-extracted with absolute
ethanol and water using the same approach. The aqueous extract was dried using a freeze-drier.

3.5 Instruments and Protocols

3.5.1 Vacuum Liquid Chromatography (VLC)

Outline of method: Fractionation is used to separate groups of active compounds according to a gradient of nonpolar: polar solvent ratios. In this study, fractions from a gradient of 100% hexane to 100% ethyl acetate were collected using vacuum liquid chromatography (VLC).

Protocol: 10-40 g of ZOR extract was subjected to VLC on silica (Silica gel 60 PF_{254+366}, MERCK) and 21 fractions were collected from gradients of a hexane: ethyl acetate mixture. The fractions were evaporated via rotary evaporator and the concentrated fractions left to dry at room temperature.

3.5.2 Solid Phase Extraction (SPE)

Outline of method: SPE is a chromatographic method used to isolate compounds by eluting them from a normal phase column. SPE uses the affinity of solutes in a liquid mobile phase, for a solid through which the sample is passed (stationary phase) to separate a mixture into desired and undesired components. The amount of sample loaded depends on the size of the column.

Protocol: 200-400 mg of sample was loaded onto a 10 g/60 mL Giga tube normal phase column (Strata SI-1 Silica, 55 μm, 70A, Phenomenex). Prior to sample loading, the column was conditioned with hexane. Once the sample was
loaded, elutions with increasing polarity from 100% hexane to 100% ethyl acetate were carried out. Each eluent was collected and evaporated. TLC and NMR were used to determine the profile of each fraction before further purification.

### 3.5.3 Preparative Thin layer chromatography (Prep-TLC)

**Outline of method:** One of the first steps in obtaining a good separation of pure compounds is the selection of an appropriate mobile phase. The use of Thin Layer Chromatography (TLC) will be discussed here to deal with the successful correlation between the TLC separation and the preparative silica column. TLC is a liquid-solid adsorption technique where the mobile phase ascends the thin layer of stationary phase coated onto a backing support, such as glass, by capillary action. There is a similar relationship with column chromatography where the solvent travels down the column’s adsorbent. This similar relationship allows TLC to be a rapid method for determining solvent composition for preparative separations. The optimum separation of compounds by TLC is usually achieved when retention values ($R_f$) are between 0.3–0.5. The $R_f$ value is defined as the ratio of the distance moved by the solute (test samples) and the distance moved by the solvent (the solvent front) from the point where the sample is initially applied to the TLC sheet.

In most cases, adjusting the compound’s $R_f$ to 0.3–0.5 is crucial for a TLC separation. For scale-up to preparative separations, the TLC solvent system’s polarity must be decreased to lower the $R_f$ to between 0.15–0.35. This $R_f$ range is optimal for a preparative separation, in terms of sample load, resolution,
residence time, and solvent usage. Several solvent systems were used in this study which ranged from 70-95% hexane in ethyl acetate and 80-98% toluene in ethyl acetate.

**Protocol:** Selected SPE fractions were purified by preparative TLC (20 mm × 20 mm, 60F254; Merck), which is essentially TLC, only on larger scale. A large developing tank and large thickly-coated plates were used. The plates used were capable of separating around 10-20 mg, depending on the separation efficiency required. Identification of an efficient solvent system which will adequately separate the compounds is crucial. Once the compounds were separated, the bands were scraped and extracted from the silica using varying ratios of chloroform and methanol. The filtrates were collected and evaporated.

### 3.5.4 Nuclear Magnetic Resonance (NMR)

**Outline of method:** NMR spectroscopy is an effective method for structural identification and orientation of compounds. It involves magnetic nuclei in magnetic fields absorb and re-emit electromagnetic energy. This energy is at a specific resonance frequency which depends on the strength of the magnetic field. Theoretically, the resonance frequency of a particular substance is directly proportional to the strength of the magnetic field.

**Protocol:** Samples were dissolved in a deuterium solvent such as CDCl₃, CD₃OD and C₃D₆O and analysed using the 400 and 500 MHz NMR spectrometer (Bruker) at the London School of Pharmacy. Data obtained were analysed using the Bruker TopSpin application.
3.5.5 Mass Spectrometry (MS)

Outline of method: MS is an analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structures of molecules, such as peptides and other chemical compounds. The technique has both qualitative and quantitative uses. These include identifying unknown compounds, determining the isotopic composition of elements in a molecule, and determining the structure of a compound by observing its fragmentation. The structure elucidated through NMR were confirmed by electro spray ionisation (ESI), accurate mass and electron impact (EI) spectrometer conducted by the Mass Spectrometry Department at the School of Pharmacy and University College London.

3.5.6 Infra-red (IR) spectrophotometer

Protocol: IR spectrum of the compounds were captured using a Perkin Elmer spectrum Express version 1.02 at the School of Pharmacy. The wavelength was scanned between 600-4000 cm⁻¹.

3.5.7 Ultra-violet (UV) spectrophotometer

Protocol: UV spectrum of the compounds were obtained using a Syngene G:Box Transluminator at the School of Pharmacy. The wavelength used was 200-800 nm.
4 Results

4.1 *Zingiber officinale* Roscoe var. *rubrum* (ZOR) was selected for determination of active compounds that exhibited anti-inflammatory effects associated with psoriasis.

In the preliminary stage of this study, twelve plants from Malaysia were screened for anti-inflammatory activity as measured by the suppression of NO and PGE$_2$ production (will be discussed in detail in chapter 3). These extracts were obtained using two methods, which are the shaking and sonicating methods. Table 2.2 summarised the potency of the extracts based on their IC$_{50}$, the concentration required to inhibit 50% of NO production by LPS-stimulated macrophages (RAW 264.7). Among these extracts, the extract from the ginger sp., *Zingiber officinale* Roscoe var. *rubrum* (Halia Bara) (ZOR) obtained via shaking method (HB01), was the most potent inhibitor of NO production with an IC$_{50}$ of $10.2 \pm 0.76 \mu$g/mL. Based on these preliminary studies, it was decided to select *Z. officinale* Roscoe var. *rubrum* (Halia Bara) (ZOR) for further characterisation in this thesis.
Table 2.2: NO inhibitory effect of the selected plants. Comparison between extracts from indigenous Malaysian plant in their IC$_{50}$ on the inhibition of NO production by stimulated RAW 264.7 cells.

<table>
<thead>
<tr>
<th>Code</th>
<th>Plants</th>
<th>NO inhibition (IC$_{50}$, µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shaking (SK)</td>
</tr>
<tr>
<td>MP</td>
<td><em>Mangifera indica</em></td>
<td>26.0 ± 6.9</td>
</tr>
<tr>
<td>KD</td>
<td><em>Archidendron bubalinum</em></td>
<td>489 ± 81</td>
</tr>
<tr>
<td>DN</td>
<td><em>Artocarpus heterophyllus</em></td>
<td>68.0 ± 10.0</td>
</tr>
<tr>
<td>MG</td>
<td><em>Morinda citrifolia</em></td>
<td>417 ± 124</td>
</tr>
<tr>
<td>DS</td>
<td><em>Artocarpus altitis</em></td>
<td>123.5 ± 21.5</td>
</tr>
<tr>
<td>UB</td>
<td><em>Ipomoea batatas</em></td>
<td>&gt;1000</td>
</tr>
<tr>
<td>DK</td>
<td><em>Piper sarmentosum</em></td>
<td>NA</td>
</tr>
<tr>
<td>DP</td>
<td><em>Pandanus amaryllifolius</em></td>
<td>NA</td>
</tr>
<tr>
<td>TS</td>
<td><em>Crinum asiaticum</em></td>
<td>129.5 ± 3.5</td>
</tr>
<tr>
<td>HB01</td>
<td><em>Zingiber officinale Roscoe var. rubrum</em> (Halia Bara)</td>
<td>10.2 ± 0.8</td>
</tr>
<tr>
<td>UBM</td>
<td><em>Maranatha arundinacea</em></td>
<td>&gt;1000</td>
</tr>
<tr>
<td>AB</td>
<td><em>Tinospora crispa</em></td>
<td>290</td>
</tr>
</tbody>
</table>

Selected plant extracts with the best NO inhibitory effects (with the lowest IC$_{50}$) are highlighted. The results represent the mean ± SD of at least three separate sets of experiments each with two replicates. *NA= not available. IC$_{50}$ of L-NAME is 7.3 ± 1.8 µg/mL
Twelve plants were extracted in 80% ethanol and tested for their effects on NO and PGE₂ inhibition.

**Zingiber officinale Roscoe var. rubrum** (ZOR) was the most active and thus selected for this study. ZOR was then extracted into 3 types of extracts and tested for their effects on NO inhibition.

- HB02 Extracted in 100% chloroform
- HB03 Extracted in 100% ethanol
- HB04 Extracted in water

Fractionated into 21 fractions in hexane:ethyl acetate solvent system, using VLC and tested for NO and PGE₂ inhibition.

Fraction 6 was selected for further fractionation using SPE and isolation using Prep-TLC (PTLC).

Two compounds were identified.

Figure 2.5: Bioassay-guided fractionation process involved a series of anti-inflammatory screening of the selected plants. A work flow of the screening process of potential plant and active compounds with potent anti-inflammatory effects relevant to psoriasis pathology.
The extraction method was optimised based on reported studies associated with ginger extraction method (97). Extract from ZOR was obtained using the solvent extraction method described in section 3.4. Extracts were obtained for comparison of biological effects using chloroform, ethanol or water (Figure 2.5).

In the first stage of the extraction, ZOR powder was obtained by sonication for 30 minutes in chloroform and left to macerate for 5 days at room temperature (Method section 3.4). The filtrate was collected and evaporated, and coded as HB02. The residue was left to dry and re-used for the next stage of extraction which was with ethanol with the same procedure and the extract coded HB03. Finally, water extraction was carried out and the collected filtrate was dried using a freeze drier and coded HB04.

Bioactivity-guided fractionation studies of these ZOR extracts were carried out by screening of the most efficacious extract (Figure 2.5) based on their effect on the inhibition of NO (Table 2.2) production by activated macrophages (discussed in Chapter 3). Among the tested extracts, HB02 was selected due to its higher potency \(\text{IC}_{50} = 10.2 \pm 0.8 \, \mu\text{g/mL}\) and, thus, further fractionated into 21 fractions using VLC (Table 2.3a). Figure 2.6 shows the TLC profiles of the VLC fractions. Undergoing the same screening procedure, the VLC fraction, (F6) exhibited the highest potency among the 21 fractions. F6 was fractionated using a solvent mixture of 80% hexane in ethyl acetate. The TLC as well as \(^1\text{H}\) NMR profiles of F6 signified the presence of major and minor compounds. Therefore, in order to determine the specific compound/s contributing to the potency of F6, it was further fractionated using SPE (Table 2.3b) with solvent of
increasing polarity. Based on the TLC and $^1$H NMR profiles of seven SPE fractions from F6 obtained, fractions 3 indicated the presence of a mixture of compounds with the solvent system of 80% hexane in ethyl acetate (Figure 2.7). Optimisation of the solvent system was carried out to obtain the best compound separation. 90-93% of hexane in ethyl acetate was the best solvent mixture for the isolation process. The purity of each of the compounds isolated from this process was assessed by NMR. Distinct separation of the NMR peaks indicates purity of a compound. Prep-TLC procedure was used to separate components from SPE fractions 3 (Figure 2.7).

Finally, two compounds were identified after several stages of isolation and purification using SPE fraction 3 of F6 (Figure 2.7). Compound 1 (brown box) was purified using a solvent system of 93% hexane in ethyl acetate with 2% acetic acid in 2 stages of isolation. The percentage yield of compound 1 was 0.017% from the crude extract (HB02). Compound 2 was isolated in two stages of prep-TLC in solvent system of 90% hexane in ethyl acetate with 2% of acetic acid. The percentage yield was 0.012% from the crude extract (HB02). Both compounds were characterised by $^1$H-, $^{13}$C-NMR, DEPT and 2D-NMR (COSY, HMQC, HMBC) analysis. The structures were validated using ESI-MS in both positive and negative ion modes, accurate mass and electron impact MS.
Table 2.3: ZOR fractionation process utilising a) VLC and b) SPE method using hexane: ethyl acetate solvent system at variable ratio.

<table>
<thead>
<tr>
<th>VLC fractions</th>
<th>Solvent system (volume in mL)</th>
<th>Hexane : ethyl acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>200 : 0</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>190 : 10</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>180 : 20</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>170 : 30</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>160 : 40</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>150 : 50</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>140 : 60</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>130 : 70</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>120 : 80</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>110 : 90</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a)

<table>
<thead>
<tr>
<th>SPE fractions</th>
<th>Solvent system (volume in mL)</th>
<th>Hexane : ethyl acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100 : 0</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>90 : 10</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>80 : 20</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>70 : 30</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>60 : 40</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>50 : 50</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>40 : 60</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(b)
Figure 2.6: TLC profiles of VLC fractions from HB02 extracts of ZOR. Twenty one fractions (F1-F21) were fractionated from HB02 extracts using VLC fractionation method as described in Method 3.5.1. Each of the fractions was spotted on TLC in hexane: ethyl acetate solvent system with a ratio of 80:20 The TLC profiles indicated abundance of compounds in each fraction. The anti-inflammatory effect of the fractions on NO production produced by macrophages was studied. The most potent fractions were further fractionated using SPE method as described in 3.5.2.
Figure 2.7: Isolation process of active compounds from ZOR. Fractionation and isolation processes of ZOR fractions (yellow) and compounds; compound 1 (brown) and compound 2 (green). VLC and SPE fractionation methods were used, utilising variable ratios of hexane and ethyl acetate mixture as the solvent system. These yielded a number of fractions. Compounds were isolated using Prep-TLC with solvent system of hexane: ethyl acetate (EtOAc): acetic acid. The structure of the isolated compounds was characterised and identified using NMR, MS, IR and UV as described in Methods.
4.2 Identification of anti-inflammatory components in F6

4.2.1 Identification of compound 1 as 6-Shogaol

Compound 1 from fraction 6 (F6) was a pale yellow liquid. Its structure was elucidated as 6-shogaol (Figure 2.8) by $^1$H and $^{13}$C NMR spectroscopy, and confirmed by comparison with data in the literature (121-123).

![Chemical Structure of 6-Shogaol]

Figure 2.8: The established structure of 6-shogaol

$^1$H NMR data of the compound showed proton signals of 1, 2, 4-substituted benzene rings (6.6-6.81, 3H), a methoxy signal (3.86, 3H), a benzyl proton signal (2.84, 2H), ketone $\alpha$-proton signal (2.84, 2H), two olefinic proton signals (6.06-6.09, 1H and 6.84, 1H), signals for methylene groups (1.42, 2H, 1.26-1.32 4H, 2.17-2.21 2H) and a methyl signal (0.89, 3H). Therefore, the compound is identified as 6-shogaol with a molecular formula of C$_{17}$H$_{24}$O$_3$ and calculated molecular weight of 276.37.
**Figure 2.9:** Electron impact (EI) MS spectrum of 6-shogaol. The spectrum indicates the presence of peaks at $m/z$ 137, 205 and 276 due to fragmentation of keto group at C-3.

![MS Spectrum](image)

**Figure 2.10:** Fragmentation of the keto group at C-2. EI fragmentation of 6-shogaol is consistent with previous findings (122).
Based on EI, the major peak was at \( m/z \) 137 (Figure 2.9). The product of fragmentation via electron bombardment was similar to the product observed by Jiang et al (2005) (Figure 2.10). Based on Figure 2.10, the fragmentation was most probably due to the keto group, a protonated group which favours ionisation to take place and thus leads to fragmentation at the \( \alpha \) position at C-2.

\(^1\)H NMR (500MHz, CDCl\(_3\)), \( \delta \): 0.89 (3H, t, H-10), 1.26-1.32 (4H, m, H-8 and H-9), 1.42 (2H, m, H-7), 2.17-2.21 (2H, m, H-6), 2.84 (4H, m, H-1 and H-2), 3.86 (3H, s, OCH\(_3\)), 6.06-6.10 (1H, dt, \( J=3,19 \), H-4), 6.60 (1H, td, \( J=2 \), H-6'), 6.70 (1H, td, \( J=1.5Hz,H-2' \)), 6.81 (1H, d, \( J=7.5 Hz, H-5' \)), 6.84 (1H, d, \( J=15.5 Hz, H-5 \)). (Please refer to Figure 1 in the Appendices).

\(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \( \delta \): 13.7 (C-10, d), 22.6 (C-9, d), 27.9 (C-7, s), 29.6 (C-8, m), 31.1 (C-1, d), 32.2 (C-6, s), 42.1 (C-2, s), 56.0 (OCH\(_3\), s), 111.0 (C-2', d), 114.2 (C-5', s), 120.5 (C-6', s), 130.8 (C-4, s), 133.4 (C-1', s), 143.8 (C-4', s), 146.4 (C-3', s), 148.3 (C-5, s), 200.2 (C-3, s) (Figure 2 in the Appendices).

DEPT, COSY, HMQC, HMBC (Figures 3-6, the Appendices)

IR Spectrum (cm\(^{-1}\)): CH: 2930 (s), 2856 (m), \( C=C: 1667 \) (m) 1693 (m), 1516 (s), Aromatic: 1431-1464(m), C-O: 1271.41(s), OH: 3200-3600 (b) (Figure 7 of the Appendices).

UV spectrum: 227 nm, 285nm (Figure 8 of the Appendices)
4.2.2 Identification of compound 2 as 1-dehydro-6 gingerdione

Compound 2 identified in F6 was characterized as a pale yellow liquid. The structure (Figure 2.11) of this compound was elucidated by $^1$H and $^{13}$C NMR spectroscopy.

$^1$H-NMR data on the isolate showed proton signals of a 1,2,4-substituted benzene ring (6.91-7.09, 3H), methoxy signal (3.90, 3H), two olefinic proton signals (6.32, 1H and 7.54, 1H), ketone $\alpha$-proton signal (5.62, 1H), ketone $\beta$-proton signal (2.38, 2H), signals for methylene groups (2.38, 2H, 1.30-1.33 4H, 1.56-1.67 2H) and a methyl signal (0.89, 3H). The compound could be identified in three tautomeric structures, based on keto-enol tautomerism. However, based on the $^1$H NMR data, it was suggested that the compound was an enol with the presence of a broad peak of an enol-OH at 15.53 ppm and CH at position 4 instead of a CH$_2$ indicated in Figure 2.11(a). In order to differentiate whether the compound was a keto or an enol, a drop of methanol-D$_4$ was added to the chloroform-D solution of the compound. The presence of the enol-OH peak was evident, whereby addition of a drop of methanol-D$_4$ to the chloroform-D solution of the compound eliminated the enol-OH peak at 15.53 ppm (compare figure 9 and 10 in the Appendices).
The UV spectrum of the compound in chloroform had two maxima at 258 and 370 nm, which are associated with the ketonic and enolic forms, respectively. Their intensity, however, varied with the solvent (112). Morton et al (1934) observed that for benzoylacetone, two peaks were observed; 247 and 310 nm that were associated with total ketone and enol content. In addition, Kimura and colleagues suggested that UV absorption at 235 and 280 nm are characteristics of keto and enol of 6-gingerdione (112). Hence, these observations suggested that the peaks at 258 and 370 nm indicate the presence of ketonic and enolic forms within the alkyl chain of compound 2. However, there are two possible structures of the enol tautomer that have to be considered, as elucidated in Figure 2.11b) and c). To determine which of the enol conformations was dominant, MS analysis was carried out.
Figure 2.11: Tautomerism in the structure of 1-dehydro-6-gingerdione. Three tautomers were considered for the structure of compound 2 which were: a) a keto, and enol tautomers b) and c) of the dehydrogingerdione.
Electrospray ionisation, (+) ESI (Figure 2.12a) and (-) ESI (Figure 2.12b) indicated peaks of relative abundance at [M+H]^+=291 and [M-H]^-=289, respectively. This was consistent with findings by Jiang et al. (2005). The accurate mass validates the peak at 291.16 (Figure 18 in the Appendices). The calculated molecular weight, however, is 290.35. This suggested that the compound might be prone to accept and donate one hydrogen atom when ionisation occurs.

The structures in Figure 2.11 indicate the tendency of the hydrogen at C-4 or the diketonic carbonyl at C-3 or C-5 to undergo ionisation in acidic and basic environments as demonstrated in Figure 2.13. Therefore, this indirectly validates the structure depicted in Figure 2.11(b) and (c). However, this was insufficient to validate the orientation of the speculated enolic diketone.

Therefore, the sample was analysed via EI-MS using a forced fragmentation approach targeting at C-4 of the elucidated structure (Figure 2.14). Fragmentation at C-4 of the ketone-α proton yielded m/z at 177.15 indicating that the broken fragment contained a hydroxyl group at C-5. Thus, implying that a carbonyl group was present at C-3 in close proximity to the benzene ring. Hence, verifying the structure as elucidated in Figure 2.11(b).
Figure 2.12: ESI-MS of 1-dehydro-6-gingerdione. ESI spectrum a) \((M+H)^+\) and b)\((M-H)^-\) indicate the presence of two major peak at 291.1 \((M+H)^+\) and 289.1 \((M-H)^-\) confirming the suggested molecular weight of 290.35 of the elucidated structure of the compound.
Figure 2.13: Enol formation via basic and acidic-catalysed processes. Adapted from http://www.chem.ucla.edu/harding/notes/notes_14D_enolates.pdf
Figure 2.14: EI-MS of 1-dehydro-6-gingerdione. The approach manipulated forced fragmentation specifically at C-4 hence, supporting the $^1$H and $^{13}$C NMR data. The EI-MS data confirmed the enolic structure of 1-dehydro-6-gingerdione as elucidated in Fig.2.12 (b).

$^1$H NMR (500MHz,CDCl$_3$), δ: 0.89 (3H, t, $J$=7Hz, H-10), 1.30-1.33 (4H, m, H-8 and H-9), 1.56-1.67 (2H, m, H-7), 2.38 (2H, m, H-6), 3.90 (3H, m, OCH$_3$), 5.62 (1H, s, H-4), 6.32 (1H, d, $J$=16, H-2), 6.91 (1H, d, $J$=8.5, H-5'), 7.02 (1H, d, $J$=1.5, H-2'), 7.09 (1H, dd, $J$=2, 8.5, H-6'), 7.54 (1H, d, $J$=15.5, H-1), 15.53 (1H, brd, enol-OH). (Figure 9 and 10, the Appendices)
$^{13}$C NMR (125 MHz, CDCl$_3$) δ: 14.3 (C-10, t), 22.6 (C-9, t), 25.5 (C-7, t), 31.7 (C-8, q), 34.1 (C-6, s), 56.1 (OCH$_3$, s), 100.4 (C-4, s), 109.6 (C-2’, s), 114.0 (C-5’, d), 120.74 (C-2, s), 122.8 (C-6’, d), 130.2 (C-1’, d), 139.8 (C-1, s), 146.93 (C-4’, s), 147.81 (C3’, s), 178.17 (C-3, s), 200.9 (C-5, s). (Figure 11, the Appendices).

DEPT, COSY, HMQC, HMBC (Figures 12-15, the Appendices).

IR spectrum (cm$^{-1}$): C-H aromatic; 3012 (w), 1592.4 (brd, m), 1513 (s), 1434-1465 (m), C-H Aliphatic; 2933.1(s), CH aldehyde: 2867.4(m), enolic β-diketone: 1640.6 (m), $\equiv$CH OOP- (out-of-plane); 756.96 (Figure 16, the Appendices)

UV spectrum: 258nm and 370 nm (Figure 17, the Appendices)
5 Discussion

5.1 Bioactivity-guided fractionation of ZOR enabled identification of two active compounds.

At the conception of this investigation, twelve plants of known traditional medicinal use from Malaysia were identified and collected. The constituent components of the plants were separately extracted using two extraction methods namely, shaking in 80% ethanol or sonication in 80% ethanol (Method 3.3).

The extracts were tested for their anti-inflammatory effects by determining their ability to suppress the production of NO (Table 2.2) and PGE$_2$ by activated macrophages (refer to Chapter 3). The results revealed that the extracts from ZOR demonstrated the strongest inhibitory effects for both inflammatory mediators. Thus, ZOR was selected for detailed analyses and to identify possible effects on inflammatory mechanisms relevant to psoriasis. Prior to this, the best solvent to use for the extraction for ZOR therapeutic components was determined. ZOR was extracted using maceration in chloroform, ethanol or water. Comparison of the inhibitory effects of the three extracts on NO and PGE$_2$ production in activated macrophages was carried out. The analyses revealed that the extract obtained with chloroform (named HB02) had the strongest inhibitory effects on NO and PGE$_2$ production and, thus, was further fractionated using VLC. This process yielded 21 fractions that were then individually analysed. Using the same procedure, these fractions were screened...
for their effect on NO and PGE\(_2\) production and fraction 6 (F6) was selected for further assessment due to its potent inhibitory effects. F6 was further fractionated and characterised using the same screening procedures. At the end of the fractionation, isolation and purification processes, two compounds from F6 were obtained and characterized and these were determined to be 6-shogaol and 1-dehydro-6-gingerdione, both gingerol-related compounds.

These fractionation and isolation approaches were bioassay-guided and that led to the isolation of potential compound/s with the most potent anti-inflammatory effects relevant to psoriasis. This approach has enabled the development of shorter screening procedures and helped to determine the most effective therapeutic compounds in ZOR. Previous studies of ginger chemistry had suggested a range of extraction and isolation methods. As mentioned earlier, solvent extraction and supercritical CO\(_2\) extraction have been proposed to be best methods to extract pungent compounds that are temperature labile. The methods used in this study avoid the loss of potential compounds with therapeutic effects which could otherwise be lost by the use of high temperature (124), which is part of the steam distillation and soxhlet extraction processes. Furthermore, by utilising the prep-TLC technique, separation of components from small amounts of sample/starting material was possible.
5.2 1-dehydro-6-gingerdione and 6-shogaol are the initial and dehydrated products of 6-gingerol biosynthesis, respectively.

The studies have identified two major compounds that were effective anti-inflammatory components in the fraction 6 (F6), obtained from the chloroform extract HB02. 6-shogaol and 1-dehydro-6-gingerdione were isolated in respective percentages 0.017% and 0.012% from the oleoresin HB02 (Figure 2.7). 6-shogaol is a pungent compound predominantly identified in dried ginger. It is a dehydrated form of 6-gingerol, a major pungent compound in ginger. In contrast, 1-dehydro-6-gingerdione is the initial product of the biosynthesis process of 6-gingerol.

6-gingerol is susceptible to chemical transformation to less pungent constituents such as 6-shogaol during handling and processing due to its instability (111). It is thermally labile due to the presence of a hydroxy keto group in its alkyl side chain and is, thus, easily dehydrated to shogaol during storage (115). In a quantitative study by Kim et al. (2002) of 6-gingerol contents in 14 samples of ginger collected in Korea, the investigators observed that 6-gingerol is approximately 0.36% in fresh ginger, which decreased during processing to 0.31% (111). Assuming that the percentile loss was due to dehydration of 6-gingerol to 6-shogaol, 0.05% could be the maximum yield of 6-shogaol during the dehydration process. 6-gingerol can also be converted to other less pungent compounds such as 6-zingerone and hexanal by base catalysis, or by heat at temperatures above 200ºC (111). In this study, however, this is not the case, since the extraction processes were carried out at room temperature and elevated temperatures were not used. The conversion of gingerols to their
dehydrated forms is an indication of consequent deterioration in quality. The longer the drying period, the higher the loss of content of pungent compounds. Therefore, there could also be a reduction in the amount of 6-shogaol. During storage, 6-shogaol can also undergo hydrogenation upon dehydration and convert to 6-paradol (Figure 2.16) (97). Therefore, the amounts of these compounds vary depending on various parameters before and after processing.

The presence of these pungent compounds can be influenced by many variables such as ginger species (varieties and variants), geographical location of the ginger being collected, cultivation parameters and variables during storage (97, 111, 124). The extraction efficiency is also dependent upon other extraction variables such as solvents used, and the duration and method of extraction (111). The major pungent compounds, gingerols, were detected after 6 hours of extraction in organic solvent and 2 hours in liquid CO₂ (125). Therefore, the percentage of these compounds in extracts is dependent on the consistency of the parameters used during pre- and post harvest, as well as extraction procedures used (115).
Therefore, considering these variables and the limited number of studies on ZOR, the exact yield of 6-shogaol in ZOR was unknown. Its presence will increase during the drying process, nonetheless, at the same time it might be converted to other gingerol-related compound, such as 6-paradol. Unlike 6-shogaol, 1-dehydro-6-gingerdione (GD) was proposed to be the initial product or precursor in 6-gingerol biosynthesis (Figure 2.3) (97, 126). This indicates that the presence of GD is an initial product in the biosynthesis of 6-gingerol in dried and processed ginger. It was proposed by Macleod et al. (1979) that in the biosynthesis of 6-gingerol (Figure 2.3), 6-dehydrogingerdione is the initial product after the incorporation of hexanoate, malonate and ferulate. Subsequently, either 6-dehydrogingerol or 6-gingerdione will be intermediate products, which then commence via C=O and C=C reduction to produce 6-gingerol (113, 127). This mechanism was supported by Harvey (1981), except
for the suggestion that the precursor prior to GD synthesis is dihydroferulate instead of ferulate (128).

Only F6 of the VLC fractions was selected based on its highest anti-inflammatory potency in comparison with the other fractions when compared with several established anti-inflammatory agents (Chapter 3). The data presented in this thesis, therefore, suggest that 6-shogaol and 1-dehydro-6-gingerdione are likely to be the active therapeutic components of ZOR.

5.3 1-dehydro-6-gingerdione isolated from ZOR exists in two forms of enolic tautomers

6-Gingerdiones and 6-dehydrogingerdione in ginger were discovered as the intermediates in the biosynthesis of 6-gingerol, the major pungent compound in ginger (113). 6-Gingerdione was shown to possess both keto and enol forms in liquid and chloroform solvents. The enol form was shown to be dominant when chloroform was used as the solvent (ca. 80%) (129). The enolate form is favoured in an excess base environment and unsubstituted at the inter-carbonyl position which gives rise to steric inhibition of resonance. Therefore, the conformation is pH-dependent. 6-dehydrogingerdione, however, was suggested to exist in through-conjugated enolate in solid state and in solution.

In neutral and acidic environments, both the keto and enol forms exist in equilibrium (130). Most carbonyl compounds exist in the keto form at equilibrium rather than the enol form which is normally present at a small percentage and difficult to isolate (131). However, increasing the acidity of the environment
enables stabilisation of the keto tautomer through hydrogen-bonding interaction with solvent molecules. In contrast, in aprotic and nonpolar solvents, the enol tautomer predominates and it is stabilised by intramolecular hydrogen bonding. This suggests that tautomerisation is strongly influenced by the hydrogen-bond donor and acceptor properties of the solvent used (130). In terms of solubility, compound 2 is soluble in non-polar solvents such as hexane and chloroform, but insoluble in 70% ethanol, which is characteristic of an enol. It could also be a chelated enol, which is associated with lower solubilities in water but higher solubility in non-polar solvents compared with ketone (132).

This suggests that compound 2 which was identified as 1-dehydro-6-gingerdione (GD) could have an enolate form rather than an open-chained enol which is characterised by higher solubility in hydroxylic solvents rather than hydrocarbons.

In reference to studies by Denniff et al. (1981), the structure and molecular mass of 6-dehydrogingerdione closely resembles compound 2 obtained in this study (129). However, the structural orientation of the enolic moiety of 6-dehydrogingerdione was similar to the structure in Figure 2.11c while the structural characterisation of compound 2 is based on Figure 2.11b. The structure elucidation described here was supported by the presence of one α-hydrogen atom at C-4 between the two carbonyl atoms as indicated by the $^1$H NMR peak at 5.62 ppm (Figure 9 in the Appendices). The hydrogen atom is prone to be protonated and lead to the formation of an enolate ion in strong base. Enolate is a resonance hybrid for both keto and enol by the delocalisation of negative charges over both carbonyl groups. In this case, the delocalisation results in the formation of enol, shown by the presence of the highly
nucleophilic double bond at C-4 which fits the structure elucidated in Figure 2.11b.

5.4 Compounds 1 and 2 demonstrate ‘drug-like’ structures.

Numerous studies have shown that ginger has a broad spectrum of anti-inflammatory activity involving multiple mechanisms of action. Several studies have demonstrated that ginger extracts possess similar effects to NSAID in inhibiting PG synthesis by inhibiting COX activity (115). Interestingly, some ginger constituents were found to have dual inhibitory effects against COX and LOX (lipooxygenase), thus, reducing the occurrence of gastrointestinal and renal side effects while expressing their anti-inflammatory effects (115). In addition, ginger extracts and its components have been shown to suppress expression of genes related to inflammation including genes encoding TNFα, IL-1β and the transcription factor, NF-κB (115, 117). Intriguingly, this highlights the potency of ginger as an anti-inflammatory agent with minimum side effects.

Recently, studies have been carried out to determine the anti-inflammatory mechanism of action of ginger constituents using structure-activity relationship (SAR). In terms of their effect on COX-2, it was suggested that the presence of the phenolic hydroxy group adjacent to the methoxy group was critical for the inhibition of PG synthesis, A (Figure 2.16); as well as the substitution pattern of the hydroxyl and carbonyl moiety on the side chains, B (Figure 2.16) and the length of the side chain itself, C (Figure 2.16) (115, 118). Length of the side chain contributes to the lipophilicity of the compound. Based on the elucidated structures of 6-shogaol and 1-dehydro-6-gingerdione isolated from ZOR, the log
P value was estimated using the software known as jlogP; http://www.vls3d.com/JME_EditorOK.dir/run_jlogp.html.

As mentioned earlier, log p is an estimated value of lipophilicity of a structure. The estimated log P value of 6-shogaol and 1-dehydro-6-gingerdione are 3.53 and 3.12 respectively. These values are similar to those of ‘drug-like’ compounds according to Lipinski’s Rules of five Ro5 (logP ≤5) (133). Based on studies on the effect of gingerol-related compounds carried out by Tjendraputra et al. (2001) using IC$_{50}$ vs. log P relationship, 6-shogaol demonstrated more potent activity than 8-shogaol (log P=4.9) with IC$_{50}$ of 2.1 ± 0.3 μM and 7.2 ± 0.4 μM, respectively. Thus, supported by the Lipinski’s Rules of five, the logP of a ‘druggable’ compound should be ≤5. This, therefore, suggests that 6-shogaol and GD isolated from ZOR have the potential as ‘druggable’ compounds with good distribution and delivery to targeted systems.

The structural orientation of 6-shogaol and 1-dehydro-6-gingerdione are differentiated by the orientation at B (Figure 2.16), which is the substitution pattern of the hydroxyl and carbonyl moiety on the side chain. Compared with 6-shogaol, GD has a hydroxyl group as part of an enolic moiety which could be easily chelated. 6-shogaol only features one free hydroxyl whilst GD has a free hydroxyl and enol-hydroxyl groups in the alkyl chain. Therefore, the activity of both compounds could be differentiated by the presence of an enolic moiety which is more reactive as an enolate ion in basic solution. Both compounds contain one oxygen atom and at least one hydroxyl group which are hydrogen acceptors and donors, respectively.
Thus, this satisfies the criteria of a ‘druggable’ compound (hydrogen-donor≤5 and hydrogen-bond acceptor≤10). However, Lipinski’s Rules of five is not an absolute indicator of the efficacy of a compound as a drug, instead, it is used as a preliminary guideline to identify drug candidates in natural product research and drug discovery.

Figure 2.16: The structural orientation of a) 6-shogaol and b) 1-dehydro-6-gingerdione. Structural orientation of 6-shogaol and -dehydro-6-gingerdione denotes by A, B and C, contribute to the activity of the compounds (112, 115, 118).
6 Conclusions

To-date, various studies have been performed to identify active therapeutic constituents and potential biological activity of various ginger species. The majority of these studies identified a number of non-volatile pungent agents as potentially the major components that potentially contribute to specific functional effects of various types of ginger extracts. The most studied are the gingerols and gingerol-related compounds. The biological efficacy and potency of these compounds are suggested to be determined by the presence of: a) the phenolic hydroxy group adjacent to the methoxy group; b) the substitution pattern of the hydroxyl and carbonyl moieties on the side chains; and c) the length of the side chain itself (115, 118).

There are, however, very limited studies on the chemical composition and biological activity of ZOR. This study has identified two constituents from the most active fraction of ZOR which are 6-shogaol and 1-dehydro-6-gingerdione. Previously, these compounds have been discovered and structurally characterised in common ginger and other ginger species (121, 134, 135). This study show that 1-dehydro-6-gingerdione isolated from ZOR is in the enolic conformation as shown in Figures 2.11(b) & 2.16c. This structure is characterised by the presence of a carbonyl group near the aromatic ring rather than a hydroxyl group as elucidated by Macleod et al. (1979) and Denniff et al (1981) (113, 129). The significance of the –OH at C-5 was discussed by Tjendraputra et al. (2001) whereby, gingerol-related constituents with this orientation were shown to exhibit inhibitory effects on COX-2 activity via H-bonding and hydrophobic interaction to the binding site of this enzyme.
Based on previous studies, 6-shogaol is known to be present in dried ginger (111) whilst 1-dehydro-6-gingerdione is present in fresh and dried ginger (121). 6-shogaol is a dehydrated form of 6-gingerol whilst 1-dehydro-6-gingerdione is an initial product in 6-gingerol biosynthesis. Assuming that 6-gingerol is essentially present in fresh ginger, 1-dehydro-6-gingerdione should be available as well. Therefore, this could be an indication of the ‘freshness’ or the ‘quality’ of the extract. It is worth noting that, the proportion of these compounds varies between species and variants, depending on the pre- and post-harvest processes.

Through the evaluation of the structure orientation of each compound, it could be deduced that they demonstrate ‘druggable’ criteria as stated by Lipinski’s Rule of Five (Ro5)(133) and match the proposed SAR characteristics of gingerol-related compounds proposed by in previous studies (112, 115, 118). Therefore, due to their ‘druggable’ potential, these compounds were studied for their effects on key mechanisms implicated in the pathogenesis of psoriasis as will be discussed in the following chapters.
CHAPTER 3

Modulatory effects of ZOR in inflammation: Inhibition of NO and PGE$_2$ production
1 Introduction

1.1 Macrophage activation by autoreactive lymphocytes promotes psoriasis pathogenesis

Numerous studies have provided evidence that the initiation of psoriatic plaques is a consequence of the activation of dendritic cells (DCs) and macrophages (136). Both DCs and macrophages serve as antigen presenting cells (APC) to T-lymphocytes during adaptive immune responses. The two cell types are differentiated partly by their migratory ability. Thus, macrophages are non-migratory cells, as they normally reside in tissues. Whilst, DC migrate and interact with naive and memory T-lymphocytes throughout their migratory routes. Further, due to the plasticity of these two cell types, their functions could only be distinctly differentiated by the cytokines they produce upon their activation (137).

In the early stages of inflammation, DCs and macrophages play significant roles in combating the source of inflammation by releasing nitric oxide (NO) and prostaglandin (PGs). However, prolonged inflammation causes the production of high levels of these pro-inflammatory mediators, leading to chronic inflammation-associated cellular and tissue damage as seen in various autoimmune diseases such as rheumatoid arthritis and psoriasis. Polymorphism in the promoter region of iNOS has been suggested to attenuate the affinity with which transcription factors bind to gene, thus, leading to various diseases (138). The role of iNOS and its products have been implicated in the pathogenesis of psoriasis. Early findings indicated that iNOS was detected in the basal epidermis of lesional skin in concert with the production of IL-8, a known
chemotactic cytokine involved in the initiation of inflammation (139). Further findings by Bruch-Gerharz and colleagues (1996) suggested that iNOS may be involved in the early phases of psoriatic lesion formation and that its presence within the lesion could be induced by IL-8 (139).

Myeloid DCs (CD11c+), which are abundant in the dermal layer, are known as TNFα- and iNOS-producing DCs, or TIP-DCs. In addition to producing high levels of TNFα and iNOS, TIP-DCs produce IL-23, IL-12 and IL-20 which activate T-cells and upregulate gene transcription in keratinocytes through activation of STAT-3. TIP-DCs are found in large numbers in psoriatic lesions and, in some cases, outnumber T-cells in psoriatic plaques (31). Cytokines produced by TIP-DCs play critical roles in initiating Th1 and Th17 T-cell differentiation and proliferation (31).

During inflammation, iNOS-induced NO production in the dermal layer is mediated by macrophages and keratinocytes. Consequently, NO potently regulates keratinocytes growth and differentiation, and is implicated in psoriasis. (45). This indicates that NO plays an important role in molecular signaling that facilitates crosstalk between macrophages, T-cells and keratinocytes in psoriasis. In addition to NO, PGE₂ has been suggested to participate in the pathogenesis of psoriasis. PGE₂ is known to induce DC to produce IL-23 which, in turn, promotes Th17 cell differentiation (8). These findings suggest a key role for DCs and macrophages during the initiation of inflammation.
1.2 NO and PGE$_2$ can play paradoxical roles in autoimmune inflammatory diseases

NO is produced as a result of the action of nitric oxide synthase (NOS) on L-arginine. There are two isoforms of NOS: 1) constitutive NOS including endothelial NOS (eNOS) and neuronal NOS (nNOS) and 2) inducible NOS (iNOS). Constitutive NOS is a key regulator of homeostasis. In contrast, iNOS plays an important role in host-defence responses. The iNOS protein is composed of two catalytic domains encoded by 27 exons; 1-13 for the oxygenase domain while 14-27 encode the reductase domain. Unlike constitutive NOS that are only present in specific cells and produce small amounts of NO, iNOS is present in various cells and produce high levels of NO for a prolonged period of time (140).

PGE$_2$, in contrast, is generated via cyclooxygenase (COX). Similarly, COX exists in two isoforms; one is constitutive (COX-1) and one is inducible (COX-2). The inducible forms of NOS and COX have been implicated in many inflammatory conditions as a component of the inflammatory response. Once these enzymes are activated, NO and PGs are produced continuously at sites of inflammation. Paradoxically, however, these inflammatory mediators play double-edged roles, both in sustaining homeostasis and exacerbating inflammation. Various studies have been carried out to understand the pathogenic and therapeutic roles of NO and PGE$_2$. 
iNOS and COX-2 are induced and regulated through a number of mechanisms and by a number of mediators. Crosstalk between these two pro-inflammatory enzymes has attracted much interest but data generated so far are conflicting. A number of studies indicate that NO could activate and regulate COX enzyme activity and, thus, regulate PGE\(_2\) production. For instance, adding IL-1\(\beta\) and NO donors, such as sodium nitroprusside (SNP), to human foetal fibroblast was shown to increase PGE\(_2\) production, which decreased with the addition of haemoglobin, a known NO scavenger (141). However, prolonged induction (>24 hours) of PGE\(_2\) with NO donor was shown to inhibit COX-2 (142). This observation was supported by D’Acquisto and colleagues, showed that NO donors SNP and S-nitroso-glutathione (GSNO) inhibited COX-2 and PGE\(_2\) production by increasing IκB-\(\alpha\) protein expression and, thus, blocking NFκB activation (143). These findings identify the potential negative feedback mechanism by which NO down-regulates COX-2 activity.

Liu and colleagues (2009) suggested that COX-2 activity is modulated by NO through transcriptional and post-transcriptional mechanisms (141). It is established that both COX-2 and iNOS are regulated by modulation of NF-κB, a transcription factor located in cytoplasm, which consists of a p50/p65 heterodimer attached to an inhibitor protein subunit, IκB. Upon stimulation by endotoxins (e.g. LPS), or by cytokines, IκB kinase (Ikk) phosphorylates the regulatory domain of the inhibitory κB (IκB) protein leading to its degradation. The degradation of IκB enables NF-κB to enter the nucleus where it initiates the transcription of many pro-inflammatory genes. It was reported that NO enhances the activity of IKK-\(\alpha\), or DNA-binding of NF-κB (142). This in turn could lead to the activation of COX. Moreover, NO was proposed to modulate
COX-2 enzymatic activity via iNOS selective binding to COX moiety which enables accessibility of NO molecule to the COX-2 active site. NO could also up-regulate COX-2 activity by nitrosylation of the cysteine residue at position 152 (Cys-152) within the active site of COX-2, or suppress COX-1 activity by nitration of tyrosine residues (Tyr385) at COX-1 active site (144). This supports the suggestion by Salvemini et al. of an inter-relationship between the two pathways (140, 145). Thus, these investigators proposed a direct interaction between NO and COX leading to increased COX activity. During acute inflammation, excess amounts of free radicals, such as superoxide, are generated that in turn scavenge NO to produce highly reactive peroxynitrite (ONOO\(^{-}\)) (Figure 3.1). Together with NO, peroxynitrite plays a role in further activating COX (Figure 3.1) which, leads to increased prostaglandin (PG) production.

However, questions were raised as to whether COX and its products could modulate the NO pathway. Earlier studies proposed that products of the COX pathway do not modulate iNOS activity as suggested by the effect seen with indomethacin (a non-selective COX inhibitor) on arthritis, whereby the drug only blocked PG rather than NO production (145). In contrast, D’Acquisto and colleagues showed that iNOS protein expression in the LPS-stimulated J774 mouse macrophage cell line was down-regulated by PGE\(_2\) and iloprost (a prostacyclin analogue) through the inhibition of NF-κB. Nevertheless, the study showed that PGE\(_2\) and iloprost were only effective at the transcriptional level and had no direct effects on iNOS enzymatic activity (146). Thus, it has been regarded as an advantage of selective iNOS inhibitors that would have a dual inhibitory effects on both NO and NO-driven COX-s activation, while
maintaining cytoprotective roles of constitutive enzymes such as cNOS against inflammation (140).

The overall picture, therefore, reveals an inter-relationship between iNOS and COX during inflammation (Figure 3.1). The presence of both enzymes is important in innate immunity during infections and inflammation. Nevertheless, when produced in excess, they cause chronic inflammation as seen in autoimmune diseases. These observations have led to the emergence of a number of non-steroidal anti-inflammatory (NSAID) drugs that modulate the COX pathway. However, although there are numerous studies on drugs targeting the NO pathway non-specifically, few drugs have been designed to specifically target this pathway, particularly targeting iNOS (141).
Figure 3.1: A sketch showing the proposed crosstalks between the iNOS and COX-2 pathways. The crosstalk involves molecular mechanisms at transcriptional and post-translational levels via the NF-κB pathway. Solid arrow indicates direct effects whilst dashed arrow indicates indirect effects. Adapted and modified from Salvemini (140).

1.3 Anti-inflammatory effects of ZOR extract, fractions and constituents

It is established that ginger extracts have modulatory effects on various mechanisms and pathways involved in inflammation. For example, a number of studies have suggested that extracts from dried ginger inhibit prostaglandin biosynthesis (112). As cited in chapter 2, phenylpropanoids, or gingerol-related, compounds isolated from ginger including gingerol, shogaol, and paradol have anti-oxidative, anti-inflammatory and anti-cancer effects. These inhibitory effects
on prostaglandin biosynthesis appear to correlate with the length of their aliphatic tail, with longer chains leading to higher affinity and enhanced inhibitory effects (117). Among these compounds, 10-gingerol, 10-shogaol and 1-dehydro-10-gingerdione (1-DH-6-GDO) show the strongest effects. Interestingly, it appears that the orientation of hydroxyl, methoxy and carbonyl groups in the structure of these compounds is important to determine the potency of the phenylpropanoid species as anti-inflammatory agents (115).

Although ginger has been shown to contain some of these constituents there have been limited studies on the effectiveness of extracts obtained from ZOR and on the potential anti-inflammatory constituents of ZOR. Previous studies have identified some potentially-active ingredients from the essential oil of this ginger species (107). Further, anti-bacterial effects of ZOR has also been studied (102). To-date, ethanolic extracts from ginger species similar to ZOR collected from Indonesia have been studied for their anti-arthritic effects in acetic acid-induced mouse writhing model (147). This study showed that the ethanolic extract had major inhibitory effects on PGE\(_2\) but minor effects on NO inhibition. The inhibitory effects on NO production were reported to be due to the presence of high levels of 6-shogaol, gingerdiol and proanthocynidin in the extract.

Studies by Frondoza and colleagues identified potential mechanisms that could underlie the effect of ginger on inhibiting COX-2 and NF-κB activity to induce PGE\(_2\) and TNFα production in human synoviocytes, respectively (148). This finding was supported by Pan and colleagues who showed that 6-shogaol obtained from ginger inhibited iNOS and COX-2 gene transcription in
macrophages through down regulation of NF-κB activation and blocking the activation of PI3K/Akt/IKK and MAPK (149). As discussed earlier, NF-κB is involved in the transcription of the genes encoding NOS and COX. Besides the effect ginger in suppressing gene induction by NF-κB, computational modelling of protein ligand docking has provided further insights that 6-gingerol and 6-shogaol, can directly inhibit COX. These compounds have been suggested to have the potential as direct inhibitors of COX-2 enzyme due to their affinity for COX-2 active sites (150). Although, by and large, this is a theoretical approach, it provides a useful possibility to understand how ginger could modulate inflammation.

2 Objectives

The objectives of the experiments in this chapter were to explore the anti-inflammatory potential of ZOR in comparison with established pharmaceutical compounds, its mechanisms of action and the specific effects of compounds isolated from this species of ginger. Twenty four crude extracts from 12 Malaysian plants were initially studied for their anti-inflammatory effects such as the inhibition of NO and PGE$_2$ production and suppression of $iNOS$ and $TNF\alpha$ gene transcription. Among the plants studied, ZOR extract was selected due to its highest efficacy in inhibiting NO and PGE$_2$ production. ZOR extracts were further refined and characterised as reported in chapter 2. Further, compounds isolated from the most active fraction of ZOR were studied in detail for their effects on mediators of inflammation that are produced by activated macrophages.
3 Methods

3.1 Samples

Concentrated stocks of extracts and HB02 VLC fractions described in Chapter 2 were prepared in DMSO at 50 mg/mL. HB02 compounds were prepared at 10 mg/mL. These stocks were diluted in DMEM and tested at various concentrations throughout the experiments. The effect of HB02, fractions and compounds on the inhibition of NO production was compared with L-NAME (L-N\(^{G}\)-Nitroarginine methyl ester)(Sigma) a known NO inhibitor. Similarly the effect of the test samples on inhibiting PGE\(_2\) production was compared with Indomethacin (Fluka), a known inhibitor of PGE\(_2\) production and with dexamethasone (Sigma).

3.2 Cell culture

The murine macrophage cell line (RAW 264.7) was used in stimulation experiments to induce NO and PGE\(_2\) production. The cell line was maintained in DMEM (BioWhittaker LONZA) supplemented with 10% FBS (GIBCO) and 1000 U/mL penicillin and 100 μg/mL streptomycin. The cells were incubated at 37\(^\circ\)C with 5% CO\(_2\) in a humidified incubator. The cells were sub-cultured when reaching 90% confluence by dilution at 1:10 ratio with fresh medium, or at approximately 2 x 10\(^5\) cells/mL in fresh medium. Generally, the cells reached confluence after 2 days of incubation. Subsequent experiments to determine
optimal conditions for using the cells showed that it was essential to make sure that the cells were in the log phase before splitting them for stimulation.

Assessment of the best conditions for the growth of RAW 264.7 cells was carried out for high cell viability (more than 80% confluence). Before subculturing, cells were detached from the plate using trypsin EDTA (170000U) (BioWhittaker LONZA) and viable cells was counted using an inverted microscope (Nikon TMS). The cells in suspension and the trypan blue solution were mixed at equal volumes and cells counted in 4 chambers of the haemocytometer. The number of cells in the suspension was determined according to the calculation:

Cells per 4 grids x dilution factor\(^*\) x \(10^{4}** = \text{number of cells/mL}

\( (*\text{Dilution factor } = 2 \text{ as the cell suspension was diluted with an equal volume of trypan blue; } **\text{the haemocytometer retained a volume of } 10^{-4}\text{mL.}

### 3.3 Optimisation of protocols used for cell culture conditions, treatment and stimulation to induce NO and PGE\(_{2}\) production

During the early stages, it was decided to optimize the methods to identify the most favourable conditions for murine macrophages to induce maximal amounts of NO and PGE\(_{2}\). In order to determine the most effective stimulation method for NO and PGE\(_{2}\) production, the RAW 264.7 cells were stimulated using two approaches: 1) the cells were stimulated with LPS; and 2) the cells were stimulated with a cocktail of pro-inflammatory cytokine and LPS. The stimulating cocktail contained 10 \(\mu g/mL\) LPS (Sigma), 20 ng/mL TNF\(\alpha\)
(Peprotech) and 20 ng/mL IL-1β (Peprotech) whereas in the LPS stimulation assay, the cells were stimulated with 0.1 μg/mL LPS. Besides using additional amounts of pro-inflammatory cytokines, in the cocktail method, higher density of cells with longer incubation periods were used. NO produced by the activated RAW 264.7 cells was compared in two stimulation protocols namely stimulation with LPS+TNFα+IL-1β (LPS + CT) and stimulation with LPS. The efficacy of these methods was assessed on the amount of NO produced. The method that produced the highest and NO was selected.

Prior to stimulation with the LPS and the cytokine cocktail, RAW 264.7 cells were cultured at a density of $1 \times 10^6$ cells/mL in 12 well plates and incubated for 24 hours at 37°C in 5% CO₂. After incubation, dilutions of the extracts, or positive control, (L-NAME, a NO inhibitor), were added to the cells and cultured for a further 24 hours before stimulating the cells with the cytokines cocktail (10 μg/mL LPS, 20 ng/mL TNF-α and 20 ng/mL IL-1β). After 24 hours of culture, supernatants were collected for NO and PGE₂ measurements and the cells harvested for RNA extraction. This approach was used in the preliminary study on the NO and PGE₂ inhibitory effects of the twelve types of plant.

In the subsequent studies on the effect of Z. officinale Roscoe var. rubrum (ZOR) (Halia Bara) extracts, fractions and compounds on NO and PGE₂ production, RAW 264.7 cells were stimulated only with LPS. Two culture conditions were used to assess the optimal activation conditions for the RAW 264.7 cells with LPS. In the first approach, the cells were cultured in 24-well plates at a density of $2.5 \times 10^5$ cells/mL with 1 mL per well. The cells were then incubated for 24 hours at 37°C in 5% CO₂. A range of plant extract
dilutions were prepared and 100 µl of each dilution added to a total volume of 1000 µl to each well and incubated for 30 minutes. The cells were then stimulated with 0.1 µg/mL LPS and cultured for a further 24 hours. Supernatants from the cultured cells were collected and centrifuged at 1700 g at 4°C to remove cell debris. NO and PGE₂ measurements were then performed. The level of pro-inflammatory cytokines was also determined in the supernatants by enzyme linked immunosorbant assay (ELISA). mRNA level of cytokines were determined using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

For the second culture condition, the cells were first stimulated with 0.1 µg/mL LPS for 12 hours before treatment with ZOR extract (HB02), fractions or compounds. The treated cells were then incubated for a further 24 hours. Supernatants were then collected as indicated previously. NO and cytokines protein levels were then quantified. The cells were used for mRNA extraction and cytokine gene transcript levels using qRT-PCR (real-time PCR).

### 3.4 Measurement of NO

The NO content of the supernatants was measured using the Griess reagent which measures nitrite, a stable product of NO. One hundred µl of culture supernatants were dispensed into 96 well plates followed by the addition of 100 µl of Griess reagent (1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylendiamine in dH₂O). Absorbance at 560 nm was measured within 10 minutes using a microplate reader (TECAN GENios). Nitrite contents were
calculated by extrapolation from a standard curve constructed using known concentrations of sodium nitrite, 0-100 μM.

### 3.5 Measurement of PGE$_2$

PGE$_2$ was measured using a PGE$_2$ ELISA kit (R & D Systems) and an HTRF PGE$_2$ kit (CISBIO). The PGE$_2$ ELISA kit was used as one of the screening tools in the preliminary study to select plants with anti-inflammatory activity for further studies. Supernatants from the cultured RAW 264.7 were collected as described above and stored at -80°C until tested.

For the PGE$_2$ ELISA kit, 150 µl of supernatants, negative control and PGE$_2$ standard dilutions (0-2500 pg/mL) were added to each well, that were pre-coated with goat anti-mouse polyclonal antibody. To the samples was added 50 µl of primary antibody with specificity to PGE$_2$ with the exception of wells designated as blank (NSB) to which only 50 µl of buffer was added. The plate was incubated for 1 hour at room temperature on a horizontal orbital shaker at 500 ± 50 rpm. Later on, 50 µl of PGE$_2$ conjugated to horseradish peroxidise (HRP) was added and the plate was incubated for a further 2 hours on the shaker. The wells were then aspirated and washed 4 times with wash buffer (PBS containing 0.05% Tween 20; PBS/T). The plate was aspirated and blotted dry on a clean paper towel to remove any residual wash buffer. Two hundred µl of the substrate was added into each well and the plate incubated for 30 minutes on the bench with minimal light exposure. Finally, 100 µl of stop
solution (2N sulphuric acid) was added and the OD measured at 450 nm (reference: 560 nm) using microplate reader (TECAN, GENios).

The HTRF PGE\textsubscript{2} kit (CISBIO) was used in the subsequent experiments to more precisely assess the therapeutic effect of ZOR extracts, fractions and compounds in regulating PGE\textsubscript{2} production by activated RAW 264.7 cells. Assays were performed in a 96-well microtitre plate format, requiring 25 μl of each reagent (which were anti-PGE\textsubscript{2} antibody labelled with cryptate (anti-PGE\textsubscript{2} cryptate), PGE\textsubscript{2}-d2 and buffer) and 50 μl of sample, standard or diluents (for negative control) to a total volume of 100 μl. PGE\textsubscript{2} standard was diluted in a range of 0-1667 pg/mL from a stock solution of 5000 pg/mL.

This assay is a competitive immunoassay based on HTRF® technology (Homogeneous Time-Resolved Fluorescence) and involves competitive binding between PGE\textsubscript{2} produced by cells and d2-labeled PGE\textsubscript{2} to monoclonal anti-PGE\textsubscript{2} antibody labelled with Cryptate. The HTRF® is based on Forster’s Resonance Energy Transfer (FRET) between a Europium (Eu\textsuperscript{3+}) Cryptate (donor) and a second fluorescent label (acceptor), d2 when both are in close proximity. The excitation of the donor at 620 nm triggers FRET toward the acceptor leading to detection of emission signal at 665 nm using a Berthold Mithras LB 940 multimode plate reader (Berthold Technologies). The signal is inversely proportional to the concentration of PGE\textsubscript{2} in the sample. PGE\textsubscript{2} levels were calculated by interpolation from a PGE\textsubscript{2} standard curve plotted using known standards and a curve fit program (GRAPHPAD PRISM).
3.6 RNA extraction

RNA was extracted in a RNase and DNase free environment and using sterile consumables. RAW 264.7 cells were harvested and lysed using lysis/binding solution of RNAqueous®-4PCR kit. Sample lysates were mixed with ethanol and applied onto the provided AMBION® column which selectively bound to mRNA and larger ribosomal RNA. The RNA extracts were treated with ultra-pure DNase I to remove any contaminating DNA. mRNA yields varied depending on the type and amount of sample. Generally, about 1 μg of mRNA was obtained from 1x10^5 cells. The yield of mRNA (ng/μl) was determined using a NanoDrop 1000 spectrophotometer, at 260 nm. Ratio of 260/280 nm was used to assess the purity of the isolated RNA. The ratio of pure RNA is ~2.00. RNA was stored at -80 °C prior to use.

3.7 RT-PCR

Twenty ng of total RNA extracted from cell lysates were reverse transcribed to single-stranded cDNA in a 20 μl reaction mixture using High-Capacity cDNA Reverse Transcription Kit. The master mix was prepared on ice prior to addition of mRNA. The master mix contained a final concentration of 1x RT buffer, 4 mM dNTP mix, 1x RT primer, RNase inhibitor (100U) and multiscript reverse transcriptase (50U). RNA samples were pre-heated at 65°C for 5 minutes prior to mixing with the master mix. The condition for reverse transcription was;
The generated cDNA was then used for PCR for assessing the level of pro-inflammatory cytokines transcripts using glyceraldehydes 3-phosphate dehydrogenase (GAPDH) gene as a control. The reactions were carried out using a PCR kit (PROMEGA). Master mixes were prepared prior to adding cDNA from the above experiments. The master mixes consisted of final concentration of 1x TAQ buffer, 1.5 mM MgCl₂, 2 mM dNTP, selected forward and reverse primers (2 mM) and GoTaq DNA polymerase enzyme (5U). Samples were subjected to 34 amplification cycles using Peltier Technology Thermal Cycler (PTC-200 MJ Research) according to these conditions:

- **Initial denaturation** 95°C 2 minutes (1 cycle)
- **Denaturation** 95°C 30 second (34 cycle)
- **Annealing** 60°C 30 second (34 cycle)
- **Extension** 72°C 30 sec/ kb (34 cycle)
- **Final extension** 72°C 10 second (1 cycle)
The PCR products were visualized with ethidium bromide (10 mg/mL, BDH) for DNA staining following agarose gel electrophoresis. The gels consisted of 1% agarose in 0.5x TAE buffer (containing 0.02M Tris acetate and 1 mM Na₂EDTA pH 8) and 5 μl ethidium bromide. Fragment sizes were confirmed by comparison with a 1 kb DNA ladder molecular weight marker (Invitrogen). The level of iNOS transcripts were assessed using primers for the murine iNOS gene (95 bp) and TNFα (175bp), and house-keeping gene, GAPDH (480 bp) (Table 3.1).

Table 3.1: Summary of PCR primers used in the experiments.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH_for</td>
<td>CACCATCTTCCAGGAGCGAGA</td>
</tr>
<tr>
<td>GAPDH_rev</td>
<td>ACGGAAGGCCATGCCAGTGA</td>
</tr>
<tr>
<td>iNOS_for</td>
<td>CAGCTGGGCTGTACAAACCTT</td>
</tr>
<tr>
<td>iNOS_rev</td>
<td>CATTGGAAATGGAAGCGTTCG</td>
</tr>
<tr>
<td>TNFα_for</td>
<td>CATCTTCTCAAAATTCGAGTGACAA</td>
</tr>
<tr>
<td>TNFα_rev</td>
<td>TGGGAGTAGACAAAGGTACACCC</td>
</tr>
</tbody>
</table>

3.8 Real time PCR (quantitative PCR)

Real time PCR enables the monitoring and quantification of changes in RNA levels. The target gene is detected when it is first amplified at a given point of time during the DNA amplification process. 5.5 μl of ABsolute SYBR Green ROX mixes (THERMO Scientific) and 0.5 μl of TAQMAN® gene expression assay probes (Table 3.2) were mixed with 6 μl of the samples cDNA. TAQMAN
gene expression consists of 3 oligonucleotides which are forward and reverse primers along with a TAQMAN probes with attached fluorosphores; reporter dye and a quencher. The primers bind to specific targets and anneal to the DNA template. The TAQMAN probe covalently bind DNA template between the two primers. During PCR, DNA polymerase adds nucleotides and removes the TAQMAN probe. This permanently separates the quencher from the reporter fluorophore and energy is then emitted by the reporter fluorophores which is detected as fluorescence. Thus, signal increase is proportional to the amount of the PCR product. DNA concentrations can be quantified by plotting fluorescence against cycle number on a logarithmic scale.

A threshold of fluorescence above background is established. The sample fluorescence which intersects the threshold is called the cycle threshold (C_T). The expression of gene of interest (GOI) in test samples was normalised to GAPDH (reference gene) and expressed as a relative value using the comparative C_T method (2^{-\Delta\Delta C_T}) (151). The reference gene is used as the baseline for the expression of the target gene and is considered a benchmark. The C_T value of the target gene is determined and normalized with the C_T values of the reference gene to obtain 2^{-\Delta\Delta C_T}. 2^{-\Delta\Delta C_T} values which are the relative quantitative expression of the target gene. This method relies on measuring the GOI relative to that of a reference gene which in these studies was the GAPDH gene (Table 3.2).
### Table 3.2: TAQMAN pre-designed primers from murine source

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amplicon length</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>TAQMAN assay ID: Mm99999915_g1  &lt;br&gt;NCBI RefSeq: NM_008084.2</td>
<td>107</td>
</tr>
<tr>
<td>iNOS</td>
<td>TAQMAN assay ID: Mm01309898_m1  &lt;br&gt;NCBI RefSeq: NM_010927.3</td>
<td>81</td>
</tr>
</tbody>
</table>

### 3.9 Bicinchoninic acid assay (BCA assay) for protein quantification

The purpose of conducting this assay was to standardise the amount of protein to be loaded on the NuPAGE gel (Invitrogen) for Western blot analyses. Cells were washed with PBS and lysed using RIPA buffer (50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulphate) and 10 µl/mL protease inhibitor cocktail (containing AEBSF, Aprotinin, Bestatin, E64, Leupeptin and Pepstatin) (Sigma). Cells were left on ice for 10-15 minutes and then centrifuged (1700 g) at 4°C for ~10 minutes to eliminate any protein residues. The supernatants were collected and either tested immediately or stored at -80°C until tested.

The concentration of proteins in the cell lysates was determined using the BCA™ protein assay kit (Thermo Scientific). Cell lysates were diluted in RIPA (1:1 ratio). The diluted cell lysates were dispensed in 96-well plate and mixed with working reagent solution (WRS) in 1:20 ratio. The mixture was incubated for 30 minutes at 37 °C on horizontal shaker. The total protein concentration was measured at 562 nm using multiwall plate reader (TECAN GENious).
Visually, the reaction is exhibited by a colour change of the sample solution from light blue to purple in proportion to protein concentration. The assay involves two stages of reaction manipulating the well-known reduction of Cu$^{2+}$ to Cu$^{1+}$ by protein in an alkaline medium which then gives a light blue colour. The cuprous cation (Cu$^{1+}$) was then reacts with bicinchoninic acid and chelated into BCA/copper complex which gives an intense purple-coloured reaction product.

### 3.10 Western blotting

Equal amount of proteins (30 μg) were mixed with reducing loading buffer (LB) in a volume ratio of 1:3 (LB: sample). The mixture was then heated at 100ºC for 5 minutes to denature the proteins, and then centrifuged briefly. Ready-made Bis-Tris 4-12% NuPAGE gels (Invitrogen) were used in these experiments. Sample LB mixtures were loaded in each well of the NuPAGE gel alongside with 10-250kDal Precision Plus protein standard. The gel was run for 2 hours at 150V in 1x NuPAGE running buffer (Invitrogen). The next stage involved blotting the gel onto a nitrocellulose membrane whereby the gel is sandwiched with sponges, filter paper and the nitrocellulose membrane in a cassette. This step was carried out carefully to avoid breakage of the gel. The blotting was carried out for 2 hours at 30V. Once proteins were transferred onto the nitrocellulose membrane, the membrane was blocked with 5% fat-free milk in PBS for 1 hour at room temperature (RT). The membrane was then incubated with the primary antibody, which was mouse iNOS specific rabbit antibody (Cell Signalling) in 5% milk (ratio of 1:1000) to detect the level of total iNOS protein. The membrane was incubated overnight at 4ºC.
The blot was then washed 4 times (for 15 min each) with PBS/T (0.05% Tween-20 in 1x PBS) and then incubated with the secondary antibody, sheep anti-rabbit IgG polyclonal antibody conjugated with horseradish peroxidase (AbD Serotec). The membrane was washed 4 times again with PBS/T and soaked in enhanced chemiluminescence (ECL) solution for 1 minute. The membrane was exposed to autoradiography film for different periods of time (1, 10 or 30 minutes) and film developed using SRX-101A (Konica Minolta). These steps were repeated for the detection of Actin using rabbit anti-Actin antibody (Sigma). Prior to incubation with the antibody, the membrane was washed with mild stripping buffer to eliminate bound iNOS antibody.

### 3.11 Statistical analysis

Data was analysed using GraphPad Prism software (GraphPad Prism, San Diego, California). Means and standard deviation were used to describe normally distributed data. The significance of differences between groups was assessed using One-way ANOVA (Tukey’s or Dunn’s multiple comparison test) or Two-way ANOVA (Bonferroni posttest). Comparison between two selected groups was performed using Student t-test. Difference of p<0.05 is considered significant.
4 Results

4.1 Assessment of optimal culture condition for the RAW 264.7 macrophage cell line

4.1.1 Assessment of the bioactivity of transfected and un-transfected RAW 264.7 cells

One aim of experiments described in this chapter was to assess the effect of plant extracts on the production of NO and PGE$_2$. The transcription of genes encoding these inflammatory mediators is under the influence of NF-$\kappa$B. Therefore, the usefulness of RAW 264.7 cells stably transfected with the Luciferase gene under the control of the NF-$\kappa$B promoter was examined. The readout of this assay was the activity of luciferase, which catalyses a reaction with luciferin to produce a change in colour to blue. However, studies of the transfected cells showed that they produced less than half the amount of NO produced by un-transfected cells upon LPS stimulation. Previous studies have shown that activation/inactivation of NF-$\kappa$B related to changes in the concentration of NO (152). This may suggest that a positive feedback mechanism may exist whereby low concentrations of NO induce NF-$\kappa$B activation to promote further NO production. Once biologically optimum levels of NO levels have been reached, excess NO could then act to reduce NF-$\kappa$B activation to turn off further NO production. It, thus, appeared possible that the transfected RAW 264.7 cells were less efficient in responding to alter NO levels than un-transfected cells. Based on these results, it was decided to use un-transfected RAW 264.7 cells for stimulation and measurement of NO production and inhibition by plant extracts.
4.1.2 Assessment of protocol for the stimulation of RAW 264.7 cells.

Experiments to identify the optimal protocol for reproducible stimulation of RAW 264.7 cells were carried out. Stimulatory optimal effects for NO production by two alternative protocols were examined, stimulation with LPS alone and LPS in combination with a pro-inflammatory cocktail (CT) of cytokines (Method in section 3.2 of this chapter).

Contrary to reports in the literature, results of the comparison revealed that LPS was more efficient (p<0.001) and more reproducible than LPS together with the CT under the culture conditions established by the earlier experiments in inducing NO production by the RAW 264.7 cells (Figure 3.2). Based on these experiments, all subsequent studies on the anti-inflammatory properties of the extracts, fractions and identified compounds were tested using stimulation with LPS.
Figure 3.2: Comparison between the level of NO produced by RAW 264.7 cells stimulated with LPS or with LPS + CT relative to untreated cells. Statistical analysis for the difference in the level of NO production (in μM) was performed using the paired t-test on data from seven experiments (n=7). Data presented as mean ± SD. LPS produced significantly higher amounts of NO compared with LPS + CT (***P<0.001). CT=pro-inflammatory cocktail of cytokines (TNF-α and IL-1β).

4.2 Anti-inflammatory effects of extracts from Malaysian plants

The part of the studies to assess the potential anti-inflammatory effects of the plant extracts started with screening experiments for 12 types of indigenous Malaysian medicinal plants. Two different extracts were obtained for each plant as described in chapter 2. One extract ionmethod relied on shaking of the dried plants in 80% ethanol in water while the other relied on sonication of the dried plants in the same solvent. The extracts were assessed for their effects on inhibiting NO and PGE₂ production. In the preliminary study, RAW 264.7 cells
were stimulated with LPS+CT. The outcome of this experiment determines the most potent plant extract against NO production, and thus, selected as the focus of this thesis.

4.2.1 Inhibition of NO: Ethanolic extract of ZOR had comparable effects to L-NAME.

Extracts from the 12 native Malaysian plants (described in Chapter 2 and Table 1 in the Appendices), were tested for their ability to inhibit the NO production by RAW 264.7 cells stimulated with LPS and CT cocktail the cells were incubated with the extracts prior to stimulation. The potency of the extracts was determined based on their IC$_{50}$, the concentration required to inhibit 50% of NO production by the stimulated cells.

This data was compared to the IC$_{50}$ of L-NAME (L-N$\text{^3}$-Nitroarginine methyl ester). Among these extracts, the extract from the ginger sp., Halia Bara or Zingiber officinale Roscoe var. rubrum (ZOR) obtained by the shaking method (HB01), was the most potent inhibitor of NO production with an IC$_{50}$ of 10.2 ± 0.8 $\mu$g/mL (Table 3.3). HB01 had comparable NO inhibitory activity to L-NAME (7.3 ± 1.8 $\mu$g/mL). This is followed by extracts from Mangifera indica (MP) that were extracted using sonication method (IC$_{50}$= 17.0 ± 1.7 $\mu$g/mL) and shaking method (IC$_{50}$= 26.0 ± 6.9 $\mu$g/mL). MP extracted using sonication (SN) was slightly more active than the one extracted using shaking method (SK). This was also demonstrated for the effect of Artocarpus heterophyllus (DN) and Archidendron bubalinum (KD). However, it was the opposite for Morinda citrifolia (MG) and Artocarpus altilis (DS), which showed higher potency when
extracted using the shaking method. Therefore, based on the inhibition of NO production by the plant extracts listed in Table 2.2, no clear distinction could be drawn on the efficiency of either type of extraction procedure as more efficient than the other. Hence, the differential NO inhibitory effects of both extraction methods indicate that the potency of the extracts are more likely influenced by the active components than the extraction protocol.
4.2.2 Inhibition of PGE₂ production

Since the plant extracts showed strong inhibition on NO production, it was decided to test the effect of some of the more promising extracts on PGE₂ production. HB01, MP and DN which were extracted using the shaking method, were tested. The concentrations of HB01, MP and DN used were approximately 10, 20 and 60 µg/mL respectively, based on the IC₅₀ values obtained in the NO inhibition experiments as indicated in Table 2.2. PGE₂ production was measured using a PGE₂ ELISA. The results of PGE₂ production by stimulated RAW 264.7 cells are summarised in Figure 3.3. The data showed that HB01 was more potent by two-fold or more than the other two extracts in inhibiting PGE₂ production (Figure 3.3). This finding is consistent with the noted potency of HB01 in inhibiting NO production.
Figure 3.3: The inhibitory effects of HB01, MP and DN on PGE$_2$ production by RAW 264.7 cells. The RAW 264.7 cells were stimulated with the LPS+ CT cocktail. Before stimulation the cells were pre-cultured with 10 μg/mL HB01, 20 μg/mL MP or 60 μg/mL DN. Their inhibitory effects on PGE$_2$ production were determined. The concentrations were based on the IC$_{50}$ obtained for inhibition of NO production (Table 3.3). The inhibitory effects on PGE$_2$ production was measured relative to untreated but stimulated RAW 264.7 cells. Values are mean ± SD of % inhibition of PGE$_2$ production from two independent experiments conducted in triplicates.

4.2.3 The inhibitory properties of plant extracts: NO versus PGE$_2$ production

The extracts of ZOR (HB01), M. indica (MP) and A. heterophyllus (DN) showed good inhibitory effects on NO and PGE$_2$ production. Amongst these 3 extracts, however, HB01 had the most potent inhibitory effects on NO, and this effect was favourable in comparison with L-NAME.
Based on these results, it was decided to further explore the mechanisms of action, extent of anti-inflammatory effects of ZOR and identify compounds that could have therapeutic potential in treating psoriasis. Results in Table 3.3 and Figure 3.3 revealed that the HB01 extract inhibited 50% NO and PGE\(_2\) production (IC\(_{50}\)) at concentration of \(\sim 10 \mu g/mL\). The observation on the ability of HB01 to inhibit both NO and PGE\(_2\) suggested that this plant possesses dual inhibitory effects on both pro-inflammatory pathways. It was previously reported that iNOS-derived NO activates COX which subsequently induces PGE\(_2\) production (153). This implies that HB01 might have an effect on iNOS expression which could explain its inhibitory effects on PGE\(_2\) production. Studies to address the effect of the ZOR on iNOS expression were, therefore, carried out and preliminary results are presented later in this chapter.

### 4.2.4 Effect of HB01 on iNOS and TNFα gene transcription.

mRNA for iNOS and TNFα genes in stimulated RAW 264.7 cells with and without pre-treatment of the cells with HB01 and L-NAME at of concentrations 10 \(\mu g/mL\) and 7 \(\mu g/mL\), respectively, was assessed using conventional PCR. mRNA for the TNFα gene was detectable in both stimulated and unstimulated RAW 264.7 cells. However, mRNA levels for the gene were less abundant in stimulated cells treated with HB01 than cells that were not pre-treated with the extract (Figure 3.4). Nevertheless, since the PCR protocol does not provide information on the amount of mRNA, it was decided to carry out quantitative real-time PCR to verify the observation. The PCR results showed that mRNA levels for iNOS did not change in cells pre-treated with the extract. This latter observation is interesting since the early data showed that HB01 has a
suppressive effect on NO production at the same concentration used for the PCR (Table 3.3). Figure 3.4 depicts, that despite the observation that L-NAME is a potent inhibitor of NO, the PCR experiment did not show inhibition of \textit{iNOS} gene transcription by the compound. This finding may suggest that the effect of L-NAME may not be through down regulating iNOS gene transcription.

Interestingly, however, the results showed that HB01 reduced the level of mRNA for the TNF\(\alpha\) gene. Previous studies have shown that TNF\(\alpha\) is a principal mediator of inflammation in most inflammatory conditions. Therefore, the ability of HB01 to down-regulate \textit{TNF\(\alpha\)} mRNA levels is interesting but more evidence is needed to verify this observation.

![Figure 3.4: PCR analysis for \textit{iNOS} and \textit{TNF\(\alpha\)} mRNA levels in stimulated RAW 264.7 cells.](image)

Lane 1 shows PCR products for mRNA obtained from unstimulated RAW 264.7 cells (negative control). Lane 2 depicts PCR result of cDNA amplification from RAW 264.7 cells stimulated with the LPS + CT cocktail. Lane 3 shows PCR products for stimulated RAW 264.7 cells pre-treated with L-NAME at 7.0 \(\mu\text{g/mL}\). Lane 4 shows PCR products for stimulated RAW 264.7 cells pre-cultured with HB01 at 10.0 \(\mu\text{g/mL}\).
4.3 Comparison of solvents for the optimal preparation of extracts with maximal anti-inflammatory properties from ZOR

The experiments described above on NO and PGE₂ production by extracts showed that the HB01 extract from ZOR had the most potent inhibitory activity on both mediators. Due to these properties, it was decided to carry out detailed studies on ZOR to further assess the extent of its anti-inflammatory effects, mechanisms of action and identify therapeutic compounds inducing such effects. For this purpose, new extracts of ZOR were prepared with 3 alternative solvents: chloroform (HB02), ethanol (HB03) and water (HB04) (Chapter 2).

ZOR rhizomes were obtained and processed as described in Method 3.4 (Chapter 2) and labelled as indicated in Table 3.4. Briefly, active components from 499g of ZOR powder were extracted with 3 L chloroform in 30 minutes using sonication and then maceration for 5 days at room temperature (RT). The extract was collected, filtered and evaporated using a rotary evaporator at 200 Pa at 45°C and labelled as HB02. The powder residue was then dried and re-extracted with ethanol (HB03) and then with water (HB04) using the same approach. The aqueous extract was dried using a freeze-drier. The same extraction approaches using chloroform and ethanol were carried out on common ginger, Z.officinale and labelled as HC01 and HC02, respectively.
Table 3.3: Preparation of ZOR extracts using different solvents.

<table>
<thead>
<tr>
<th>Code</th>
<th>Extract Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB01</td>
<td>ZOR extracted in ethanol using the shaking method*</td>
</tr>
<tr>
<td>HB02</td>
<td>ZOR extracted in chloroform using the soaking (maceration) method</td>
</tr>
<tr>
<td>HB03</td>
<td>ZOR extracted in ethanol using the soaking (maceration) method</td>
</tr>
<tr>
<td>HB04</td>
<td>ZOR extracted in water using the soaking (maceration) method</td>
</tr>
<tr>
<td>HC01</td>
<td>*officinale (common) extracted in chloroform using the soaking (maceration) method</td>
</tr>
<tr>
<td>HC02</td>
<td>*officinale (common) extracted in ethanol using the soaking (maceration) method</td>
</tr>
</tbody>
</table>

*Prepared in the preliminary study
4.3.1 Extraction of ZOR with chloroform (HB02) produces an extract with potent inhibitory effects on NO production.

The inhibitory effects of ZOR and the common ginger extracts (Table 3.4) on NO and PGE$_2$ production by stimulated RAW 264.7 cells were compared. The extracts were dissolved in DMSO. DMSO was chosen due to its ability to dissolve lipophilic compounds and its use in most *in-vitro* assays (154). The concentration of DMSO most widely-used is 0.1%, which was also used in these studies. The potential effect of using DMSO on the viability of the stimulated RAW 264.7 cells was determined. For this purpose, a range of DMSO concentrations were tested for the effect on cultured RAW 264.7 cells. Viability of the cells was determined using the MTT viability assay. The results showed that the concentration of DMSO that could be used without affecting cell viability should be less than 1.7 ± 0.01% so that >50% macrophages remain viable. The best DMSO concentration which enables 90% macrophage viability was found to be in the range of (0.4 ± 0.02)%.

The findings from these experiments are consistent with previous observations on DMSO concentration which suggested that its concentration for culture cells should not exceed 2% (154). These investigators used human cryo-preserved hepatocytes and rat primary cultured hepatocytes to assess the expression of 1137 sets of gene probes for drug-metabolising enzymes at a range of DMSO from 0.1-2%. The investigators have observed that DMSO concentration of up to 0.75% can be tolerated with the expression of only a minority of the genes studied affected (154). Therefore, the maximum percentage of DMSO used in this study was 0.5%.
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ZOR extracts with the three different solvents and common ginger extracts were dissolved in DMSO and at a final maximum concentration of 0.2% when tested for inhibition of NO production by LPS-stimulated RAW 264.7. The results showed that HB02, HB03, HC01 and HC01 were all more potent than L-NAME in inhibiting NO production (Figure 3.5). These findings also showed that the chloroform and ethanol extracts of both gingers were effective in inhibiting NO production and more potent than L-NAME. However, HB01, the ethanol extract of ZOR prepared using shaking method was similar to L-NAME but less potent than HB02 and HB03. The least effective extracts were obtained in aqueous extraction, HB04, which was incapable of 50% inhibition of NO production even at the concentration of 1 mg/mL. HB04 was, therefore, not studied further. It was also noted that L-NAME and HB01 showed less potency (higher IC$_{50}$ values) on NO production by RAW 264.7 stimulated with LPS compared with the previous studies on LPS+CT-stimulated RAW 264.7 cells (Table 3.3). Although different stimulation protocol resulted in different IC$_{50}$ values, the effect of HB01 and L-NAME were comparable.

The efficacy of ZOR extracts, HB01, HB02 and HB03 were variable. HB02 was significantly more effective in inhibiting NO production when compared with L-NAME (p<0.001). HB02 was also significantly more effective than HB01 and HB03. This suggests that chloroform extract of ZOR yielded more potent compound/s than of ethanol plus shaking. Chemically, chloroform extracts non-polar compounds from ZOR since it is a non-polar solvent, whereas, ethanol is a polar solvent and extracts mainly polar compounds. As discussed in chapter 2, recent studies indicate that a majority of anti-inflammatory properties of ginger are attributed to the non-polar components, such as the
phenylpropanoids. This might suggest that the potent NO inhibitory effect of the chloroform extract, HB02, could be attributed to the presence of more non-polar compound/s from ZOR.

In addition to the effect of solvent on the efficiency of extracting therapeutic compounds from ZOR, the results also indicate that the extraction protocol itself has an influence. HB02 and HB03 were extracted using a 5 day-maceration protocol while HB01 was extracted using the shaking at 40ºC for 24 hours. Comparison between the ethanol extracts, HB03 and HB01, which were extracted using different protocols showed a significant difference (p<0.05), wherein HB03 (IC₅₀ = 21.1 ± 2.1 μg/mL) was about twice as active as HB01 (IC₅₀ = 35.0 ± 5.0 μg/mL) in inhibiting NO production. This data shows that gradual extraction at room temperature is more efficient than fast extraction at 40ºC in extracting anti-inflammatory compounds. Theoretically, extraction of therapeutic compounds at higher temperature might lead to the destruction of some active ingredients (111). Storage, can also lead to gradual degradation of active compound/s (114). This may explain the reduced activity of HB01, which was prepared prior to the other extracts and stored until tested.

Based on these observations, HB02 extract was selected for detailed studies to assess its anti-inflammatory effects. In addition, the anti-inflammatory effects of HB02 were compared with those obtained for common ginger to determine if variation between close species existed. In the results shown in Figure 3.5, ZOR had similar inhibitory activity on NO production to common ginger when using similar extraction protocols. Thus, no significant differences were observed between the two ginger species (HB and HC) based on the IC₅₀ value.
The chloroform extracts of both species (HB02 and HC01) showed similar potency in inhibiting NO production. This was also seen for the ethanol extracts of both species. This finding may imply that both ginger species share the same or similar anti-inflammatory compound/s acting on NO production by macrophages. To test this proposition the extracts were assessed for their inhibitory effects on PGE₂ production.

Figure 3.5: IC₅₀ for inhibition of NO production by ZOR and common ginger extracts obtained using different solvents. The effect of the extracts in inhibiting NO production was tested at a range of concentrations (0.5-100.0 µg/mL). NO inhibitory effects of the extracts were assessed relative to untreated LPS-stimulated RAW 264.7 and the IC₅₀ (in µg/mL) was obtained. The histograms represent the IC₅₀ (µg/mL) for the different extracts. The inhibitory effects of ZOR extracts were compared with L-NAME using One-way ANOVA Dunnet’s test. Probability values (p) are indicated as: * for p <0.05 and ** for p<0.01. The data is mean ± SD of at least three independent experiments (n=3).
4.3.2 HB02 inhibition of PGE$_2$ production.

PGE$_2$ production was measured using a kit based on Homogenous Time-Resolve Fluorescence (HTRF) technology (CISBIO). The effects of the extracts (for both types of gingers) and known COX inhibitors, indomethacin (INDO) and dexamethasone (DEX) at 10 $\mu$g/mL on PGE$_2$ production by stimulated macrophages were determined. The concentration was chosen based on the IC$_{50}$ value for the most potent extract (HC01) in inhibiting NO production as shown in Figure 3.5. The results revealed that HB02 had the most consistent and potent inhibitory effects on PGE$_2$ production (Figure 3.6). The inhibitory effects of HB02 on PGE$_2$ production were higher than those of HB03, HC01 and HC02 although differences were not statistically significant. The extracts showed comparable effects with indomethacin and dexamethasone when tested at the same concentration (10 $\mu$g/mL) in inhibiting PGE$_2$ production,
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Figure 3.6: The inhibitory effects of extracts obtained from ZOR (HB-) and common ginger (HC-) on PGE\(_2\) production. Extracts were assessed for their ability to inhibit PGE\(_2\) production by RAW 264.7 stimulated with LPS. The columns represent mean ± SD of % inhibition of PGE\(_2\) production of at least three independent experiments. Comparison of the inhibitory effect of all the extracts with respect to the positive controls, indomethacin (INDO) and dexamethasone (DEX), was performed using One-way ANOVA (Tukey’s test). No significant difference was observed.

Based on this data on NO and PGE\(_2\) production (Figure 3.6), it was decided to further study the composition of HB02 and identify potential therapeutic compound(s). In the first stage, HB02 was to be further fractionated and the inhibitory properties of the fractions examined in detail.
4.3.3 Vacuum liquid chromatography (VLC) fractionation of HB02 and assessment of anti-inflammatory properties of individual fractions.

As described in chapter 2, HB02 was fractionated using VLC. The individual fractions were then screened for their inhibitory effects on NO production by LPS-stimulated RAW 264.7. Twenty one fractions were obtained and each fraction was tested at 20 μg/mL, a concentration of approximately the IC\textsubscript{50} of HB02 in inhibiting NO (Figure 3.5). As a control, L-NAME was used at 50 μg/mL, an approximate concentration of its IC\textsubscript{50} value (Figure 3.5).

Figure 3.7 depicts the data obtained for the inhibitory effects of the 21 fractions on NO production. Fractions 5, 6, 7 and 10 (column in black) at tested concentration of 20 μg/mL, inhibited NO production by LPS-stimulated RAW 264.7 cells by >80%. The inhibitory effects were clearly higher than that of HB02 and L-NAME, but the differences were not statistically significant. F5 and F6 exhibited high inhibitory activities. This observation was consistent as indicated by the relatively low SEM values obtained for the repeat experiments. F5, 6, 7 and 10 were selected for further studies and molecular characterisation.
Figure 3.7: The inhibitory effects of HB02 and its fractions on NO production. HB02 was fractionated by VLC as described in chapter 2. The concentration of the fractions were adjusted to 20 μg/mL and fractions were tested for their inhibitory effects on NO production by RAW 264.7 cells stimulated with 0.1 μg/mL LPS. L-NAME at 50 μg/mL was used as a positive control and for consistency and reproducibility of the assays. The columns represent mean ± SD from two sets of independent experiments (n=2).

4.3.4 Selected HB02 fractions inhibit NO production without reducing macrophage cell viability

The MTT assay was used to assess the effect of the fractions on cell viability (Figure 3.8). Based on the viability assay results shown in Figure 3.8, F7 appeared to be the most toxic to the RAW 264.7 cells at the higher concentration range (50 and 100 μg/mL) with the viability reduced to 53.0 ± 2.8% at 50 μg/mL whilst culture with HB02 and its other fractions
resulted in more than 80% viability. The effect of DMSO in the culture medium on RAW 264.7 macrophage cell viability was also studied. The results showed 50% (IC$_{50}$) and 90% (IC$_{90}$) viability at percentage of 1.7 ± 0.01% and 0.4 ± 0.02%, respectively. The maximum concentration of DMSO used was 1% at 100 μg/mL of HB02 extract and fractions. Thus, these results indicate that the % of DMSO which was used to prepare the stocks of HB02 and the fractions did not affect viability of RAW 264.7 cells. Based on these results, the concentration of HB02 and fractions used in all subsequent studies were ≤50 μg/mL.

Further studies to determine the IC$_{50}$ of the HB02 and its fractions on the inhibition of NO production showed that these were significantly lower (p<0.05) than the IC$_{50}$ of L-NAME (Figure 3.9). The fractions had lower IC$_{50}$ than the crude HB02 extract. F6 was particularly effective in inhibiting NO production (IC$_{50}$= 8.5±1.1 μg/mL), two-fold lower than HB02 (16.0 ± 1.9 μg/mL). Importantly, the IC$_{50}$ for the fractions were significantly lower than their potential toxicity levels (>50 μg/mL) (Figure 3.8).
Figure 3.8: Viability of RAW 264.7 cells in response to treatment with HB02 extracts and fractions from VLC fractionation. RAW 264.7 cells were cultured and treated with the ZOR HB02 extract or with fractions at a range of concentration from 0.5-100 μg/mL and incubated for 24 hours. The maximum concentration of DMSO used was 1% at 100μg/mL of HB02 extract and fractions. The viability of the RAW 264.7 cells was determined using the MTT assay. The results represent the mean ± SD of three independent experiments (n=3). The effect of DMSO in culture medium on RAW 264.7 macrophage cell viability resulted in 50% (IC$_{50}$) and 90% (IC$_{90}$) viability were: 1.7 ± 0.01% and 0.4 ± 0.02%, respectively.
Figure 3.9: IC$_{50}$ of HB02 and its fractions obtained by VLC fractionation for inhibition of NO production. HB02 and fractions at a range of concentration (0.5-100 µg/mL) were tested for the inhibitory effects on NO production by LPS-stimulated RAW 264.7 cells. The maximum DMSO % used when tested was up to 0.2%. The data is the mean ± SD of six independent experiments (n=6). One-way ANOVA (Dunnet’s test) analysis indicates that HB02 and its fraction demonstrate very significant difference (***p<0.001) compared with L-NAME.
4.3.5 F6 and its isolated compounds show comparable inhibitory effects on NO production.

The next sets of experiments examined the effect of compounds isolated from F6, 6-shogaol (6S) and 1-dehydro-6-gingerdione (GD) on NO production by the stimulated macrophages. The inhibitory effects of 6S and GD on NO production (IC$_{50}$) were compared with F6 (Figure 3.10). Both compounds showed comparable effects to F6. Although not significant (p>0.05), F6 is better NO inhibitor than its compounds as suggested by the lower IC$_{50}$ value (8.5 ± 1.1 µg/mL) while 6S showed better inhibitory effect than GD.

**Figure 3.10: IC$_{50}$ of F6 and its isolated compounds 6S and GD, in inhibition of NO production.** Two major compounds were isolated from F6 by Prep-TLC. These were identified as 6S and GD. The effect of 6S and GD in inhibiting NO production was tested at a range of concentration and the IC$_{50}$ (in µg/mL) was determined. The data is the mean ± SD of at least five independent experiments (n=5).
4.4 HB02 fractions and 6S and GD show potent inhibitory effect on PGE\(_2\).

Since F6 and its compounds; 6S and GD have variable but significant effects on NO production at 8-12 µg/mL (Figure 3.10), the effect of HB02, the fractions and the compounds from F6 on PGE\(_2\) production was assessed. The fractions were tested (all at 10 µg/mL) for their ability to inhibit PGE\(_2\) production by RAW 264.7 cells before their stimulation with LPS. HB02 and its fractions had inhibitory effects on PGE\(_2\) production comparable to indomethacin and dexamethasone (Figure 3.11a). They reduced PGE\(_2\) production by >80% compared with stimulated but untreated cells. Amongst the fractions, F6 had the most potent inhibitory effect on PGE\(_2\) production.

Assessment of 6S and GD showed that 6S had similar potent inhibitory effects to F6 on PGE\(_2\) production (Figure 3.11b). GD had lower inhibitory effects. The potency of F6 and both compounds was comparable to indomethacin and dexamethasone (p>0.05). This observation complements the effect of F6 and its compounds on NO production (Figure 3.10).
Figure 3.11: The inhibitory effects of HB02, selected fractions and compounds on PGE$_2$ production. RAW 264.7 cells were pre-treated with a) HB02 and fractions b) 6S and GD. Dexamethasone (DEX) and Indomethacin (INDO) at 10 µg/mL, were the positive controls. The inhibitory effect on PGE$_2$ production were measured relative to untreated but LPS-stimulated cells. The data represents four set of independent experiments and was analysed using One-way ANOVA (Tukey’s Test). The effect of the test samples was compared to INDO and DEX. The differences were not significant.
4.5 The effect of the ZOR extract and fractions on the expression \textit{iNOS} gene transcripts

The experiments described so far assessed the potential anti-inflammatory effects of ZOR extracts, fractions and compounds for their suppressive effects on NO and PGE\textsubscript{2} production. To determine the basis for the inhibition of NO production and extent of effects, their effects on the expression of iNOS at mRNA level before and after stimulation of RAW 264.7 cells with LPS, were examined. The rationale for these experiments was to determine the potential mechanism of action and whether inhibition was at the transcriptional level of the gene encoding iNOS or the release of NO from the stimulated RAW 264.7 cells.

4.5.1 Conventional RT-PCR does not show altered levels of iNOS gene expression by HB02 or its fractions.

The production of NO is regulated by iNOS and production is increased in activated macrophages during inflammation. Figure 3.9 shows that HB02 and the tested fractions effectively suppressed the production of NO by at least 50% at concentrations <20 μg/mL when added to the cultured RAW 264.7 cells before stimulation with LPS. To assess whether this observation was due to reduced iNOS expression, a conventional RT-PCR was carried out. \textit{iNOS} gene expression by LPS-stimulated RAW 264.7 untreated or treated with 20 μg/mL of HB02 or tested fractions F5, F6, F7 and F10 were compared with cells treated with L-NAME at 54 μg/mL (200 μM) The concentrations are based on IC\textsubscript{50} value.
reported in figure 3.9. The results showed no significant difference in the level of iNOS mRNA between RAW 264.7 cells treated or untreated with HB02 or the fractions before stimulation with LPS (Figure 3.12). The results also showed that L-NAME did not affect iNOS mRNA levels at 54 μg/mL. These results suggest that the suppressive effects of HB02 and its fraction on NO production may not occur at the level of iNOS gene transcription. However, it was also possible that the conventional RT-PCR used was not capable of detecting small changes in iNOS mRNA levels.

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Figure 3.12: The effect of HB02 and fractions (F5, F6, F7 and F7) on iNOS mRNA by conventional PCR. The effect of HB02 and fractions at 20 μg/mL (lane 3-7) on the level of iNOS mRNA expression by stimulated RAW264.7 cells was assessed in comparison to the effect of L-NAME(LN) at 54 μg/mL (lane 8). The figure depicts the results of analysing RT-PCR products on a 1% agarose gel in the presence of ethidium bromide. RT-PCR for mRNA for the housekeeping gene GAPDH was used as an internal control.
4.5.2 Quantitative RT-PCR reveals that HB02 and its fractions reduce iNOS mRNA level before and after LPS stimulation

To further explore the question of whether HB02 and its fractions influence iNOS mRNA transcription, quantitative RT-PCR (qRT-PCR) was used. The reasons for exploring this issue were to determine mechanisms that underpin the inhibitory effects of HB02 (fractions and compounds) on NO production. It was hoped that the results could determine whether ZOR could be effective after inflammation starts and thus, be used as therapy. The experiments were based on the knowledge that the ability to inhibit gene expression before macrophage activation indicates that HB02 acts at transcription levels, i.e. more effective before inflammation starts while inhibition after macrophage activation implies that HB02 could inhibit transcription and also iNOS catalytic activity (146).

Comparisons between the level of iNOS mRNA in the presence of HB02/its fractions before and after LPS stimulation of RAW 264.7 cells showed reductions in both settings (Figure 3.13a) compared with the control (untreated but LPS-stimulated cells). F6 was particularly effective in down regulating the level of iNOS mRNA when added before or after RAW 264.7 cell stimulation with LPS wherein it was a better iNOS inhibitor that L-NAME (P<0.001). Interestingly, F7 was significantly more effective than L-NAME (p<0.01) and marginally more effective than F6 and F10 when added to the cultured cells after stimulation. HB02 and F5 were as effective as L-NAME when added before and after LPS stimulation. Thus, these results indicate that HB02 is likely to contain compounds which inhibit both iNOS transcription or message stability.
and enzymatic activity which are comparable to L-NAME. These findings are consistent with the ability of HB02/fractions to inhibit NO production (Figure 3.9, Figure 3.13b).

To verify these findings, the level of inhibition of NO production by RAW 264.7 cells treated with HB02/its fractions at 20 μg/mL before and after stimulation were determined. Figure 3.13(b) shows the inhibitory effects of HB02/its fractions on NO production by RAW 264.7 cells before and after LPS stimulation. The results show that HB02/its fractions had stronger inhibitory effects when added to the cells before stimulation than after (Figure 3.13b). Even though HB02 and the fractions strongly reduced iNOS mRNA level when added after LPS stimulation, this was not reflected on inhibiting NO production. Interestingly, the results also show that F7 possess more suppressive effects on NO production than other fractions. L-NAME showed consistency in its effect on iNOS mRNA level and NO production before and after LPS stimulation. The inhibitory effects of HB02, F5, F6 and F10 were comparable to that of L-NAME before LPS stimulation but not after LPS stimulation, particularly for HB02 (p<0.05). These results, therefore, indicate that HB02 and the fractions act favourably in regulating iNOS before and after LPS stimulation, but reduce NO production more effectively when the cells are treated before LPS stimulation.
Figure 3.13: The effect of HB02 and its fractions on iNOS mRNA level and NO production. The effect of treating RAW 264.7 cells with HB02 and its fractions at 20 μg/mL either before or after stimulation with LPS on the level of (a) iNOS mRNA was determined by qRT-PCR (i) Level of iNOS mRNA expression when the RAW 264.7 cells were pre-treated with HB02/fractions 30 minutes before LPS stimulation (ii) Level of iNOS mRNA expression in the presence of HB02/fractions added 12 hours after the cells were stimulated with LPS. Comparison is relative to untreated LPS-stimulated RAW 264.7 cells (control). (b) NO production was determined using the Griess. The results are presented as the mean ± SD of at least 3 independent experiments. Statistical analysis were carried out using Two-way ANOVA Bonferroni posttest to compare the effect of HB02 and its fractions with the effect of L-NAME(LN) at 54 μg/mL, denotes * p<0.05.
4.5.3 HB02 and its fractions down-regulate iNOS protein levels more efficiently when the cells are treated before LPS stimulation.

As shown above, F6 was the most efficient inhibitor of iNOS mRNA level whether the cells were treated with the fraction before or after stimulation (Figure 3.13a). HB02, F5, F6 and F7 were similarly effective as L-NAME in inhibiting NO before LPS stimulation but less effective than L-NAME when used after LPS stimulation (Figure 3.13b).

In light of these observations, the effect of HB02 and its fractions on the level of iNOS at protein level was evaluated. The level of iNOS protein was assessed using Western blotting and results semi-quantified by assessing the intensity of the resulting bands relative to an internal standard (β-actin) (Figure 3.14(a)(b)). Surprisingly, L-NAME at 54 µg/mL did not affect iNOS protein level despite its effect on iNOS mRNA. However, the effect of HB02 and its fractions on iNOS protein is consistent with the effect on NO production (Figure 3.13b). There was a significant down-regulation of iNOS protein when the cells were pre-treated with HB02/its fractions before stimulating the RAW 264.7 cells with LPS. When the cells were pre-treated with F5, F6 or F7 the level of iNOS protein level was up to seven-folds lower than when the cells were treated with L-NAME (Figure 3.14b(i)).
In contrast to the strong effects of HB02 and its fractions when added to the cells before stimulation, none had a significant down-regulatory effect on iNOS protein levels after LPS stimulation, except F7 (Figure 3.14(b)(ii)). These findings suggest that whilst HB02 and its fractions had a profound effect on suppressing the level of *iNOS* mRNA they did not have such a strong effect on secretion. Surprisingly, however, F7 reduced *iNOS* protein secretion when added before and after LPS stimulation. The level of inhibition of *iNOS* protein secretion with F7 which was much more pronounced than the effect of L-NAME (Figure 3.14(a)(b)). Figure 3.14b shows that the inhibitory effect of F7 on *iNOS* protein secretion is about ten-folds higher than L-NAME. This issue, however, needs to be further tested.

The results of these experiments show that the suppressive effects of HB02 and its fractions on *iNOS* mRNA level and protein secretion correspond with their suppression of NO production when the cells are pre-treated before stimulation. In contrast, it appears that HB02 and its fraction reduce mRNA levels but have little effects on secretion or the half life of the pre-synthesised *iNOS* but inhibit their enzymatic activity when the cells are treated after stimulation with LPS. These experiments were repeated but using higher concentrations of HB02 and the fractions (50 µg/mL) to assess whether they could exert toxic effects on the cells (Figure 3.15(a)(b)). The results showed that whilst F5, F6 and F7 significantly inhibited *iNOS* protein secretion at 50 µg/mL when the cells were treated before and after LPS stimulation, that only F5 and F7 had toxicity at this concentration. F6 was not toxic as could be seen from the intensity of the β-actin bands (Figure 3.15a).
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(b)

Figure 3.14: Western blotting analysis of iNOS protein levels in RAW 264.7 cells treated with HB02 and its fractions. RAW 264.7 cells were treated with 20 µg/mL of HB02 or its fractions (lane 3-7) either (i) before or (ii) after stimulation with LPS. L-NAME (LN) (lane 8) was used at 54 µg/mL or 200 µM. Cells were removed from the wells, washed and lysed with lysis buffer and loaded onto the pre-cast NuPAGE gel and separated as described in the Methods section. iNOS protein levels were assessed in (a) and in (b) iNOS protein levels semi-quantified relative to β-actin using ImageJ. The film was scanned and the intensity of the protein band of the treated cells was analysed and compared relatively to the negative control (NC)(untreated cell)(lane1) and control (stimulated with LPS only)(lane 2).
(a) Before stimulation

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(b) Western Blot analysis of iNOS protein levels in RAW 264.7 cells treated with HB02 or fractions at 50 µg/mL. The experiments were carried out as in the legend to Figure 3.14. The effect of 50 µg/mL of HB02 or its fractions (lane 3-7) and L-NAME (LN) (lane 8) on iNOS protein secretion revealed in (a) and in b) iNOS protein levels were semi-quantified relative to β-actin using ImageJ. The film was scanned and the intensity of the protein band of the treated cells analysed relative to that of negative control (NC) (lane 1) and control (lane 2).
4.6 The effect of F6 and compounds 6S and GD on iNOS mRNA and NO production.

Results of the experiments described above showed that F6 had significant inhibitory effects on iNOS mRNA level and NO production. Furthermore, F6 effectively reduced iNOS protein secretion. Despite the strong effects, F6 even at concentrations of 50 µg/mL did not significantly affect cell viability (Figure 3.15(a) (b)).

Therefore, the chemical composition of F6 is determined in order to gain insights into the potential anti-inflammatory compounds in ZOR. As described in Chapter 2, detailed experiments resulted in the identification and purification of two major compounds from F6, 6S and GD. After isolation and characterisation, the cytotoxic potential of both compounds on RAW 264.7 cells at concentrations of 0.05-20 µg/mL was determined using MTT assay. These studies revealed that at these concentrations the macrophage cells line remained >90% viable for a 24 hours incubation.

A series of experiments were carried out to determine the anti-inflammatory effects of 6S and GD. The two compounds were tested for their inhibitory effects on NO production when added before LPS stimulation. These experiments showed that the IC$_{50}$ for 6S and GD were 9.6 ± 2.3 and 10.4 ± 2.3 µg/mL, respectively (Figure 3.10). Based on the IC$_{50}$ values (approximately 50 µM) obtained, the effect of both compounds at 50 µM on iNOS mRNA expression was determined and compared to L-NAME at 200 µM (54 µg/mL).
These experiments showed that 6S had a significant inhibitory effect on the level of \textit{iNOS} mRNA whether it was used to treat cells before or after LPS stimulation (Figure 3.16a). The inhibitory effects were comparable with the effect of L-NAME at a higher concentration. The experiments showed that 6S had more potent effects when cultured with the cells before stimulation (Figure 3.16a (i)). GD, unexpectedly, showed no inhibitory effects on \textit{iNOS} mRNA levels when cultured with the cells before LPS stimulation. Indeed, the results showed an increase of \textit{iNOS} mRNA level. In contrast, the level of \textit{iNOS} mRNA was reduced when the cells were treated with GD after LPS stimulation (Figure 3.16a(ii)). These results indicate that the inhibitory effect of GD on NO production maybe through affecting \textit{iNOS} activity rather than its gene transcription.

The effect of the both compounds on NO production at 50 $\mu$M was also studied. Both compounds showed potent suppressive effects on NO production when cultured with the cells prior to stimulation with LPS (Figure 3.16b (i)). The inhibitory effects of both compounds were about two-fold higher than L-NAME in the pre-treatment protocol (p<0.05). Somewhat surprisingly, both 6S and GD did not have potent inhibitory effects on NO production when they were used to treat the cells after stimulation with LPS (Figure 3.16b (ii)). These findings suggest that 6S is more potent in inhibiting NO production by acting mainly at \textit{iNOS} gene transcription. GD, in contrast, appeared to act on the enzymatic activity of \textit{iNOS} but was less effective than expected, possibly, due to the relatively high transcription rate of the gene after stimulation of the cells with LPS (Figure 3.17b (ii)).
Figure 3.16: The effect of 6S and GD on iNOS mRNA levels. RAW 264.7 cells were cultured as described in the Methods section and either treated with 6S or GD before or after stimulation with LPS as indicated. Both 6S and GD were used at 50 μM. Both were compared to L-NAME at 200 μM. Percent reduction in the level of iNOS mRNA were measured relative to the level in stimulated but otherwise untreated RAW 264.7 cells (control). The columns represent data from 3-4 sets of independent experiments. Statistical analysis were carried out using Two-way ANOVA Bonferroni posttest to compare the effect of HB02 and its fractions to L-NAME (LN) * denotes p<0.05.
4.6.1 6S influences iNOS protein secretion and activity before and after LPS stimulation but GD may suppress its enzymatic activity.

To verify the observations of the effects of 6S and GD on iNOS expression and activity the level of protein production was analysed by Western blotting. LPS-stimulated RAW 264.7 cells were treated with the two compounds at 50 μM (Figure 3.17). The results showed that 6S reduced the production of iNOS protein before and also after LPS stimulation of the cells. This observation is consistent with the noted effects of 6S on iNOS mRNA level (Figure 3.17a). In contrast, the results showed that GD marginally decrease the level of iNOS protein when the cells were treated with the compound before and after LPS stimulation (Figure 3.17b (i)), which is similar to the effect of L-NAME (200 μM).

These results indicate that GD does not have a significant effect on iNOS mRNA and protein levels but is likely to have an inhibitory effect on its enzymatic activity. In contrast, 6S may have modulating effects on iNOS mRNA and protein levels. However, further experiments are required to confirm these findings.
(a) Table showing the effects of 6S and GD on iNOS protein levels before and after LPS stimulation.

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(b) Bar graph showing the iNOS protein secretion (relative to β-actin) for each sample at 50 µM.

Figure 3.17: Effects 6S and GD on iNOS protein level. 6S or GD (lane 3/4) was added to RAW 264.7 cells either before or after stimulation with LPS. Cells treated with L-NAME (at 200 µM) were used as control. Proteins separated by electrophoresis were electroblotted onto nitrocellulose membranes and probed with conjugated mAb to iNOS and protein levels revealed as described in the Methods section. (a) is a photograph of the protein contents in the cell lysate from each experiment (b) depicts semi-quantitative levels of proteins in each sample expressed as band intensity relative to the bands representing β-actin in each cell sample (i) before and (ii) after stimulation with LPS. Protein band of the treated cells was analysed and compared relatively to the negative control (NC)(untreated cell)(lane1) and control (stimulated with LPS only)(lane 2).
5 Discussion

5.1 F6 reduces the level of iNOS mRNA and iNOS protein activity.

The experiments described in this chapter show that ZOR modulates NO and PGE₂ production by activated macrophages. The experiments were carried out with a number of objectives in mind. First, to provide experimental evidence that ZOR and common ginger ameliorate important pro-inflammatory events mediated by macrophages. Second, to provide insights into potential mechanisms through which both plants achieve their anti-inflammatory effects. Third, isolate and characterise the anti-inflammatory compounds in ZOR.

The results showed that both ZOR and common ginger possessed potent anti-inflammatory effects comparable with currently-used synthetic pharmaceutical compounds (L-NAME, Indomethacin, and dexamethasone). The results also provided data on potential methods of extraction of the anti-inflammatory compounds from both plants. The results showed that both plant extracts efficiently suppressed the production of NO and PGE₂ by activated macrophages. Further, ZOR inhibited iNOS expression and its enzyme activity. Subsequent analyses of ZOR identified compounds with distinct mechanisms of action on iNOS expression and/or activity. Further, studies assessed the potential beneficial effects of using the extracts for prophylactic and therapeutic effects. Fractionation of ZOR identified a number of fractions with therapeutic effects amongst which F6 was shown to be the most potent. F6 was, thus, shown to suppress NO and PGE₂ production with IC₅₀ values lower than HB02.
Further, F6 was shown to down regulate \textit{iNOS} at mRNA level as well as iNOS catalytic activity.

Detailed assessment of all 21 fractions obtained for HB02 showed that in addition to F6, F7 also displayed potent effects on reducing \textit{iNOS} mRNA and protein secretion. However, in contrast to F6, F7 was more effective in down regulating \textit{iNOS} mRNA levels when the macrophage cell line was treated after LPS stimulation. Paradoxically, perhaps, F7 was extremely more effective at reducing \textit{iNOS} protein levels when the cells were treated before and after LPS stimulation compared with F6. This finding was in agreement with its effect on inhibition of NO production. The somewhat similar effects noted for F6 and F7 could be attributed to both fractions sharing similar, though not identical components. One caveat for suggesting similarities between F6 and F7 (and possibly also F5) is that F7 (and F5) have also reduced the level of the control housekeeping protein, \textit{β}-actin which may imply that F7 and F5 are toxic to the cells. In contrast, F6 was not noted to be toxic, which supports the notion that although F6 and F7 could have similar mechanisms of actions, both also have differences in terms of composition or level of active compounds,

Further studies of F6 showed that it was efficient in down-regulating iNOS levels both before and after the cells were stimulated with LPS. Further, it was notable that F6 had lower IC$_{50}$ than L-NAME, the specific inhibitor of NO used in the studies as a control. L-NAME is a L-arginine analogue and is a non-selective iNOS inhibitor (155). It acts by competing with L-arginine for binding to iNOS. Taken together, these data imply that F6 inhibits NO production by acting as an iNOS inhibitor. Although further studies will be required to directly assess the
specificity of action of HB02, fractions and compounds, the results suggest that they act by influencing iNOS both at transcriptional and post-transcriptional levels.

5.2 6S and GD have distinct effects on iNOS but both suppress NO production.

To gain insights into mechanisms of the anti-inflammatory effects of ZOR, F6 was further fractionated and key components were identified. Two major compounds, 6-Shogaol (6S) and 1-dehydro-6-gingerdione (GD) were identified and their structures were determined (detailed in Chapter 2). Both compounds are known and have been identified in common ginger. However, the mechanisms of action on inflammatory mechanisms associated with psoriasis have not been explored. Moreover, to-date, there are insufficient studies on compounds isolated from the oleoresin of ZOR.

To address these issues, the effect of 6S and GD on NO and PGE₂ production and on iNOS protein and mRNA levels before and after macrophage stimulation with LPS were studied. 6S was shown to have a major inhibitory effects on NO production, causing >60% reduction in NO production at 50 μM when the RAW 264.7 cells were treated with the compound before stimulation with LPS (Figure 3.36b). Treatment of the cells with GD also resulted in >60% inhibition of NO production. However, the degree of inhibition of NO production was significantly less when the compound was added after stimulating the cells. Interestingly, however, there was a dichotomy in the effect of both compounds on iNOS mRNA levels (Figure 3.16a).
Thus, whilst 6S significantly reduced the level of *iNOS* mRNA level when the cells were pre-treated with the compound GD had no inhibitory effects and, instead, increased *iNOS* mRNA level (Figure 3.16a (i)). When the compounds were added to the RAW 264.7 cells after stimulation, they both reduced the mRNA level of iNOS (Figure 3.16a (ii)). Interestingly, there was no correlation between the level of message and the effect on NO production which suggested that all of the effect noted for GD on NO production is likely to be due directly to affecting the enzymatic activity of the iNOS protein. 6S, in contrast, is likely to have reduced NO expression by a combination of its effect on reducing iNOS production and an inhibition of its enzymatic activity. However, these questions need to be further studied to provide direct experimental evidence regarding the exact mechanism of action.

5.3 ZOR’s active compounds display dual inhibitory effects on NO and PGE₂ production.

As cited earlier, previous studies have suggested that links between the iNOS and COX-2 pathways exist. iNOS and its products have been shown to influence the COX pathway, particularly, COX-2 activation and PG production (140-143, 145). Among the PGs, PGE₂ is the most studied for its key role in inflammation. With this in mind, it was decided to study the effect of HB02 fractions and the two active compounds 6S and GD from F6 on PGE₂ production. The effect on PGE₂ production by RAW 264.7 cells was studied before LPS stimulation.
The experiments showed that the fractions, 6S and GD had potent inhibitory effects on PGE$_2$ production which were comparable to indomethacin and dexamethasone (both at 10 μg/mL) (Figure 3.11). These results are consistent with the effects seen for F6 and 6S on iNOS mRNA and protein levels and on NO production. Based on the proposed link between the iNOS and COX pathways, these results suggest that F6/6S affect both pathways. However, it remains unclear whether either F6 or 6S are specific in their action on iNOS and/or COX1/2. The anti-inflammatory effects of 6S has been studied before and there is evidence to show that 6S has potent inhibitory effects on iNOS and COX (121, 149, 156). There is also evidence that 6S down regulates iNOS and COX-2 gene expression in macrophages by down regulating of NF-κB activation and through blocking the activation PI3K/Akt/IκB kinases IKK and MAPK (149). The experimental data presented in this chapter are in line with these observations and as shown by the ability of 6S to preferentially down regulate iNOS mRNA level when the cells were treated before stimulation with LPS. GD, in contrast, did not reduce the level of iNOS mRNA and showed marginal effect on iNOS protein level when the cells were treated before stimulation. However, GD still demonstrated inhibitory effects on NO and PGE$_2$ production. Thus, this data suggests that GD is most likely to affect the enzymatic activity of iNOS possibly by influencing the crosstalk between the iNOS and COX pathways (140, 141, 144, 145). Another possibility is that GD may have more substantial effects on COX compared with iNOS. Nevertheless, this hypothesis needs further studies.
6 Conclusions

The experiments described in this chapter provide experimental evidence for the anti-inflammatory effects of ZOR and has enabled the identification of compounds that could mainly underlie these properties. Further, the data provide insight into potential mechanisms of action and the beneficial effects of this plant in inflammatory conditions including psoriasis.

The murine macrophage cell line RAW 264.7 was selected for the screening process as it has been widely used in studies aimed at understanding the role of iNOS in inflammation and its role in NO and PGE\textsubscript{2} production. This is particularly relevant as murine macrophages are known to be better responders to LPS stimulation resulting in NO production than human monocytes and macrophages (157). Thus, this gives consistency in the evaluation of ZOR’s fractions and compounds.

As for the implication of results described in the chapter, it is known that iNOS, COX and their products can be modulated through various mechanisms at transcriptional, post-transcriptional, translational and post-translational levels (158). The results presented in the chapter showed that HB02 and its active fractions, particularly F6, have promising effects as potential anti-inflammatory agents. This proposition can be justified by the potent inhibitory effects of F6 on NO and PGE\textsubscript{2} production and by its ability to down regulate iNOS at transcriptional and enzymatic levels. In this respect, the results suggest that the effect of F6 on iNOS mRNA level can be attributed to the effect of 6S rather
than GD. GD, in contrast, appeared to influence iNOS at post-translational level by modulating its enzymatic activity although this needs further studies.

Figure 3.18 provides a summary on the potential mechanisms through which ZOR extract (HB02), its selected fractions (F5, 6, 7 and 10) and compounds from F6 (6S and GD) modulate the production of NO and PGE$_2$. Both compounds appear to have dual inhibitory effects on both NO and PGE$_2$. This is particularly important in inflammation since both mediators are key inducers of the inflammatory cascade in psoriasis. Both NO and PGE$_2$ are also linked to the initiation and expansion of Th1 and Th17 lineage cells through by the induction of cytokines such as TNF$\alpha$, IL-23 and IL-12, which are prominent in psoriasis pathogenesis. The next stage will, therefore, focus on studying the effect of these compounds on the production of IL-12, IL-23 and TNF$\alpha$ by activated macrophages.
Figure 3.18: A proposed mechanism of ZOR constituents on iNOS and COX-2 activities. The cartoon depicts possible mechanisms of ZOR action on inflammation through 6S and GD. 6S acts by reducing iNOS mRNA and protein levels resulting in reduced NO and PGE$_2$ production. GD, in contrast, appears to act by modulating the enzymatic activities.
CHAPTER 4

Effects of ZOR on the production of pro-inflammatory cytokines
1 Introduction

1.1 The IL-12 /IL-23 axis of cytokines in inflammation

Dendritic cells (DCs) and macrophages are important players in the initiation of immunity. Both cells function partly by producing IL-12 and IL-23, that, in turn, promote the differentiation of Th1 and Th17, respectively (136). Both immature (CD1a⁺) and mature (CD11c⁺, DC-LAMP⁺) DCs are capable of producing IL-12 and IL-23. Interestingly, both IL-12 and IL-23 have been shown to be expressed in dermal DCs at the locality of inflammation. Within the context of psoriasis immunopathology, the detection of these two cytokines, and in particular IL-23, is important as Th17 are predominant in psoriatic lesions (159). The production of IL-12 leads to the differentiation of Th0 cells to Th1 cells which produce INFγ, a cytokine necessary for innate immunity and also the induction of T-cell homing to the skin (Figure 4.1). IL-23, in contrast, is important for the differentiation of Th17 cells that produce IL-17. IL-17 induces a chemotactic environment for the attraction of PMNs mainly through the production of CXC chemokines, such as CXC1 and CXCl5 along with the presence of LTB₄. This promotes PMNs migration to sites of inflammation and, in chronic inflammation, leads to the exacerbation of disease. IL-23 and IL-12 share some common, though not identical, biological effects. For example, both activate STAT4, but IL-23 also, preferentially induces STAT3. This is demonstrated by the ability of IL-23 to weakly induce IFNγ production, whereas IL-12 is a strong inducer of IFNγ production (160). Thus, besides strongly activating Th17 cells, IL-23 also contributes to the induction of Th1 cells.
Various studies have highlighted the existence of crosstalks between APCs and T-cells through the release of inflammatory mediators by APC, particularly PGE₂, IL-12 and IL-23. For example, it has been shown that the IL-23 /IL-17-axis induces PMN recruitment which is enhanced by the presence of PGE₂ which, in turn, augments the production of IL-23 and IL-17 but dampens IL-12 and IFN-γ production (161) (Figure 4.1). This depicts the indirect effect prostaglandins have in modulating Th1 and Th17 cell induction and expansion by synergising or inhibiting with IL-12 and IL-23, which are released by macrophages. Consequently, this regulates inflammatory cascades promoted by Th1 and Th17.

**Figure 4.1: Crosstalks between the IL-12 and IL-23 axis and inflammatory mediators produced by DCs and macrophages.** This interaction leads to enhanced Th1 and Th17 cell differentiation and expansion. The diagram indicates positive (blue arrow), negative (red arrow) and indirect (blue dotted arrow) interactions between the inflammatory mediators in the different cascades. Adapted and modified from Zakharova et al (2005) and Lemos et al. (2009) (161, 162).
1.2 The correlation between the IL-12 /IL-23 axis and TNFα

There is evidence that TNFα, which is mainly produced by activated macrophages, inhibits Th1 cells by acting as a negative regulator of IL-12 and IFN-γ production. Within this scenario, TNFα would act by limiting the length of inflammatory response. TNFα has, thus, been suggested to play an important role in the regulation of the IL-12 /IL-23 axis (162). Numerous studies have been carried out to understand the paradoxical roles TNFα plays in immune responses, in the sense that it could function both as an inflammatory and also anti-inflammatory mediator.

Generally, TNFα is known to play an important role in inducing many inflammatory cascades upon infection. Upon LPS stimulation, macrophages and DCs are activated through the NF-κB transcription factor which leads to the production of inflammatory cytokines, including IL-12 (163), IL-23 (164) as well as TNFα (165). TNFα acts in a temporal manner to specifically inhibit IL-12p40 production by macrophages stimulated with LPS, IFNγ and CpG DNA (162). Consequently, inhibition of IL-12p40 reduces IL-12 and IL-23 production by macrophage to a similar degree. Therefore, TNFα could act through a negative feedback response to regulate Th1 and Th17 induction. This, indeed, has been associated with the regulatory role of TNFα during the late stages of infection. This may, therefore, explain that despite the beneficial anti-inflammatory effects of anti-TNFα therapy (166) there could be side effects associated with the therapy such as cutaneous eruption or psoriasis rashes in patients diagnosed with rheumatoid arthritis, psoriatic arthritis, seronegative spondyloarthropathy, and with inflammatory bowel disease (167, 168).
Because of the increased awareness of the central role played by IL-12 and IL-23 axis in immune-mediated disease, it has become a therapeutic target in recent years. In this context, it has been suggested that the inflammatory activity of IL-12 and IL-23 is connected to the presence of the shared p40 subunit. Regulation of the p40 subunit has been shown to influence IL-12 and IL-23 roles in activating the Th1 and Th17 pathways, which are predominant in psoriasis. Further, clinical studies have shown that the p40 and IL-23p19 subunits are promising targets to suppress the pathogenesis of psoriasis. For example, treatment with Ustekinumab, a monoclonal antibody with specificity for the p40 subunit, results in the resolution of psoriatic lesions in patients (34).

1.3 The p40 subunit of IL-12 and IL-23 as a novel target for the treatment of psoriasis

The success of Ustekinumab in the resolution of psoriatic lesions has become a benchmark in employing specific and selective targeting of cytokines in psoriasis. Ustekinumab binds to, and inhibits the p40 subunit of IL-12 and IL-23. Early studies indicate that IL-23 is predominant in psoriasis compared with IL-12. Thus, expression of p19 and p40 but not p35 has been shown to predominate in psoriatic lesions (169, 170). The important role of IL-23 has also been shown in mice. Intradermal injection of IL-23 into the skin of normal mice was shown to result in lesions that closely resemble psoriatic plagues. In contrast, injection of IL-12 did not cause psoriasis (171).

In summary, there is good evidence for the involvement of IL-23 in the pathogenesis of psoriasis and that targeting of p40 ameliorates psoriasis. This
appears to result from down regulation of the activity of Th1 and Th17 lineage cells which, in turn, suppress TNFα-induced inflammation and indirectly inhibit IL-23-induced PMN migration. Both of the latter events are important in the induction of inflammatory cascades that underpin the pathology of psoriasis. Thus, understanding and designing new strategies to manipulate the IL-12 and IL-23 axis could prove to be highly beneficial in treating patients with psoriasis.
2 Objectives

Although ginger has been widely used in traditional medicine, there have been few studies aimed at understanding, at the molecular level, how it modulates inflammation. Whilst there are some reports on the effect of common ginger and its constituents on leukocyte adhesion and migration (172), iNOS and COX (117, 123, 147, 173), limited studies have explored its effects on cytokines such as IL-23, IL-12 or TNF-α. In line with current therapeutic strategies of targeting selective inflammatory pathways and molecules, the treatments of psoriasis have benefited from the use of biologics such as TNFα antagonists and anti-IL-23 Ab. The studies in this chapter, therefore, focus on another side to the immune-modulatory effects of ZOR and that is on IL-12, IL-23 and TNFα production by macrophages.

These three pro-inflammatory cytokines are implicated in the inflammatory cascades that underpin immunopathology of psoriasis and the involvement of activated macrophages. In addition, by studying the effect of ZOR, its fractions and constituents of these key cytokines, their effects on leukocytes activation and recruitment could also be explored.
3 Methods

3.1 Samples

ZOR extract (HB02), fractions obtained by VLC fractionation (fractions 5, 6, 7 &10) and isolated compounds from fraction 6 (6S and GD) were tested at two concentrations, 20 μg/mL for the HB02 and fractions and 50 μM for the compounds for the effect on the production of key pro-inflammatory cytokines. These tested samples were dissolved in DMSO (Sigma) to give a final concentration of DMSO in the test solutions of 0.2%. The inhibitory effects of the test samples were compared with the anti-inflammatory agent L-NAME. Besides its ability to inhibit NO, L-NAME has been shown to inhibit TNFα production from LPS-stimulated macrophages (174).

3.2 Cell cultures and bioassays

All experiments were carried out by methods described in Method 3.3 (chapter 3) and using the RAW 264.7 cell line. The cell line was treated with HB02, the selected VLC fractions and active compounds, prior to and after stimulation of the cells with LPS, as described in the previous chapter. The level of mRNA for IL-12 p35, IL-12p40, IL-23p19 and TNFα in the stimulated RAW 264.7 cells was determined by real-time PCR. The corresponding levels of TNFα protein in the collected supernatant were determined using MULTI-ARRAY™ 96-Well Small Spot ELISA kit.
CHAPTER 4

3.3 RT-PCR

RNA was extracted as described in Method 3.6 (Chapter 3). In preliminary experiments, the level of \textit{TNF}\textalpha\ mRNA was also detected in HB02-treated RAW 264.7 cells before LPS stimulation using conventional PCR (Method 3.7 of Chapter 3). The following primers were used for the conventional PCR:

Table 4.1: Summary of PCR primers used.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH_for</td>
<td>CACCATCTTCCAGGAGCGAGA</td>
</tr>
<tr>
<td>GAPDH_rev</td>
<td>ACGGAAGGCCATGCCAGTGA</td>
</tr>
<tr>
<td>TNF\textalpha_for</td>
<td>CATCTTCTCAAAATTCGAGTGACAA</td>
</tr>
<tr>
<td>TNF\textalpha_rev</td>
<td>TGGGAGTAGACAAAGGTACAACCC</td>
</tr>
</tbody>
</table>

Subsequent studies described in this chapter relied on using real-time PCR to accurately quantify the level of gene expression for cytokines of interest indicated above.

3.4 Quantification of cytokine mRNA using real-time PCR

Absolute qPCR ROX mixes and pre-designed TAQMAN® probes were used (Table 2). The relative quantitative method was used for this set of experiments. This method relies on measuring the concentration of mRNA for the gene of interest (GOI) in unknown samples relative to mRNA of the reference gene, which was GAPDH (Table 2). Level of the reference gene is the baseline for
expression of the target gene and is considered a benchmark. The Ct value of the target genes was determined and normalized with the Ct values of the reference gene to obtain $2^{-\Delta\Delta C}$. $2^{-\Delta\Delta Ct}$ values that represent the relative quantitative expression level of the target gene.

**Table 4.2: Pre-designed primers for real-time PCR.** Descriptions of the real-time PCR primers (TAQMAN gene expression assay pre-designed by Applied Biosystems) for mouse pro-inflammatory cytokine genes and house-keeping gene (GAPDH) used in the study. The sequences were not available due to manufacturer confidentiality.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amplicon length</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>TAQMAN assay ID: Mm99999915_g1 &lt;br&gt;NCBI RefSeq: NM_008084.2</td>
<td>107</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TAQMAN assay ID: Mm99999068_m1 &lt;br&gt;NCBI RefSeq: NM_013693.2</td>
<td>63</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>TAQMAN assay ID: Mm00434174_m1* &lt;br&gt;NCBI RefSeq: NM_008352.2</td>
<td>75</td>
</tr>
<tr>
<td>IL-12 p35</td>
<td>TAQMAN assay ID: Mm00434165_m1 &lt;br&gt;NCBI RefSeq: NM_001159424.1, NM_008351.2</td>
<td>68</td>
</tr>
<tr>
<td>IL-23p19</td>
<td>TAQMAN assay ID Mm00518984_m1* &lt;br&gt;NCBI RefSeq: NM_031252.2</td>
<td>61</td>
</tr>
</tbody>
</table>
3.5 MULTI-ARRAY™ 96-Well Small Spot assay for cytokine protein measurement

This protocol was used to measure the level of TNFα protein produced by activated RAW 264.7 cells either with no inhibitors or in the presence of HB02, fractions and isolated compounds. The kit was from Meso Scale Discovery (MSD; Gaithersburg, MD, USA) and assay was carried out according to the manufacturer instructions. The MSD platform is based on the proprietary combination of electrochemiluminescence detection and patterned arrays. The assay is very sensitive and can simultaneously provide quantitative data on single or multiple cytokines using small volumes of supernatant (25 μL). The assay has a detection limit 10,000 to 1.2 pg/mL. Briefly, 25 μL of supernatants (and recombinant mouse TNFα standard) were incubated for 2 hours on a pre-designed mouse TNFα 96-well microtiter plate bearing capture antibodies to TNFα on the bottom of the wells. After washing, the detection antibody (at 1 μg/mL) conjugated to the ruthenium-based MSD Sulfo-TAG™ reagent was added. Plates were washed and the MSD Read Buffer T (2x) added and results read on a Sector Imager (MSD). TNFα concentrations were determined with Softmax Pro Version 4.6 software, using a curve fit model (log-log or four-parameter log-logit) as suggested by the manufacturer.

Prior to the assay, TNFα standards were prepared in DMEM (medium used for the samples) from a stock concentration of 1 μg/mL into concentrations of 10,000, 2500, 625, 256, 39, 9.8, 2.4 and 1.2 pg/mL. Supernatants were tested without dilution.
3.6 Statistical analysis

Data were analysed using GraphPad Prism software (GraphPad Prism, San Diego, California). Means and standard deviation were used to describe normally distributed data. The significance of differences between groups was assessed using Two-way ANOVA (Bonferroni posttest). Difference of $p<0.05$ is considered significant.
4 Results

4.1 The effects of ZOR extract, fractions and compounds on TNFα mRNA and protein levels.

Targeting TNFα with biologic antagonists has revolutionized the approach to treatment of autoimmune inflammatory disorders, such as psoriasis, rheumatoid arthritis and Crohn’s disease. The success of this approach to therapy has confirmed the pivotal role of this cytokine in chronic inflammatory condition. For this purpose, the effect of ZOR on TNFα production by RAW 264.7 cells both before and following stimulation with LPS was studied.

4.1.1 Pre-treatment of RAW 264.7 cells with HB02, or its fractions had no suppressive effects on TNFα mRNA level.

The effect of pre-treatment of RAW 264.7 cells with HB02 or its fractions before stimulating the cells with LPS was originally studied using conventional PCR. HB02 and its selected fractions were all tested at 20 μg/mL prior to LPS stimulation. This concentration was chosen based on the average IC₅₀ value in the NO inhibition experiments described in Chapter 3. Results of the PCR showed that HB02 and its fractions at 20 μg/mL had no, or negligible effects on detecting TNFα mRNA in the RAW 264.7 cells (Figure 4.2). Although the data showed slight reductions in the intensity of PCR bands representing TNFα mRNA for the cells when pre-cultured with the fractions, these results were not
reproducible. Therefore, it was decided to establish and use qRT-PCR to relatively quantified TNFα level.

Figure 4.2: The effect of pre-treating RAW 264.7 cells with HB02 or its fractions on the level of TNFα mRNA using conventional PCR. The cells were cultured as described in the Methods and pre-treated with 20 µg/mL of HB02 or its fractions 5, 6, 7 or 10 (Lane 3-7) for 30 minutes before the cells were stimulated with LPS. The effect of the samples were compared with L-NAME(LN) at 54 µg/mL (lane 8). The treated and stimulated cells were incubated for 24 hours and then lysed for RNA extraction. RNA was extracted from the cells and used to reverse transcribe to cDNA which were used for conventional PCR. The results were then analysed using a 1% Agarose gel and DNA bands revealed with ethidium bromide.

4.1.2 Real-time PCR reveals that HB02 and its fractions increase the level of TNFα mRNA in LPS-stimulated RAW 264.7 cells.

The real-time PCR protocol provided quantitative data showing that pre-treatment of the RAW 264.7 cells with HB02 and its fractions increased the level of mRNA for TNFα. Interestingly, the fractions had differing effects with the mRNA level, most enhanced by fraction 10 (Figure 4.3). HB02 and fractions 6 and 10 added after stimulating the RAW 264.7 cells with LPS also had an
enhancing effect on TNFα mRNA level (Figure 4.3ii). Interestingly, fractions 7 and, to a lesser extent fraction 5 had inhibitory effects on TNFα mRNA when they were added to the cells after stimulation with LPS. The data indicated that F7 and, to a lesser extent, F5 may contain active compounds that suppress TNFα mRNA levels after stimulation. L-NAME, however, did not significantly affect TNFα mRNA when added before LPS stimulation. Moreover, it increased TNFα mRNA level when added after LPS stimulation. F5 and F7 showed better effects than L-NAME on inhibiting TNFα mRNA. In order to validate these data, the level of TNFα at protein level was determined.
Figure 4.3: The effect of HB02 and fractions (F5, F6, F7 & F10) on the level of TNFα mRNA. The level of TNFα mRNA was determined in RAW 264.7 cells that were stimulated with LPS. The extract, HB02 and fraction at 20 μg/mL were either (i) added to the cells 30 minutes before stimulating the cells with LPS, or (ii) added 12 hours after stimulation. The outcomes of the treatment were compared with L-NAME (54 μg/mL/ 200 μM) for changes when the cells were treated before and after LPS stimulation. Data are presented as the mean ± SD of % of TNFα mRNA level relative to control. Statistical comparisons were carried out using Two-Way ANOVA (Bonferroni posttest). No significant difference was observed between samples in each set of approaches (before or after LPS stimulation).
4.1.3. 1-dehydro-6-gingerdione (GD) identified in F6 suppresses TNFα mRNA level.

The results presented in the previous section showed that F6 increased the level of TNFα mRNA. Further, effect of its constituents, 6S and GD (at 50 µM) on TNFα mRNA was determined (Figure 4.4). This concentration was chosen based on the IC\text{50} of the compounds when tested for NO inhibition. The effect of both compounds on TNFα mRNA was compared with that of L-NAME at 200 µM.

The experiments on TNFα mRNA level showed that GD reduced the level of mRNA when the cells were pre-treated with the compound before LPS stimulation (Figure 4.4i). 6S, in contrast, increased the level of TNFα mRNA, again when the cells were pre-treated. The effect of 6S is, thus, similar to that seen for the crude fraction, F6. This finding shows that GD behaves in a contradictory manner to both 6S and F6. The findings on the effect of 6S is in agreement with previous findings (123) that showed that 6S was unable to inhibit TNFα production and that this relates to the inability of the compound to suppress TNFα gene transcription. However, there is a caveat to the study by Lantz and colleagues in that their study was based not on purified 6S but on a mixture of compounds containing 6S.
Figure 4.4: The effect of 6S and GD on the level of $\text{TNF}\alpha$ mRNA. The level of $\text{TNF}\alpha$ mRNA was determined in RAW 264.7 cells that were stimulated with LPS. 6S and GD at 50 μM were added to the cells either (i) added 30 minutes before stimulation with LPS, or (ii) 12 hours after stimulation. Changes in the level of mRNA level were determined using real-time PCR. GD reduced the level of $\text{TNF}\alpha$ mRNA by about 40% when the cells were pre-cultured with the compound before stimulation. Data is in mean ± SD. Statistical comparison between all samples was carried out using Two-Way ANOVA (Bonferroni posttest) on at least three independent experiments. The outcomes of the treatment were compared with L-NAME (200 μM).

As shown earlier, L-NAME did not reduce the level of $\text{TNF}\alpha$ mRNA when the cells were either treated before stimulation with LPS or added after stimulation. In these experiments, 6S and F6 that contains the compound were shown to have similar effects to L-NAME. However, no studies are available showing the effect of GD on $\text{TNF}\alpha$ mRNA.
4.1.4 HB02, tested fractions, 6S and GD have no effect on the level of TNFα protein.

To confirm whether any of the tested products of ZOR could or could not affect TNFα, their effects on the production of the protein produced by LPS-stimulated RAW 264.7 cells was assessed. These experiments also aimed to verify the results obtained for their effect on TNFα mRNA levels. The level of TNFα in supernatants of the activated RAW 264.7 cells was quantified MSD kit. The extract, HB02, tested fractions and compounds 6S and GD were all tested at 20 μg/mL. The results showed that none of the test samples used had significant effects on TNFα protein levels detected in the tested supernatants (Figure 4.5). This data confirms the real-time PCR data on the lack of effect on TNFα mRNA levels. However, there were slight reductions in the level of TNFα protein by the fractions, especially when the test samples were added after the cells were stimulated with LPS (Figure 4.5ii). F5, F7 and F10 marginally reduced the level of TNFα protein production by activated RAW 264.7 cells by 1-2% before and after LPS stimulation.

These results, thus, confirm that neither HB02, its fractions nor the two isolated compounds are capable of influencing TNFα gene transcription/mRNA stability or protein production despite their significant inhibitory effects on iNOS mRNA and protein expression. This may suggest that, the test compounds influence the activation of different transcription factors or different regulatory pathways. Theoretically, this could be due to different turnover rates of the respective mRNAs (175). However, the modest ability of F5, F7 and GD in down-regulating
TNFα mRNA level after LPS stimulation could be therapeutically relevant as it is in line with its inhibitory effect on iNOS.

Figure 4.5: The effect of HB02, fractions and compounds on TNFα protein production. The level of TNFα protein production by RAW 264.7 cells was determined using the MULTI-ARRAY™ 96-Well Small Spot MSD kit. HB02, F5-F10 were tested at 20 µg/mL while 6S and GD were tested at 50 µM. The effects were assessed when the cells were either pre-treated with the test inhibitors or added after stimulation with LPS. L-NAME was tested at 200 µM. No significant differences were observed for the test inhibitors when the cells were treated before or after stimulation. The comparison is based on one experiment.
4.2 The effects of HB02, fractions and active compounds on *IL-23p19* mRNA level.

The results presented earlier in this chapter indicated that treatment of the macrophage cell line RAW 264.7 with HB02 or with its fractions (except for F5 and F7) and compounds 6S had either no effect or even increased the level of *TNFα* mRNA. To further explore the effect of ZOR on inflammation, the effect of HB02, its fractions and compounds 6S and GD on the level of *IL-23p19* mRNA was studied.

4.2.1 HB02 and fractions down regulate *IL-23p19* mRNA levels.

Experiments revealed that *IL-23p19* mRNA level was down-regulated when RAW 264.7 cells were pretreated with HB02 and its fractions prior to stimulation with LPS (Figure 4.6i). Of the fractions tested, F7 showed the most potent inhibitory effect (~60% inhibition). Interestingly, the results showed that pre-treatment of the cells with L-NAME, increased the level of *IL-23p19* mRNA levels (Figure 4.6i). This effect is consistent with the noted effect of L-NAME on *TNFα* mRNA level. This finding highlights the fact that ZOR has a wider spectrum of anti-inflammatory effects compared with L-NAME (p<0.001). These results highlight that the effect HB02/its fractions have on *IL-23p19* mRNA may be therapeutically beneficial in psoriasis. However, when the extract and fractions were tested for their effects on *IL-23p19* mRNA after the RAW 264.7 cells were stimulated with LPS, the opposite affect was observed in that HB02 and the fractions all enhanced the level of *IL-23p19* mRNA to varying degrees (Figure 4.6ii).
The explanation for this observation is unclear but this could relate to differences in the transcription factors, or even components of the same transcription factor, involved in steady state induction of IL-23p19 than those involved in activated cells and which of these is or is not inhibited by the selected ZOR samples. In this respect, it is known that the promoter for IL-23p19 has a number of sites for NF-κB binding but interestingly it appears that the different members of the transcription factor family have different effects on the level of IL-23p19 gene transcription. For example, deficiency in NF-κB c-Rel protein abolishes p19 production while Rel-A deficiency only partly inhibits the production of p19 in macrophages (164). In contrast, deficiency in Rel-B enhances p19 production. It could, therefore, be that ZOR inhibits, or promotes the binding of the different components of the NF-κB complex, or other transcription factors, at each stage of IL-23p19 gene transcription.
Figure 4.6: The effect of HB02 and its fractions on the level of IL-23p19 mRNA. The level of IL-23p19 mRNA was determined using real-time PCR. The cells were either treated with the test samples 30 minutes before stimulation with LPS or treated 12 hours after stimulation. Data are presented as the mean ± SD from more than three independent experiments. Statistical comparisons between all samples were carried out using Two-Way ANOVA (Bonferroni posttest). The outcomes of the treatments were compared with L-NAME for changes when the cells were treated before and after LPS stimulation. All selected ZOR samples showed more potent effects compared with L-NAME when added before LPS stimulation (**p<0.001).

4.2.2 The effect of 6S and GD on IL-23p19 mRNA level.

Using the same approach as in the previous section, the effect of 6S and GD on IL-23p19 mRNA levels in LPS-stimulated RAW 264.7 cells was then compared. The results showed that both 6S and GD reduced the level of IL-23p19 mRNA when the RAW 264.7 cells were treated with the compounds before stimulation.
(Figure 4.7i). In contrast, treatment of the cells with GD, and to a lesser degree 6S, increased the level of IL-23p19 mRNA when added after LPS stimulation (Figure 4.7ii). L-NAME increased the level of IL-23p19 mRNA irrespective whether the cells were treated before stimulation or after. Hence, the inhibitory patterns of these compounds are comparable to that of F6 (Figure 4.6).

**Figure 4.7: The effect of 6S and GD on the level of IL-23p19 mRNA.** The level of IL-23p19 mRNA was determined using real-time PCR. The cells were either treated with 6S or GD at 50 μM (i) 30 minutes before stimulation with LPS or (ii) treated 12 hours after stimulation with LPS. Data are presented as the mean ± SD from more than three independent experiments. Statistical comparisons between all samples were carried out using Two-Way ANOVA (Bonferroni posttest). Both compounds showed significant effects than L-NAME (p<0.01) when the cells were treated before stimulation.
4.3 The effect of HB02, fractions, 6S and GD on IL-12p40 mRNA level.

IL-23 and IL-12 share a common protein subunit, p40 that forms a heterodimeric complex with p19 and p35, respectively. The heterodimeric complexes are biologically active in the differentiation of Th17 and Th1 cells, and important in the pathogenesis of psoriasis. Based on these facts, the next set of experiments examined the effect of HB02, its fractions, and 6S and GD on the regulation of IL-12/IL-23p40 production by the RAW 264.7 macrophage cell line.

4.3.1 The effect of HB02 and fractions on IL-12/IL-23p40 mRNA levels.

Experiments to assess the effect of HB02 and its fractions were carried out as described in the previous section using the RAW 264.7 cells treated either before LPS stimulation or after. The results showed that HB02 and its fractions significantly reduced the level of IL-12/IL-23p40 mRNA levels when the RAW 264.7 cells were treated before stimulation with LPS (Figure 4.8i). Interestingly, the results showed that HB02 and its tested fraction had more potent inhibitory effects on IL-12/23p40 mRNA when the cells were treated after stimulation with LPS (Figure 4.8ii). L-NAME also reduced the level of IL-12/IL-23p40 mRNA when the cells were added to cells before LPS stimulation but in contrast increased the level when added after stimulating the cells with LPS. These data suggest that HB02 and its fractions reduce IL-12/IL-23p40 gene transcription and/or mRNA stability before and after cell stimulation with LPS. This finding may indicate that ZOR could have therapeutic potential in reducing the level of IL-12 and IL-23 in pathological conditions including psoriasis.
Figure 4.8: The effect of HB02 and its fractions on the level of IL-12 / IL-23p40 mRNA. The level of IL-12 /23p40 mRNA was determined using real-time PCR. mRNA levels were determined using LPS-stimulated RAW 264.7 cells. The cells were either treated with HB02/fractions (i) 30 minutes before stimulation with LPS or treated (ii) 12 hours after stimulation. Data are presented as the mean ± SD of IL-12/23p40 mRNA level with the indicated treatments. Statistical comparisons between all samples were carried out using Two-Way ANOVA (Bonferroni posttest). Comparisons are based on at least three independent experiments. Both treatment approaches reduced IL-12 /23p40 mRNA levels and no statistically significant differences between the two approaches were noted. All samples showed very significant changes compared with L-NAME. The significance is indicated as *** = p<0.001.
4.3.2 The effect of 6S and GD on IL-12/IL-23p40 mRNA.

In the next stage of this study examined the effect of 6S and GD on IL-12/IL-23p40 mRNA level. Both 6S and GD were tested at 50 μM and inhibitory effects compared with L-NAME at 200 μM.

Both 6S and GD consistently showed inhibitory effects on the level of IL-12/IL-23p40 mRNA when the cells were treated before stimulation with LPS (Figure 4.9i). The inhibitory effects of both compounds were superior to the effect of L-NAME. Interestingly, however, whilst 6S also reduced the level of IL-12/IL-23p40 mRNA when the cells were treated after stimulation with LPS, GD in contrast increased the level of IL-12/IL-23p40 mRNA. The inhibitory effect was significant when compared with that of L-NAME (Figure 4.9ii). This may suggest that in so far as the effect of suppressing ongoing inflammation in which IL-12/IL-23 play a role is concerned, 6S but not GD, has the potential to be used as a therapeutic agent.
Figure 4.9: The effect of 6S and GD on the level of IL-12/IL-23p40 mRNA.

The level of IL-12/IL-23p40 mRNA was determined using real-time PCR. The effect of treatment with 6S and GD on the level of IL-12/IL-23p40 mRNA was determined using LPS-stimulated RAW 264.7 cells. The cells were either treated with 6S and GD 30 minutes before stimulation with LPS or treated 12 hours after stimulation. Statistical comparisons were carried out using Two-Way ANOVA (Bonferroni posttest). Data are presented as the mean ± SD of % of IL-12 /IL-23p40 mRNA level relative to control. The results for the effect of 6S vs. GD on IL-12 /IL-23p40 mRNA are based on at least three independent experiments. 6S showed very significant inhibition of IL-12/IL-23p40 when added after LPS stimulated (p<0.001).
4.4 The effects of HB02, fractions, 6S and GD on \textit{IL-12p35} mRNA.

In order to determine the spectrum of the effect of HB02, fractions and compounds on the two cytokines relevant to psoriasis immunopathogenesis, IL-12 and IL-23, experiments were carried out to determine their effect on \textit{IL-12p35} mRNA level. Besides IL-12/IL-23p40, IL-12 is composed of a heterodimer containing IL-12p35. The experiments to assess the effect of ZOR on IL-12, however, were hampered by the finding that LPS stimulation of the RAW 264.7 resulted in the production of low and inconsistent levels of \textit{IL-12} p35 mRNA. In this respect, there is some evidence that in contrast to IL-12p40 and IL-23p19 the induction of \textit{IL-12} p35 mRNA requires the activation of additional transcription factors to complement LPS stimulation (176). Studies have suggested that such transcription factor could be induced by treating macrophages with IFN\textgamma (177, 178). Time constraints did not allow for detailed assessment of the optimal conditions for using IFN\textgamma in the system to study the kinetics of IL-12 p35 production by the RAW 264.7 cells and to assess the effect of ZOR.
5 Discussion

5.1 ZOR’s extract and active constituents reduce $IL-12p40$ mRNA level.

There is strong evidence that IL-12 and IL-23 play important roles in the pathogenesis of psoriasis. In addition to finding high levels of both cytokines in psoriatic skin lesions, recent therapeutic targeting of IL-12/IL-23p40 has proven to be very beneficial. With this in mind, the effect of HB02, its fractions, 6S and GD on the IL-12 and IL-23 was studied. The results showed that HB02 and its fractions reduced the level of $IL-12p40$ mRNA whether the RAW 264.7 cells were treated before or after stimulation with LPS. Although examining the effect of ZOR and its fractions showed they down regulated $IL-23p19$ mRNA only when the cells were treated with HB02 or the fractions before the cells were stimulated but not after, their effect on IL-12/IL-23 could be highly relevant therapeutically. This is because IL-12/IL-23p40 constituents functionally active heterodimeric IL-12 and IL-23 cytokines. This, in turn, implies that HB02 and its fraction are capable of indirectly suppressing the differentiation of Th1 and Th17 lymphocytes. Interestingly, HB02 and its fractions also down-regulated $IL-23p19$ mRNA levels which further supports their potential anti-inflammatory effects. However, their effect on IL-12p35 could not be determined due to inefficiency of LPS in inducing its expression in LPS-activated RAW 264.7 cells (176-178). The fact that IL-12p35 is mainly expressed constitutively and not enhanced in psoriatic lesions compared with IL-12p40 and IL-23p19 (169, 170), suggested that focus should be placed on the effect of the ZOR on IL-23 and Th17 cells.
It was interesting to note that among the tested fractions, F7 down-regulated TNFα mRNA levels after the cells were stimulated with LPS but had no effects on protein levels. This suggests either that the TNFα protein produced after stimulation represents pre-synthesised proteins or that the turnover rate of production is too strong to be inhibited by F7. Nevertheless, these findings suggest that F7 may contain compound/s that modulate the level of TNFα mRNA transcription, or stability once transcribed, which could be identified in future studies.

Of interest regarding the effect of HB02 fractions was the inability of F6, shown in chapter 3 to have the most potent inhibitory effect on NO and PGE₂, to modulate TNFα levels. This fraction had no effects, or had enhancing effects on TNFα mRNA levels. Despite this observation, however, assessing the effects of compounds 6S and GD showed that the latter had the ability to reduce the level of TNFα mRNA. The fact that 6S resembled F6 in its effect on TNFα mRNA may have a stronger influence than GD when in a mixture (in F6). It is noteworthy, however, that both 6S and GD up-regulated TNFα mRNA when added to the cells after stimulation with LPS and also had little effect on TNFα protein production. All these results indicate that, neither HB02, its fractions (with the exception of F7) nor compounds; 6S and GD have significant inhibitory effects on inflammation mediated by TNFα.
5.2 The potential regulatory effects of 6S and GD on Th1/Th17 differentiation.

There is evidence for a link between the IL-12 /IL-23 axis and TNFα production. IL-23 has a central role in the differentiation of Th17 cells. Further, IL-23-dependent Th17 differentiation is involved in various autoimmune inflammatory conditions (179). The presence of IL-23p19 along with IL-17 enhances the production of TNFα (180). In turn, TNFα and IL-17 synergistically enhance IL-23p19 production (179) and lead to unrelenting progression of inflammation. The interaction between IL-23 and TNFα has been suggested by the noted effect of Etanercept in patients with RA whereby reduction in TNFα consequently leads to a decrease in IL-23 production (181).

One interpretation of these results is that a correlation between the effect of GD on TNFα expression when the cells are treated before stimulation and a reduction in IL-23p19 mRNA exists. The reduction of about 40% in TNFα mRNA (Figure 4.4a) equates with a similar reduction in IL-23p19 mRNA (Figure 4.7i). This may suggest that GD acts by directly modulating a common transcriptional pathway(s) involved in the production of both IL-23 and TNFα.

The available evidence indicates that LPS-induced TNFα gene transcription is mediated by the binding of Egr-1, CRE and NF-κB (κB3/RelA) to promoter regions within the gene (165, 182). LPS-induced IL-23p19 gene expression is mediated by signaling through the MAPK (ERK, JNK, and p38) pathway leading to the activation of NF-κB (183). Both cytokines are, therefore, induced by processes that lead to the activation of NF-κB. This may explain, at least partly,
the inhibitory effect of GD on the level of mRNA of both cytokines. A number of studies have shown that Rel-A and particularly c-Rel bind to the proximal NF-κB site in the promoter region of the \textit{IL-23p19} gene (164, 184). Thus, the transcription of TNFα and IL-23p19 genes could be linked to Rel-A binding to sites within their promoter regions. Down-regulation of both \textit{IL-23p19} mRNA and IL-12p40 mRNA by GD and 6S, in contrast, may suggest that the transcription of these two genes are connected through c-Rel binding (163).

In contrast to GD, 6S demonstrated similar effects to F6 and HB02. Thus, 6S down regulated mRNA levels for both \textit{IL-23p19} and \textit{IL-12p40} but enhanced \textit{TNFα} mRNA when the cells were treated with 6S before stimulation with LPS. After stimulation, 6S reduced the level of \textit{IL-12p40} mRNA and, thus, indirectly inhibited IL-12 production. These results may, therefore, suggest that GD has advantages over 6S in that the former has the ability to inhibit IL-12 /IL-23 and TNFα production when the cells are treated before stimulation. It is not clear at this stage, however, whether this would have any beneficial therapeutic applications.
6 Conclusions

The data presented in this chapter provides an overview for possible mechanisms of how ZOR’s extract, fractions and compounds might influence inflammation mediated by IL-12 /IL-23 and TNFα (Figure 4.10). The results, thus, showed that HB02, its fractions (F5, F6, F7 and F10) and 6S had similar effects on IL-12p40 mRNA whether the cells were treated before LPS stimulation or after. However, they only had suppressive effects on the level of IL-23p19 mRNA when used to treat the cells before stimulation but not after. In addition, all except F7 and GD had no effects on the level of TNFα mRNA whether the cells were treated before or after stimulation. The exact implication of these findings is not available at the stage. However, it is possible that the lack of inhibitory effects when the extract, fractions or compounds are added after stimulation may relate to the enhanced transcription/turnover rate of the genes, or the involvement of additional transcriptional pathways not inhibited by the test samples on the cells once stimulated. With this point in mind it may be possible to suggest that the ability of F7 to down regulate TNFα mRNA level after stimulating the cells may relate to inhibition of signalling pathway/transcription factors that are induced after activation but not before.

In contrast to F7, GD was effective in reducing the level of TNFα mRNA level before LPS-stimulation of the cells but not after. This, again, may be attributed to stronger gene transcription by the same signalling pathway/transcription factor or involvement of different ones after activation that is/are not inhibited by GD. However, overall these data indicate that ZOR contains compounds that target multiple signalling pathways and transcription factors. Some of the
findings regarding the effect of HB02, fractions and compounds on the cytokines studied in this chapter are consistent with findings by Lantz and colleagues who showed that ginger extracts, which contained shogoals and gingerols, were unable to reduce TNFα production but potently down-regulated COX-2 in LPS-stimulated U937 cells (123).

Figure 4.10: A cartoon depicting proposed mechanisms for the effects of ZOR on TNFα, IL-12/IL-23p40 and IL-23p19 mRNA levels before and after stimulation of macrophages. The plus (+) and minus (-) signs indicate stimulatory and inhibitory effects on the designated cytokine mRNA level, respectively. All ZOR samples regulate IL12/IL23 mRNA level when added before LPS stimulation. Of interest, was the ability of F5, F7 and GD in regulating TNFα may have a beneficial impact on the treatment of psoriasis. The mechanisms outlined indicate that ZOR contains compounds that could target multiple signalling pathways and transcription factors involved in inflammation.
CHAPTER 5

Modulatory effects of ZOR on immune cell responses
CHAPTER 5

1 Introduction

1.1 The role of leukocytes in psoriasis

In addition to the role Th1 and Th17 T-lymphocytes play in psoriatic inflammation, leukocytes such as polymorphonuclear PMNs (PMN) have been shown to be involved in the early stages of psoriasis. It is generally thought that leukocytes play a key role during the ‘acute’ phase of the inflammatory response in psoriasis. This is manifested by the accumulation of PMN and parakeratotic hyperkeratosis which is characterised by abnormal keratinisation or incomplete formation of keratin in the stratum corneum, loss of granular layer and increased cell turnover. The ‘acute’ inflammatory phase is severe and resistant to treatment compared with the ‘chronic’ phase inflammation. ‘Acute’ and ‘chronic’ inflammations are histological terms to characterise and categorise the phenotype of lesions within the same lesional area (185). Chronic inflammation is characterised by epidermal hyperplasia and T-lymphocyte infiltration without the presence of PMNs as seen in plaque psoriasis. The characteristics of ‘acute’ inflammation, however, are seen in guttate and pustular psoriasis, which are due to viral and bacterial infection. It is thought that autoimmune mechanisms trigger the recruitment of PMNs to the site of parakeratosis in the epidermis to cause the appearance of Munro’s microabcesses (subcorneal abcessess) (186). Thus, it is suggested that the accumulation of PMNs at the site of inflammation is not a passive event and that they act as stimulators of T-cell-mediated immune responses which subsequently boost T-cell-associated-inflammation-sustaining loop through the activation of both epidermal keratinocytes and T-cells (185).
In the initial stages of inflammation, the transmigration of PMNs to sites of inflammation is facilitated by the interaction of cellular adhesion and co-stimulatory signalling molecules. These molecular signals guide the interactions between PMNs and activated endothelial cells to enable the former to travel through the vascular lining from vessel lumen to inflamed tissues. Cell recognition and interaction depend on several adhesion molecules constitutively expressed, or induced, on the surface of PMNs and endothelial cells. These adhesion molecules are the selectins, the integrins and the immunoglobulin-like molecules that operate in a sequential manner (Figure 5.1). The first stage of inflammation involves PMN tethering and rolling, and these are facilitated by L-selectin, PSGL-1 and E-selectin ligand on the surface of PMNs. They interact transiently with their cognate counter-receptor ligands, including GlyCAM-1. PSGL-1, hyaluronan, E- and P-selectin, on activated endothelial cells. P-selectin/PSGL-1 interaction enables leukocyte rolling, E-selectin controls the rolling velocity for binding signal surveillance on the endothelium and L-selectin/PSGL-1 enables leukocyte capture on the endothelium. Available evidence shows that PSGL-1 mediates rolling and tethering of leukocytes through interaction with all three selectins through leukocyte-endothelium and leukocyte-leukocyte interactions (187, 188). PSGL-1 directly interacts with L-selectin through PMN-PMN interaction. PMN-PMN interaction during free flowing allows the PMNs to bind to the adherent PMNs resulting in amplification of recruitment rate at the site of inflammation (189). Therefore, selectins and their ligands are useful indicators of the initiation stage of inflammatory processes.
L-selectin (CD62L) is a homing receptor that enables leukocytes to enter secondary lymphoid tissues. When L-selectin binds to its cognate ligand on activated endothelial cells, leukocyte movement slows down. This enables leukocytes to adhere to high endothelial venules and subsequently transverse into secondary lymphoid tissues for priming. During this interaction L-selectin will be disengaged, or shed from the cell surface of PMNs upon activation by chemokines. Among the selectin family, L-selectin is constitutively expressed on all subsets of leukocytes, whilst E- and P-selectin are expressed on activated endothelium (134). Previous in vivo studies have shown that the blockade of L-selectin reduces PMN accumulation at sites of inflammation in the peritoneum, skin and the lung (134). This suggests that L-selectin plays an important role in the initiation of inflammation. It mediates the earliest interaction between leukocytes and activated vascular endothelium and allows for leukocytes rolling in search of inflammatory signals.

In severe psoriasis, decreases in L-selectin expression on monocytes, PMN, B-cells and CD4+ T-cells as well as increase of serum L-selectin have been observed (190). It has been suggested that L-selectin expression levels on some leukocyte subset, particularly CD4+ T-cells corresponds to the severity of disease (190). Chemoattractant signaling by chemokines produced upon infection/inflammation induce firm adhesion of leukocytes. This is facilitated by conformational changes in β2 integrin, such as Lymphocytes function-associated antigen-1 (LFA-1) and macrophage-1 antigen (Mac-1). LFA-1 is comprised of two integrins, CD11a and CD18. CD11a, or integrin-αL (also known as ITGAL), is involved in cellular adhesion and costimulatory signaling. CD18 integrin is the prime mediator in leukocytes/endothelium interaction (191).
Mac-1, which is also known as heterodimeric integrin alpha-M beta-2 (αMβ2) molecule (also as complement receptor 3; CR3), is a protein integrin subunit of CD18 and CD11b (Integrin-α M or ITGAM). LFA-1 plays an important role in firm adhesion whilst Mac-1 is crucial in leukocytes intravascular crawling and transmigration to inflamed sites. Mac-1 enables leukocytes to crawl perpendicularly against the blood flow, which is an advantage as it allows leukocytes to detect endothelial junctions in a short period of time and transmigrate through them (192).

These evidence, therefore, indicate that both CD11a and CD11b integrins are important markers of leukocyte transmigration. During leukocytes transmigration, LFA-1 and Mac-1 interact with receptor ligands, ICAM-1 and/or ICAM-2. Both ligands are intracellular adhesion molecules (ICAM) expressed on the surface of the endothelial cell. This interaction facilitates recruitment of leukocytes to sites of inflammation upon activation by chemokines, such as IL-8. IL-8 is known to induce a chemoattractant environment by binding to chemokine receptors, such as CXCR-1/CXCR-2. In psoriasis, CXCR-1 is expressed on infiltrating cells in the dermal layer of psoriatic skin, whilst CXCR-2 is expressed on both infiltrating cells and suprabasal layers of psoriatic keratinocytes (193). In inflammatory diseases, continuous release of inflammatory mediators such as cytokines, chemokines and secondary messengers create a chemotactic environment that favours the recruitment of more APCs, leukocytes and T-lymphocytes that consequently exacerbate the inflammatory response.
Figure 5.1: The interaction between leukocytes and endothelial cells during transmigration. The adhesive interaction between leukocytes and endothelial cells is involved in promoting leukocyte tethering and rolling, activation, firm adhesion and transmigration to sites of inflammation. PMN tethering, rolling and activation are facilitated by selectins. Integrin, such as LFA-1 and Mac-1 take charge for PMN firm adhesion and transmigration. Migrated PMNs localise to inflamed tissues and interact with activated antigen presenting cells (APCs), which release cytokines and chemokines which, in turn, exacerbate the inflammatory response.

1.2 Activation of T-lymphocytes in psoriasis

One of the characteristics of an autoimmune disease is the activation of auto-reactive T-lymphocytes that is partly mediated by the production of pro-inflammatory cytokines and chemokines. TNFα, IL-1 and IL-2 are all known cytokines that promote T-lymphocyte activation and proliferation. IL-1 is secreted by many cells including macrophages, monocytes, T- and B-lymphocytes, and known for its function as co-stimulator for T-lymphocyte
activation. IL-1 induces activated T-lymphocytes to produce IL-2, which subsequently induces further T-lymphocyte activation and proliferation. According to previous studies, this co-stimulatory role of IL-1 is attributed to two complementary effects which are 1) by enhancing IL-2 transcription and secretion (194) and 2) by stimulating expression of membrane receptor for IL-2 (195, 196). Activated T-lymphocytes express activation markers on their surface and these include CD25, CD69 and HLA-DR. Generally, after T-cell activation, CD69 is the earliest marker to be upregulated followed by CD25 and HLA-DR. CD25 is the α subunit of IL-2 receptor (IL-2R), This receptor plays an important role in inducing T-cell activation and is critical for the activation of Th1 cells in psoriasis. CD69 is a receptor that is transiently expressed after T-cell activation; nonetheless, its functional importance in T-lymphocyte activation in psoriasis is not entirely clear.

A previous study using flaky skin mice (fn/fn) whose disease resembles psoriatic skin showed that high levels of CD69 expression was biased towards CD8⁺ whilst CD25 was dominantly expressed on CD4⁺ T-lymphocytes (197). In patients, this notion is supported by a study showing that CD25 expression is prevalent in the epidermal and dermal layer of psoriatic skin as well in the circulation whilst CD69 and HLA-DR are significantly increased on circulating T-cells (198). Interestingly, a recent study suggested that ligation of CD69 can inhibit Th17 differentiation by activating Jak3 signal transducer and STAT5 (199). This, in turn, prevents STAT3 translocation to the nucleus and blocks STAT3-mediated RORyt induction by activating FoxP3 (199). In this context, CD69 expression could modulate the Th17 cell expansion, which is pathogenic in psoriasis.
As cited in chapter 1, Th1 and Th17 have been shown to play significant roles in initiating and sustaining inflammation in psoriasis. Besides the described roles played by helper T-lymphocytes there is evidence that cytotoxic CD8$^+$ cells also produce TNF-α and IFN-γ as well as Th17-related cytokines such IL-17, IL-21 and IL-22 in psoriatic plaque (200). This highlights the contribution of CD8$^+$ subsets in complement with the roles played by helper T-lymphocytes in psoriasis.

1.3 Modulation of leukocyte migration and T-lymphocyte activation as therapeutic options.

Over the years, therapeutic approaches targeting leukocyte recruitment and trafficking by inhibiting expression of selectins, integrins and chemokines has been used to treat inflammatory diseases, including psoriasis. Thus far Efalizumab has been shown to be effective in treating some psoriatic patients by binding to the α-subunit (CD11a) of LFA-1 and inhibiting T-cell activation. LFA-1/ICAM binding has been known to facilitate the activation and migration of T-lymphocytes from the circulation into dermal and epidermal layers and, consequently, leading to their reactivation (201).

Despite the emergence of effective biologic treatments (described in chapter 1), other types of therapy with enhanced efficacy, safety and convenience are needed in the treatment of psoriasis. There are studies on potential synthetic antagonists that inhibit the interaction between selectins and their carbohydrate ligands. Among the successful ones is Efomycine, which mimics the structure of
sialyl Lewis\textsuperscript{x} (sLe\textsuperscript{x}), which has shown significant beneficial effects in experimental psoriasis by acting to impair leukocyte rolling and adhesion (202). Designing inhibitors against sLe\textsuperscript{x} carbohydrate epitope is based on the fact that all the three selectins bind to sLe\textsuperscript{x}. Therefore, sLe\textsuperscript{x} mimetic behaviour of Efomycine enabled it to impair all the three selectins which contribute to its higher potency compared with antibody specific to individual selectins (203). This implies that sequential inhibition of the selectins is required for profound effects against leukocyte transmigration and lymphocytes homing.

Modulation of T-lymphocyte activation and expansion has also been shown to be effective in the treatment of psoriasis. This was demonstrated by the effectiveness of Daclizumab in resolving psoriatic lesions within six weeks of administration in a trial involving 19 psoriatic patients (204). It was, therefore, proposed that Daclizumab attenuates CD25, which, in turn, blocks IL-2/IL-2R binding and consequently inhibits T-lymphocyte activation.

Recently, it was shown that several phenolic compounds from methanolic extracts of \textit{Z. officinale} (common ginger), which included 6-shogaol, 8-shogaol, 10 gingerol and dehydro-6-gingerdione had inhibitory effects on T-cell migration (172). This study concluded that the compounds inhibited direct binding between sICAM and LFA-1 of THP-1 cells (monocytes), whereby, 6-shogaol (IC\textsubscript{50} =27.1\textmu M) showed most potent activity and better than Lovastatin (IC\textsubscript{50} =57.2 \textmu M). Furthermore, it was suggested that 6-shogaol and dehydro-6-gingerdione regulated the late phase of monocyte migration by inhibiting direct binding between sVCAM-1 and VLA-4 (172). Thus, these findings indicate that both compounds inhibit binding during and after activation of monocytes.
Another study by Nogueira de Melo et al. (2010) demonstrated that the ginger essential oil (GEO) causes significant reduction in leukocyte chemotaxis towards casein stimuli in an *in vitro* chemotaxis assay (205). The study indicated that direct and systemic effects of the GEO on leukocyte migration as an important mechanism of the anti-inflammatory action of ginger (205). Moreover, previous studies on the effect of ten commonly used herbs on T-lymphocytes proliferation indicated that ginger was among the most potent immune-suppressors against mitogenic- and allogenic-stimulated-T-lymphocyte proliferation in mouse splenocytes (206). Another study using volatile oil of *Z. officinale* Roscoe, showed that it inhibited proliferation of total T-lymphocyte and T-helper lymphocytes and suppressed IL-1α production (207). Therefore, in general, ginger has the ability to modulate T-lymphocyte activation and proliferation.
2 Objectives

In the previous chapters the chloroform extract of ZOR (HB02), its fraction 6 (F6) and the identified compounds (6-shogaol and 1-dehydro-6-gingerdione) were shown to have inhibitory effects on NO and PGE$_2$ production as well as IL-12/IL-23 axis. In light of these findings, it was decided to study the regulatory effects of HB02, F6 and its compounds on PMN recruitment through the endothelium and the expression of adhesion molecules on PMNs in vitro. Moreover, the effect of the compounds on CD$^+$ T-lymphocyte activation was explored in preliminary experiments using mouse splenocytes. These experiments evaluated the effects of HB02, F6 and compounds on the expression of CD25 and CD69 on anti-CD3/CD28-activated T-lymphocytes. The results provide some insight into the mechanisms of ZOR action during the early stages of inflammation in psoriasis. The findings could provide a foundation for future more detailed studies to understand how ZOR could modulate auto-immune mediated inflammation.
3 Methods

3.1 PMN adhesion and migration

3.1.1 PMN isolation protocol

Peripheral blood from three healthy individuals was collected into 50 mL falcon tubes containing sodium citrate at a 1:10 ratio (citrate: blood) and then diluted 1:1 in RPMI1640 medium. Prior to the PMN isolation process, a mixture of 3 mL 11191 histopaque (Sigma) and 3 mL of 10771 histopaque (SIGMA) was dispensed and prepared in 15 mL falcon tube. The two histopaque solutions make two distinct visible layers. Next, 6 mL of the blood-RPMI1640 mixture was layered onto the histopaque mixture. The tube containing the mixture was then centrifuged at 1337rpm for 30 minutes without the brake being on. Five visible layers formed in the blood-histopaque mixture after centrifugation (Figure 5.2a). The forth layer containing PMNs was extracted out using plastic pipette and placed in a new 15 mL falcon tube in a volume of less than 6 mL. RPMI1640 was then added to make the volume up to 12 mL and then the mixture was inverted to homogeneous. The mixture was centrifuged at 1500rpm for 15 minutes. The supernatant was decanted and pellet re-suspended gently. Contaminating erythrocytes were removed by lysing with 7.5 mL of ice-cold water and 25 mL of 3.6% NaCl. This step was carried out twice and gently to avoid cell clumping which is an indication of PMN activation. The solution was centrifuged for 10 minutes at 1200rpm and supernatant removed. The pellet was re-suspended in PBS without calcium (containing 0.1% BSA) to a final volume of 2 mL. The number of cells was counted using Turk’s solution.
3.1.2 Viability assay

To determine the toxicity level of ZOR on isolated PMNs viability. Alamar Blue viability assay was carried out. PMNs in the RPMI medium (containing 10% FBS, 2 mM L-glutamine, 100 U/mL Penicilin and 100 µg/mL Streptomycin) were plated in 96 well plates at a cell density of 2x10^4 cells/well. The cells were left to adhere for 30 minutes before being treated with HB02, F6, 6S or GD at a concentration range of 0.5-100 µg/mL. The effect of DMSO was also tested at a percentage range of 0.005-5% based on its content in the culture medium. After 4- and 24-hour incubation at 37ºC, the medium was removed and replaced with 10% Alamar Blue in fresh medium. The cells were incubated for another 4 hours at 37ºC and absorbance at 560 and 595nm were measured using a microplate reader (TECAN GENios). Cell viability was calculated according to supplier's manual (AbD Serotec) as shown below;

Cell viability:

\[
\frac{PR(\text{sample})}{PR(\text{medium control})} \times 100
\]

PR value for untreated cells (control) or cells treated with ZOR samples was calculated as below;

Percentage reduction (PR) of Alamar Blue:

\[
\frac{(117216 \times A1)-(80586 \times A2) \times 100}{(155677x N2)-(14652 x N1 )}
\]

Molar extinction coefficient (E) of oxidized Alamar Blue at 560 nm = 80586
E of oxidized Alamar Blue at 595 NM = 117216
E of reduced Alamar Blue at 560 nm = 155677
E of reduced Alamar Blue at 599 nm = 14652
A1 = absorbance of test wells at 560nm
A2 = absorbance of test wells at 595nm
N1 = absorbance of negative control (Alamar Blue in media without-cells) at 560nm
N2 = absorbance of negative control at 595nm

3.1.3 PMN adhesion and migration study using flow chamber

The migration assay was carried out using a flow chamber assay, developed and assisted by members of the Biochemical Pharmacology Department at the William Harvey Research Institute (WHRI). Prior to the experiment, a confluent human vascular endothelial cell (HUVEC) monolayer (maximum of passage 4) was stimulated with 10 ng/mL TNFα for 4 hours. The monolayer was kindly prepared and provided by Dr. Dianne Cooper from the Biochemical Pharmacology Department.

PMNs at 1×10^6 cell/mL were incubated with HB02, F6, 6S and GD at 1:6 dilution in PBS (supplemented with calcium, magnesium and 0.1% BSA) at 37 °C for 10 minutes in a water bath. In the preliminary experiments, PMNs were incubated with HB02 and F6 at a final concentration of 50 μg/mL. In all subsequent experiments, the concentration of HB02, F6, 6S and GD were reduced to 3.3 μg/mL. The treated PMNs were then perfused over the TNFα-stimulated HUVEC monolayers in the flow chamber at a constant rate at 1dyne/cm² using a syringe pump (Figure 5.2b).
After 8 minute of perfusion, six random fields were captured for 10s each at 40x magnification using JVC TK-C1360B digital colour video camera. The number of PMNs captured, rolling and migrating was counted and analysed using ImagePro Plus software (Media Cybemetic, Wokingham, Berkshire). The number of PMNs was determined as an initial cell capture and then categorised as rolling, adhering and migrating. Among the captured PMNs, those which have firmly adhered remained stationary for the 10s of observation while others rolled or migrated through the endothelial junction.

Figure 5.2: The separation and assessment of PMN adherence, rolling and migration. (a) PMNs were isolated from the blood of 3 healthy donors (n=3) as described in section 3.1.1 and (b) perfused onto TNFα-stimulated HUVEC monolayers in the flow chamber under a constant rate of 1 dyne/cm² using a syringe pump. The flow chamber was placed under a microscope with 40x magnification. After 8min perfusion, six random fields were recorded for 10s each using JVC TK-C1360B digital colour video camera.
3.1.4 Expression of adhesion molecules by flow cytometry

Flow cytometry (or FACS) analysis was carried out to determine the effect of treatment with HB02, F6, 6S and GD on the expression of CD11b, L-selectin (62L) and CXC chemokine receptor-1 (CXCR-1) on PMNs. The assessment was carried out with the help of Drs. Dianne Cooper and Lucy Norling from the Biochemical Pharmacology Department. In brief, peripheral blood PMNs were suspended in PBS (supplemented with calcium and magnesium) containing the samples at a final concentration of 50 μg/mL or 3.3 μg/mL, at 37°C for 10 minutes in a water bath. The cells were then plated out at a density of 2x10^6 cells per well in 96-well plates and incubated with purified mouse mAbs to: human L-selectin, human CD11b and CXCR-1 (Table 5.1) for 1 hour on ice. The expression of each of the adhesion molecules was recorded and analysed with FACSCalibur using CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Table 5.1: Monoclonal antibodies (mAbs) used for assessing the expression of adhesion molecules on PMNs.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD62L Pe-cy5</td>
<td>DREG56</td>
<td>1:100</td>
</tr>
<tr>
<td>CD11b APC</td>
<td>ICRF44</td>
<td>1:100</td>
</tr>
<tr>
<td>CXCR1 FITC</td>
<td>8F1-1.4</td>
<td>1:10</td>
</tr>
</tbody>
</table>

The mAbs used were diluted to final concentration as indicated. All mAbs used were from eBioscience.
3.2 Activation of T-Lymphocytes

This study was carried out with the kind help of Dr. Fulvio D’Arquisto from the Biochemical Pharmacology Department. In brief, 10^6 cells/mL of isolated T-cells obtained from splenocytes of normal Balb/c mice aged between 6-8 weeks, were cultured and stimulated in 24 well culture plates pre-coated with mAbs with specificity for mouse CD3 alone or with CD3 and CD28. Thirty minutes prior to stimulation, the cells were treated with 6S or GD at 0.1 µM. Treated and untreated cells were cultured for 24 hours in RPMI1640 medium supplemented with 10% FCS, 2 mM L-glutamine and 100U/mL gentamycin. Supernatants were collected for determination of secreted TNFα and IL-17 levels.

T-cells treated with 6S or GD were then re-suspended in FACS buffer (PBS containing 1% FCS and 0.02% NaN2) and incubated with CD16 FcγR3/CD32 FcIIR blocking antibody at 4°C for 30 minutes to prevent non-specific binding of the mAbs. To assess expression of CD25 and CD69 on CD4 and CD8, the cells were stained with PE and FITC-conjugated mAb, respectively (Table 5.2). The stained cells were analysed with FACSCalibur using CellQuest software (Becton Dickinson, Franklin Lakes, NJ).
Table 5.2: mAbs used for assessing the expression of CD25 and CD69 on CD4* and CD8* T-lymphocytes.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Isotype</th>
<th>Final Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD25 FITC</td>
<td>PC61.5</td>
<td>Rat IgG1</td>
<td>0.5</td>
</tr>
<tr>
<td>Anti-CD69 PE</td>
<td>H1.2F3</td>
<td>Armenian hamster IgG</td>
<td>0.4</td>
</tr>
<tr>
<td>Anti-CD4 PE (L3T4)</td>
<td>GK1.5</td>
<td>Rat IgG2b</td>
<td>0.4</td>
</tr>
<tr>
<td>Anti-CD8 PE FITC</td>
<td>53-6.7</td>
<td>Rat IgG2a</td>
<td>0.5</td>
</tr>
<tr>
<td>Anti-CD16/32</td>
<td>93</td>
<td>-</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The mAbs used were diluted to final concentration as indicated. All mAbs used were from eBioscience.

3.3 Measurement of TNFα and IL-17 levels.

The level of TNFα and IL-17 proteins in culture supernatants was measured using Meso Scale Multiarray™ 96-well kits. The Meso Scale Discovery (MSD; Gaithersburg, MD, USA) platform is based on the proprietary combination of electrochemiluminescence detection and patterned arrays. Kits used in this study are highly sensitive and can simultaneously provide quantitative data on multiple cytokines using small volumes of supernatant (25 µl). The assays have detection limits of 4000 to 1.2 pg/mL. Briefly, 25 µl of supernatants were added to the purchased 96-well microtiter plate coated with capture antibodies to mouse TNFα or IL-17 and incubated at room temperature for 2 hours. After washing the plate, specific anti-TNFα or anti-IL-17 antibodies (each at 1 µg/mL) conjugated with the ruthenium-based MSD Sulfo-TAG™ reagent were added. The plates were washed and the MSD Read Buffer T (2x) added and results
read on a Sector Imager 6000 incorporating a charge-coupled device (CCD) camera. TNFα and IL-17 concentrations were determined with Softmax Pro Version 4.6 software, using curve fit models (log-log or four-parameter log-logit) as suggested by the manufacturer for the specific cytokine. Concentrations of TNFα and IL-17 in the tested supernatants were determined by extrapolation from standard curves constructed for the following concentrations for both recombinant TNFα and IL-17: 10000, 2500, 625, 156, 39, 9.8 and 2.4 pg/mL.

3.4 Statistical analysis

Data was analysed using GraphPad Prism software (GraphPad Prism, San Diego, California). Means and standard deviation were used to describe normally distributed data. The significance of differences between groups was assessed using Two-way ANOVA (Bonferroni posttest). Difference of p<0.05 is considered significant.
4 Results

4.1 HB02, F6 and compounds, 6S and GD are non toxic to PMNs at concentrations $\leq 50 \mu g/mL$ after 4 hours incubation.

To determine whether the test samples are toxic to the isolated PMNs, viability of the cells after incubation with HB02, F6, 6S and GD using AlamarBlue assay was determined. The samples were tested using PMNs isolated from three individuals and with incubation for 4 and 24 hours (Figure 5.3). Evaluation of toxicity level was based on concentrations that result in 50% and 90% PMN viability, IC$_{50}$ and IC$_{90}$, respectively.

The results showed that more than 80% of the PMN cells were viable after 4 hours incubation with the test samples at concentrations of $\leq 50 \mu g/mL$ (Table 5.3). The IC$_{90}$ was within the range of 30-50 $\mu g/mL$ of all samples. Incubation of the cells with DMSO resulted in $>80$% viability at 0.005-5.0% DMSO content. This suggests that the ideal concentration of the test samples to be studied for PMN migration should be at 50 $\mu g/mL$ or less. F6 was noted to be more toxic than HB02, 6S and GD, with an IC$_{50}$ of 70±45 $\mu g/mL$ (Table 5.3). The IC$_{50}$ of HB02, 6S and GD were above 100 $\mu g/mL$.

Viability of the PMNs after 24 hours incubation with 50 $\mu g/mL$ of HB02, F6, 6S and GD were also determined. Figure 5.3 shows reduction of 50% viability after 24 hours incubation relative to 4 hour incubation period. No significant differences were observed between culture for 4 and 24 hours for viability of the cells with DMSO (Figure 5.3e).
Figure 5.3: Effect of HB02, F6, 6S and GD on PMN cell viability. The test samples were assessed at the following concentration: 0.5-100 μg/mL for HB02 and F6 and at 0.5-50 μg/mL for 6S and GD. The effect of DMSO on viability was tested at 0.005-5%. Viability was assessed after 4 hours incubation (open circles) and 24 hours (black squares) incubation at 37°C. Viability was assessed using Alamar Blue assay. Results are in mean ± SD of three independent experiments (n=3).
Table 5.3: IC$_{50}$ and IC$_{90}$ of HB02, F6, 6S and GD on PMN cell viability after 4 hours incubation.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration that allows 50% cell viability (IC$_{50}$, μg/mL)</th>
<th>Concentration that allows 90% cell viability (IC$_{90}$, μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB02</td>
<td>121± 64</td>
<td>47±33</td>
</tr>
<tr>
<td>F6</td>
<td>70± 45</td>
<td>40.2±47.8</td>
</tr>
<tr>
<td>6-Shogaol (6S)</td>
<td>166± 23</td>
<td>31.4±40.4</td>
</tr>
<tr>
<td>1-dehydro-6-gingerdione (GD)</td>
<td>117± 4.2</td>
<td>0.5±41.8</td>
</tr>
</tbody>
</table>

*Peripheral blood PMNs isolated from three healthy donors. Data presented in mean ± SD.

Based on these data all experiments carried out using PMNs to test the effects of HB02, F6, 6S and GD were carried out after an incubation period of 4 hours and test samples at ≤50μg/mL of the test samples.

4.2 The effect of HB02, F6, 6S and GD on PMN adhesion and migration.

In view of the inhibitory effects of HB02, F6, 6S and GD reported in this thesis on NO production and on the IL-12/IL-23 axis, I studied their effects on PMN adhesion onto TNFα-activated HUVEC. For this purpose, I studied PMN capture, rolling, adhesion and migration using an in vitro flow chamber assay (section 3.1.3). Further, the effect of the test samples on the expression of CD62L, CD11b and CXCR-1 on PMNs using FACS (Method 3.1.4) were also determined.
4.2.1 HB02 and F6 inhibit PMN capture, rolling, adhesion and migration.

The effect of HB02 and F6 on the capture, rolling, adhesion and migration of PMN using TNFα-stimulated HUVEC was studied at 50 μg/mL using the flow chamber assay. The results showed that HB02 and F6 strongly inhibited the capture, rolling and migration of PMNs. HB02 had the strongest inhibitory effects on the PMNs. Thus, HB02 inhibited capture by 90.3%, rolling by 57%, adhesion and migration by ~100%, relative to control. In contrast, F6 caused 48% reduction in PMN capture, 80% adhesion and 89% PMN migration. Surprisingly, F6 augmented PMN rolling by about two-folds (Figure 5.4b). This may suggest that F6 could stimulate margination of PMN rolling, nevertheless, F6 strongly inhibited PMN adhesion and migration.

These data suggest that HB02 and F6 inhibit PMN-endothelium interactions and, thus, inhibit PMN tethering and transmigration. The higher potency of HB02 compared with F6 may be due to the presence of active compounds in HB02 which are not present in F6.
Figure 5.4: The effect of HB02 and F6 on PMN adhesion and transmigration. HB02 and F6 were tested at 50 μg/mL for their effect on PMN a) captured, b) rolling, c) adhesion, and d) migration on TNFα-activated HUVEC in flow chambers. The effects of HB02 and F6 were compared to control PMNs incubated in PBS (supplemented with calcium and magnesium). The results represent one experiment.
To further explore how HB02 and F6 influence PMN biology, the effect of both test samples on morphological changes on PMNs that could have unfavourable effects on endothelial contact, was examined. The results showed that incubation of PMNs with HB02 and F6 at 50 µg/mL did not alter nor deform the shape of PMNs. Instead the treated PMNs maintained their rounded shape and had a tendency for free flowing (Figure 5.5(b)(c)). This observation indicates that HB02 and F6 modulate the biology of PMNs to make them less likely to adhere and transmigrate rather than impair their biological responses. The specific effects, therefore, appear to reduce the interaction between PMNs and HUVEC surfaces to promote free flowing and reduce PMN tethering and adhesion. The results, thus, eliminate the possibility that the observations are due to PMNs morphological defects caused by a toxicity effect.
Figure 5.5: Treatment with HB02 and F6 does not alter the morphology of PMNs. The morphology of PMNs treated with 50 µg/mL of HB02 or F6 was assessed microscopically. The figure shows: a) control PMNs incubated on HUVEC cells treated with TNFα; b) PMNs treated with 50 µg/mL HB02; and c) PMNs treated with 50 µg/mL F6. No visible morphological changes are seen under a microscope with 40x magnification.
4.2.2 HB02, F6 and 6S at low concentrations modulate PMN biology.

Due to limitations on the amount of purified 6S and GD and for comparative purposes, it was decided to assess the effect of both compounds and HB02 and F6 at much lower concentrations (3.3 μg/mL). These experiments revealed that even at these lower concentrations, HB02, F6 and 6S reduced PMN capture, rolling, adhesion and migration by up to ~46% relative to control (containing 0.03% DMSO) (Figure 5.6 a-d). GD, however, augmented PMN adhesion by 34.2% (Figure 5.6c). It is indicated in Figure 5.7(e) wherein higher margination of PMNs in medium containing GD. Nevertheless, GD reduced PMN rolling by 27.6% and migration by 29.2%. Thus, GD has some inhibitory effect against PMN migration suggesting that this compound may act to inhibit the adhesion of PMN to HUVEC.

Figure 5.6 shows that F6 and 6S at the lower concentration of 3.3 μg/mL had comparable effects on PMN capture, rolling, adhesion and migration, that were more effective than HB02 (Figure 5.6). 6S had significant inhibitory effects (p<0.05) on PMN capture (~34.2% inhibition), whilst F6 significantly affect PMN rolling (44% inhibition) compared to control (Figure 5.6a and b). These data may suggest that both F6 and 6S may have inhibitory effects during early stages of inflammation through preventing PMN tethering.
Figure 5.6: The modulatory effects of low concentrations of HB02, F6, 6S and GD on PMN biology. The effect of HB02, F6, 6S and GD relative to control (DMSO 0.03%) on PMN biology were tested at 3.3 μg/mL. The effects shown are for: a) capture, b) rolling, c) adhesion, and d) migration of PMN using TNFα-activated HUVEC in flow chambers. The interactions were assessed in six random fields from three sets of independent experiments. Statistical comparison between the samples and control were carried out using Student t-test. *=p<0.05
Figure 5.7: The interaction of treated PMNs with HUVEC during perfusion. The interaction between PMNs and TNFα-activated HUVEC during the 8 minutes perfusion into the flow chamber. Prior to perfusion, the PMNs were treated with (a) DMSO control (at 0.03%), (b) HB02, (c) F6, (d) 6S and (e) GD at 3.3 µg/mL, for 10 min. HB02, F6 and 6S reduced PMNs margination and recruitment on the endothelial cell, except GD. This observation represents three independent experiments.
4.3 The effect of HB02 and F6 on L-selectin, CD11b and CXCR-1 expression.

Results of the previous sections suggested that treatment with HB02, F6, 6S and GD specifically modulated PMN biology to restrict migration. To verify these data and explore further how these tested ZOR components modulate the migration of PMNs, the effect of the test samples on the expression of CD11b and L-selectin (CD62L) was examined. The expression of these molecules was analysed using flow cytometry as described in method 3.1.4.

4.3.1 HB02 and F6 enhance L-selectin (CD62L) shedding and CD11b expression.

Results of the flow cytometry showed that HB02 and F6 both at 50 µg/mL caused a dramatic decrease of L-selectin expression (Figure 5.8b) but enhanced CD11b expression on PMNs (Figure 5.8c). F6 had a stronger effect than HB02 on reducing L-selectin and upregulating CD11b expression. These data may suggest that HB02 and F6 enhance PMN activation rather than promoting inactivation.

These results suggest that the increased L-selectin shedding and/or decrease of L-selectin expression explain the noted reduced rolling of PMNs on the HUVEC layer. The increase in CD11b (Mac-1) expression, in contrast, may suggest that HB02 and F6 enhance adhesion and, ultimately, transmigration of PMNs. However, this is not supported by results of the flow chamber showing reduced rolling, adhesion and migration of the treated PMNs (Figure 5.4). The
possible reasons for the upregulation of CD11b and implication are not clear and further experiments may be necessary.

4.3.2 HB02 and F6 have no significant effects on CXCR-1 expression

The effect of HB02 and F6 on the expression of CXCR-1 was determined (Figure 5.8d). CXCR-1 is the receptor for IL-8, a key chemoattractant cytokine involved in inflammatory responses (208). The results showed that both HB02 and F6 at 50 µg/mL did not have a significant inhibitory effect on CXCR-1 expression. This observation may suggest that HB02 and F6 at 50 µg/mL are less likely to regulate PMN activation through inhibition of chemoattraction of PMNs.
Figure 5.8: Flow cytometric analysis of the effect of HB02 and F6 on the expression of CD62L, CD11b and CXCR-1 on PMNs. (a) Depicts the FSC/SSC of purified PMNs used in the experiments. Flow cytometry results showing that HB02 and F6 (b) down-regulated the expression of CD62L (L-selectin), (c) up-regulated CD11b, and (d) have negligible effect against CXCR-1 expression on PMNs relative to control (CON) treated PMNs (PMNs treated with PBS supplemented with calcium and magnesium. The grey line is for staining the PMNs with the isotype control.
4.4 The effects of 6S and GD on CD4$^{+}$ and CD8$^{+}$ T-lymphocyte responses.

Results presented in the previous chapter highlighted the inhibitory effects of F6 and 6S on the production of iNOS, IL-12 and IL-23 by activated macrophages. However, the overall effects of the two compounds on T-lymphocytes remained to be studied. Therefore, experiments were carried out to assess the effects of 6S and GD on T-cell activation. For this purpose the expression of CD25 and CD69, membrane proteins associated with T-lymphocyte activation, were assessed following treatment with 6S and GD (Figure 5.9).

4.4.1 6S and GD had no inhibitory effects on CD25 and CD69 expression by CD4$^{+}$ T-lymphocytes.

The effect of 6S and GD on CD25 and CD69 expression was studied at 0.1 μM, a concentration that is ten-fold lower than the tested concentration used for the PMN adhesion and migration study. The inhibitory effects of 6S and GD was tested using primary mouse CD4$^{+}$ T-lymphocytes isolated from the spleen and activated in vitro with anti-CD3/CD28 monoclonal antibodies. Expression of CD25 and CD69 was assessed by flow cytometry (Figure 5.9b). The results showed that neither 6S nor GD had any inhibitory effects on the expression of CD25 and CD69 expression at 0.1 μM (Figure 5.9(b)).
4.4.2 GD and 6S reduce CD25 and CD69 expression on activated CD8\(^+\) T-lymphocytes.

The effect of 6S and GD on anti-CD3/CD28-activated CD8\(^+\) T-lymphocytes was next examined to complete the overall assessment of the effect on T-lymphocyte responses. The same approach as described in the previous section was used. Expression of CD25 and CD69 was assessed using flow cytometry (Figure 5.9c). The results showed that 6S and GD at the low concentration of 0.1 \(\mu\)M had marginal suppressive effects on the expression of CD25 and CD69 relative to control (Figure 5.9c). This suppressive effect was stronger on CD69 than on the expression of CD25. Further, GD had slightly more suppressive effects.
Figure 5.9: The effect of 6S and GD on the expression of CD25 and CD69 on activated murine CD4$^+$ and CD8$^+$ T-lymphocytes. One million primary T-lymphocytes obtained from the spleen of normal Balb/c mice were stimulated with anti-mouse CD3 and anti-mouse CD28. (a) Shows the gating strategy to study the expression of CD25 and CD69 on mouse CD4$^+$ and CD8$^+$ T-lymphocytes. The upper panel shows the gating strategy to identify live cells (in gate R1) while the lower panel depicts the gating strategy for identifying T-lymphocytes (gate R2). (b) Columns representing the mean fluorescence intensity (MFI) values for CD25 (grey columns) and CD69 (black columns) expression on CD4$^+$ T-lymphocytes in gate R2. (c) MFI for CD25 and CD69 expression on CD8$^+$ T-lymphocytes. The cells were treated with either PBS (control), 6S or GD. 6S and GD used were tested at 0.1 μM. CD25 and CD69 expression were determined by flow cytometry (n=1).
4.5 6S suppresses IL-17 production by activated CD8\(^+\) T-lymphocytes.

Based on the noted effect of GD on CD25 and CD69 expression on CD8\(^+\) T-lymphocytes it was further explored whether the two compounds also influence cytokine production by this subset of T-lymphocytes. One further reason for measuring the effect of 6S and GD on cytokine production was the observation that CD8\(^+\) are involved in psoriasis pathogenesis (200).

The results showed that 6S marginally inhibited TNF\(\alpha\) (Figure 5.10a) but significantly reduced IL-17 (Figure 5.10b) production by 33.2% relative to control (p<0.05). In contrast, however, GD did not affect both cytokines. The observation that 6S inhibit IL-17 production is consistent with the results presented in the chapter 4 wherein HB02, F6 and 6S down-regulated \(IL-23p19\) mRNA levels. Further, the differential effects of the two compounds on TNF\(\alpha\) production may also partly explain the previous observation that ZOR extract, fractions and compounds enhanced the production of this cytokine. These observations, however, need further study.
Figure 5.10: The effect of 6S and GD on the production of (a) TNFα and (b) IL-17 by activated mouse CD8⁺ T-lymphocytes. One million primary T-lymphocytes obtained from the spleen of normal Balb/c mice were stimulated with anti-mouse CD3 and anti-mouse CD28. The cells were either treated with 6S or GD or non-treated (control). The effect of 6S and GD at 0.1 µM on the production of TNFα and IL-17 by primary CD8⁺ T-lymphocytes after 24 hours of activation with anti-CD3/CD28 was determined using the MESO scale kit. (* p<0.05) using paired t-test. Results represent one experiment conducted in triplicates.
5 Discussion

5.1 HB02, F6 and isolated compounds reduce PMN adhesion and migration.

The results presented in this chapter reveal that HB02, F6 and compounds 6S and GD modulate margination of PMNs by inhibiting their rolling, adhesion and migration through vascular endothelium. The data has shown that the tested samples increase L-selectin shedding from the membrane of treated PMNs leading to the modulation of their interaction with activated HUVEC. However, the results also showed that there was an increase in the expression of CD11b on PMNs following treatment with the test samples from ZOR. This implies that treatment of PMNs with ZOR test samples resulted in the activation of PMNs and that the inhibitory effect on migration is actively induced. However, further insights into how HB02, F6, 6S and GD modulate PMN migration further studies will be required to fully address this important question.

The available evidence indicates that once PMNs are activated, rolling is restricted by L-selectin (CD62L) shedding and that the CD11a/CD18 complex and/or LFA-1 will translocate to the endothelial surface via interactions with endothelial proteins such as ICAM-1 which then promotes adherence (209). In addition to CD11a/CD18, CD11b/CD18 also contributes to PMN adhesion to the endothelium by interacting with ICAM-1. Both CD11a and CD11b work together and sequentially whereby CD11a/CD18 plays a role in PMN tethering to allow CD11b/CD18 to stably bind to the endothelium (210). This, eventually, promotes PMN migration.
Therefore, given that CD11b was upregulated while L-selectin was downregulated on PMNs treated with HB02 and F6 and that there were more freeflowing PMNs, there is the possibility that inhibition of PMN tethering andadhesion is due to orchestrated modulation of PMN functions. Previous studiesutilising the interaction of PMNs with immobilised fibrinogen/fibrin under flowconditions have shown that pre-activated PMNs with lower L-selectin levels hadimpaired abilities to adhere (211). This is demonstrated by the effect ofleumedins, an anti-inflammatory compounds when pre-incubated with PMNs, resulted in tendency of PMN rolling and reduced adhesion on the endothelialunder flow (212). This was also reflected in the inhibitory effect of aspirin(acetylsalicylic acid or ASA) on T-lymphocytes adhesion on activated HUVEC innon-static and VLA-4/VCAM-1 independent system (213). It is, therefore,possible that the increase in L-selectin shedding and CD11b upregulation by HB02 and F6 (Figure 5.8), are due to the promotion of free flowing PMNs duringPMN-PMN interactions that are facilitated by PSGL-1/L-selectin interaction. LowL-selectin expression on pre-activated PMNs resulted in reduced adhesion tothe HUVEC layer through interaction with P-selectin. The results presented inthis chapter suggest that HB02 and F6 as well as 6S and GD could influenceinflammation by weakening the interaction between PMNs and endothelial cellsthrough reducing adhesion and, eventually, migration.

PMN activation occurs when these cells are induced by chemoattractant signalsand PMN/endothelial cell interaction. IL-8 is a known chemokine thatcontributes to PMN activation and migration and IL-8 mRNA is found in freshlyisolated PMNs. However, whether IL-8 is constitutively expressed or is inducedby the isolation procedure is still debated (214). The experiments reported in
this chapter showed that HB02 and F6 had no significant effects on CXCR-1 expression. This finding suggests that HB02 and F6 do not influence chemoattraction of PMN that is mediated by the IL-8/CXCR-1 axis. However, it remains possible that HB02 and F6 may still influence PMN chemoattraction to sites of inflammation through another chemokine receptor, CXCR-2, that also mediates IL-8 chemoattraction (208), which was not examined in this study. Overall, the studies reported in this chapter provide evidence that ZOR contains compounds that modulate the early phases of PMN migration and inflammation.

5.2 6S modulates the activity of CD8+ but not CD4+ T-lymphocytes

The expression of CD69 and CD25 on T-cells indicate their activation and proliferation in response to inflammatory stimuli. Based on this notion, the effect of 6S and GD on the expression of these two membrane proteins in anti-CD3/CD28-induced CD8+ and CD4+ T lymphocyte activation was studied. The experiments revealed that 6S and GD at low concentrations down regulated CD25 and CD69 expression on CD8+ but not on CD4+ T-lymphocytes. This somewhat unexpected observation is difficult to understand and it remains to define mechanisms of action. However, the observation may suggest that both compounds could be beneficial therapeutically, as CD8+ has been shown to over produce IL-17A and IL-22 in psoriatic skin lesions (215).

As cited earlier, CD8+ T lymphocytes complement the action of Th1 and Th17 in response to infections. Further, CD8+ T-lymphocytes produce cytokines such as TNFα and IL-17 and can, thus, contribute to inflammation. Therefore, the effect of 6S and GD on the production of TNFα and IL-17 by activated CD8+ T
lymphocyte was assessed. The results revealed that 6S down-regulated IL-17 production and had a marginal inhibitory effect on the production of TNFα. Unlike 6S, GD, however, did not affect both TNFα and IL-17 production (Figure 5.10). The noted effect of 6S in modulating IL-17 and TNFα production by CD8+ T lymphocyte is consistent with its overall anti-inflammatory effects on reducing iNOS, PGE₂, IL-12 and IL-23 production by activated macrophages. However, further studies will be required to assess the broader effects of 6S on other mediators of inflammation and range of potential side effects.

The studies reported in this chapter are the first to directly examine the effect of ZOR on T-lymphocytes. An earlier study by Tripathi and colleagues showed that 6-gingerol (6G) selectively down regulated pro-inflammatory cytokines but did not affect antigen presenting functions of macrophages such as upregulation of MHC-II and co-stimulatory molecules (216). Unlike 6G, the alcoholic ginger extract (GE) (1 μl/mL) inhibited macrophage activation and its antigen presenting functions which indirectly inhibited T-cell proliferation. GE decreased co-stimulatory interaction between CD28 on T-lymphocytes and CD80-CD86 molecules, MHC II and B7 molecule on activated peritoneal macrophages and thus, indirectly dampened the activation of CD4⁺ T-cell proliferation (217). Synergistic effects of a mixture of compounds in the GE may, thus, contribute to the multi-faceted anti-inflammatory mechanisms.

In comparison to 6G obtained from GE (216), 6S and GD from ZOR did not affect the activation of CD4⁺ T-lymphocytes but, instead, inhibited the activation and cytokine production by CD8⁺ T-cell. However, direct comparison between GE and ZOR is not possible due to variability of the ginger species, composition
of the extracts and test systems used in both studies. Nonetheless, the current study demonstrates that 6S and GD, which are the dehydrated forms of 6G show a tendency to modulate CD8$^+$ whilst 6G influence CD4$^+$ T-cells.

Finally, it would be intriguing to determine how structural differences of gingerol-related compounds contribute to the activation of different subsets of T-cells. Based on data discussed in chapter 2, it is possible to speculate that the potency of these compounds could be related to the substitution patterns of the hydroxyl and carbonyl moieties on the side chains of the compounds (Figure 2.16). Conceivably, this may suggest that compounds which are less saturated as indicated by the presence of double bonds (6S and GD), may possess higher tendency to inhibit the activation of CD8$^+$ T-lymphocytes while 6G which is more saturated influences CD4$^+$ T-lymphocytes. These possibilities, however, need to be directly studied.

6 Conclusions

Experiments described in this chapter have shown that HB02, F6 and 6S, actively modulate PMN migration by modulating the expression and shedding of adhesion molecules. The results showed that treatment of PMNs with HB02, F6 and 6S prior to their activation and interaction with endothelial cells reduced affinity of the adherent PMNs to the endothelial cells and, thus, inhibited PMN migration. This assumption is based on the increase of L-selectin shedding and CD11b expression on the treated-PMNs despite reduction in capture, rolling, adhesion and migration. Furthermore, the data suggests that GD displayed
inhibitory effects during PMN/endothelial interactions due to the fact that GD increased PMN capture and rolling but inhibited PMN adhesion and migration.

In addition, preliminary study on the regulatory effect of 6S and GD on T-cell activation indicates that both compounds may have modulatory effects on CD8⁺ T-lymphocyte activation. Thus, both compounds down-regulated CD25 and CD69 expression on CD8⁺ T-cells at the relatively low concentration of 0.1 μM. The effect of 6S on CD8⁺ T-cells activation is further supported by its ability to reduce TNFα and IL-17 production while GD did not affect the production of both cytokines. The ability of 6S and GD to modulate PMN recruitment as well as CD8⁺ activation may support the presumption that both compounds could be therapeutically beneficial during the early phases of inflammation. Thus far, no similar study has been carried out on the effect of crude ZOR extracts, albeit a number of in vivo and in vitro studies on leukocyte migration and leukocytes/endothelial cell interactions, utilising volatile (205) and non-volatile fractions (172) from common ginger, Z. officinale Roscoe have been reported.
CHAPTER 6

Modulatory effects of ZOR on keratinocytes proliferation and skin inflammation
1 Introduction

Skin epidermal layer is comprised of 95% differentiating keratinocytes and 5% of non-keratinocytes, namely melanocytes, Langerhans and Merkel cells. Skin renewal involves stages of epidermal growth and differentiation events. The earliest stage involves cell proliferation at the epidermal basal layer resulting in the establishment of an extensive network of keratin filaments such as K1 and K14. The proliferating cell starts to differentiate as it moves outwards to the spinous layer whereby it produces keratins such as K1 and K10, protein envelope and membrane-coating granules as part of epidermal building blocks. Once it reaches the granular layer, the cell stops differentiating and undergoes ‘apoptosis-like’ phase, known as terminal differentiation. This forms the cornified layer of the stratum corneum, which is depicted as flattened and anucleated squames which will eventually slough from the epidermal layer but is continuously replaced by new differentiating cells. It is crucial to maintain orderly cycle of keratinocytes proliferation and differentiation since keratinocytes play an essential role as epidermal barrier in host defence. Formation of a strong layer of the stratum corneum provides protection against microorganisms as well as maintaining bodily fluid.

In contrast to healthy skin, psoriatic skin is characterised by accelerated and vicious cycle of skin renewal. It is depicted by keratinocytes hyper-proliferation and abnormal differentiation overlying a skin inflammation reaction. Rapid cell proliferation retards complete keratinisation of the epidermis and, thus, results in rapid and disorganised stacking of corneocytes. Manifestations of psoriatic lesion are facilitated by the crosstalk and instantaneous interactions between
activated immune cells such as APCs, cytokine-secreting T-cells, polymorphs and hyper-proliferating keratinocytes. Activation of these immune cells is stimulated by environmental triggers and genetic defects that lead to secretion of an array of cytokines, chemokines and antimicrobial peptides, keratinocytes differentiation and migration.

Recent findings by Guilloteau and colleagues (2010) have identified key cytokines that are most likely to stimulate formation of psoriatic lesions. Combination of IL-17A, OSM, TNFα, IL-22, and IL-1α (M5) has been shown to work synergistically in an in vitro inflammatory keratinocyte model which resembles features of lesional psoriatic skin (43)(Figure 6.1). This is confirmed by the correlation of the transcriptional profiles of inflammation mediators present in lesional psoriatic skins compared with the in vitro inflammatory keratinocytes model. In comparison to the unstimulated normal human epidermal keratinocytes (NHEK), M5-stimulated NHEK had significantly higher PMN chemotactic activity and antimicrobial peptides expression for skin innate defence (43). This in vitro human model of inflammatory epidermis depicts a synergistic effect of Th1, Th17 and Th22 in inducing psoriatic-like characteristic

It has been widely suggested that Th1 and Th17 T-cell subsets are potent mediators of psoriasis pathogenesis. Th1 secretes TNFα while Th17 produces IL-17, IL-22 and induces PMN-attracting chemokines (CXC), such as CXCL-1, -5 and -8 as well as CCL-20 by keratinocytes. TNFα and IL-17 have been reported to work synergistically in inducing keratinocytes’ to produce IL-8 (218). Furthermore, stimulation by both cytokines also induces many inflammatory
products, additively or synergistically, compared with single cytokines in keratinocytes (218). Apart from that, IL-1α which plays a major role in IL-1 activity in innate immune response, by activating T-cell, macrophages and leukocytes function, is predominant in psoriatic keratinocytes (219). This creates cytokines milieu that stimulates continuous inflammatory response by the keratinocytes.

Recently, Th22, another subset of T-cells has been described and suggested to act in parallel with Th1 and Th17 cells in psoriasis (32). Th22 exclusively produced IL-22, which is structurally related to IL-10 as well as IL-19, IL-20, IL-24, and IL-26. However, unlike IL-10, IL-22 demonstrates pro-inflammatory properties. There is also evidence that IL-22 induces activation of STAT3 in keratinocytes and, thus, promotes the generation of cytokines involved in skin inflammation (220). Moreover, IL-22 induced the release of IL-20, a downstream cytokine produced by the keratinocyte. A large body of evidence indicates that IL-22 and IL-20 play key roles in keratinocytes alterations observed in psoriasis (221, 222). IL-22 and IL-20 drive keratinocyte hyperplasia by attenuating terminal keratinocyte differentiation and down-regulate the production of proteins associated with keratinocytes differentiation, including filaggrin, loricrin, involucrin, calmodulin-like skin protein (CLSP) and calmodulin-related protein NB-1 (220, 223) (Figure 6.1). Furthermore, there is evidence that IL-20 receptors, IL-20R1 and IL-20R2 protein, are up-regulated in epidermis of psoriatic skins compared with healthy skins (224). The upregulation of these receptors leads to higher binding of IL-20 to keratinocytes.
In addition to these properties associated with IL-22, this cytokine along with Oncostatin M (OSM) up-regulates anti-microbial peptides and other proinflammatory molecules belonging to the S100 family of calcium-binding proteins (42). These include S100A7, S100A8 and S100A9. OSM is a cytokine related to the IL-6 family and is mainly produced by activated T-cells, monocytes and dendritic cells. These antimicrobial peptides have been shown to be extensively expressed in psoriatic keratinocytes but produced in negligible amounts in the epidermis of healthy individuals (42). The microbial peptides can act as chemokines or induce chemokines stimulating chemotaxis of PMNs, monocytes as well as CCR6-expressing Th17 cells that facilitate positive feedback loop that exacerbate the inflammation. Therefore, IL-22, IL-17 and OSM share similar or overlapping roles in stimulating keratinocytes hyper proliferation and inflammatory reactions associated with psoriasis.

Based on these observations most of the above cytokines (IL-17A, OSM, TNFα, IL-22, and IL-1α) appear to be inter-related and in combination they provide the closest framework of inflammation-mediating molecules in psoriatic skin. Hence, these cytokines could be highly useful targets for drug discovery for the treatment of skin inflammation.
Figure 6.1: Synergistic effects of IL-1α, IL-17A, TNFα, IL-22 and Oncostatin M (OSM) in inducing inflammatory cascades in keratinocytes. IL-1α, IL-17A and TNFα are involved in the induction of innate immune responses whilst IL-22 and OSM induce epidermal differentiation. Over expression of these cytokines lead to the up-regulation of chemokines (CXCL1, CXCL8, CCL20) and antimicrobial peptides (β-defensin, psoriasin, LL37) by keratinocytes. These stimulate chemotactic activities, such as the recruitment of PMNs as well as CCR6-expressing Th17 cells that facilitate positive feedback loops. Modified from Guilloteau et al. (43).
2 Objectives

This chapter explores the effect of HB02, F6 and compounds 6S and GD on keratinocyte responses in vitro. The studies are aimed at gaining insights into the effect of ZOR on keratinocyte proliferation and differentiation, important components of the pathophysiology of psoriasis. In the experiments detailed in the chapter I explore an in vitro model of inflammatory epidermis as first reported studies by Guilloteau and colleagues (2010). The experiments examine the effect of HB02, fraction 6 (F6) and compounds 6S and GD on cytokine activated-normal human epidermal keratinocytes (NHEK). Apart from observing morphological changes that affect keratinocytes during an inflammatory response modelled to reflect psoriasis inflammation, cell proliferation and the production of IL-20 and IL-8 cytokines were studied. These important cytokines released by activated keratinocytes are known to be involved in promoting cell proliferation and inflammation, respectively. The overall objectives of the studies described are to assess the effect of ZOR on the components of the inflammatory response mediated by keratinocyte.
3 Methods

3.1 Keratinocytes

Two sources of keratinocytes were used in the experiments described here. The cells were immortalised human cutaneous keratinocyte, Human Adult low Calcium Temperature (HaCaT) cell line and normal human epidermal keratinocytes (NHEK) from foreskin. NHEK was obtained from three individuals (A-page, SFK1 and SFK2) and cultured from passage 3. Irradiated mouse fibroblast-cell line (3T3) was used as feeder cells for culturing the NHEK cells. Both the NHEK and 3T3 cells were obtained courtesy of Professors M. Philpott and H. Navasaria at the Centre for Cutaneous Research, Blizard Institute, Queen Mary University of London. HaCaT was used for assessing the effect of selected ZOR constituents on proliferation whilst NHEK was used to establish a psoriatic skin inflammatory model.

3.1.1 Culture conditions of HaCaT keratinocytes cell line

The HaCaT-cell line used in this study was cultured and maintained in DMEM medium supplemented with 10% FBS (GIBCO), 100U/mL penicillin and 100 µg/mL streptomycin and 2 mM L-glutamine. The cells were incubated at 37°C with 5% CO₂. The cells were sub-cultured when reaching 90% confluence by dilution of 1:4 in medium. Confluency of the cell line was calculated to take a week-duration, thus, medium was changed once every 3 days in order to maintain consistent growth of the cells. The cells were detached from the flask surface using 5 mL of trypsin-EDTA with tapping of the flask. The number of
cells was counted and viability was assessed using trypan blue and an inverted microscope (Nikon TMS).

3.1.2 Culture conditions for NHEK

NHEK cells were obtained from 3 healthy individuals (coded as: A-page, SFK1 and SFK2) and each at their third passages. The cells were cultured onto irradiated 3T3 cells in cell culture medium (DMEM F12) containing RM++ supplement (obtained courtesy of Prof. Philpot’s group), 2 mM L-glutamine, 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. Briefly, 1 x 10^6 cells/mL of irradiated 3T3 cells were seeded in T75 flasks and incubated for 1 hour in 5% CO₂ at 37ºC for the cells to attach to the flasks. Once the 3T3 cells were attached, 2 x 10^6 cells/mL of NHEK cells were seeded onto the 3T3 cell layer. The cells were incubated in a humidified incubator with 5% CO₂ at 37 ºC for 1-2 days for NHEK-3T3 cell firm attachment. After two days, the medium was changed and the cells were incubated until 70-80% confluence. Prior to splitting the NHEK cells, the feeder cell (irradiated 3T3 cells) were first detached by washing with PBS and then with 5 mL Versene (EDTA) 0.02%. Next, NHEK cells were trypsinised using Trypsin-EDTA and sub-cultured for the next passage to be used in assays for determining the effect of ZOR samples on proliferation and overall role in inflammation in the test model used.
3.2 Preparation of HB02, fractions, 6S and GD compounds for assessing their effects on keratinocytes.

Stock concentration of HB02 fractions (F5, 6, 7 and 10) and compounds were prepared in DMSO at a concentration 10 mg/mL. The samples were then diluted in medium (DMEM) to a concentration range of 10-1000 μg/mL for the HaCaT proliferation study. For NHEK cell proliferation study, HB02 extract, Fraction 6 and compounds 6S and GD were diluted to range of 5-100 μg/mL. Cyclosporin A (CsA)(Neoral®) was dissolved in DMSO and diluted in medium to a range of concentration of 2-20000 ng/mL. For the study using psoriatic-like human model, HB02, F6, 6S and GD were diluted at the concentration of 5, 20 and 50 μg/mL and CsA at a concentration of 20 ng/mL.

3.3 Proliferation assay

The anti-proliferative effects of extract, fractions and compounds from ZOR on HaCaT and NHEK cells were studied using AlamarBlue and MTT assays to quantitatively measure proliferation.

3.3.1 AlamarBlue Assay

AlamarBlue assay measures cell proliferation by detecting the REDOX reaction in viable cells after 24, 48 and 72 hours. Change of colour of culture medium containing the AlamarBlue stain from blue to reddish purple is a measure of cell proliferation. Briefly, $1 \times 10^4$ cells/mL were cultured in 12 well plates and
incubated for 24 hours at 37°C in 5% CO₂. HB02, fractions were tested at a selected range of concentration on cells and incubated for 24, 48 and 72 hours. At each time point (24, 48 and 72 hours), the medium was replaced with 10% AlamarBlue (AbD Serotec) in DMEM and incubated for 4 hours. The colour change was measured at 560 nm and 595 nm at each time point using a multi-well plate reader (TECAN GENios). Cell proliferation was calculated according to the manufacturer's manual as below;

Cell viability:

\[ \text{*PR (sample)} \times 100 \]
\[ \text{*PR (medium control)} \]

PR value for untreated cells (control) or cells treated with ZOR samples was calculated as below;

\[ \begin{align*}
\text{*Percentage reduction (PR) of Alamar Blue:} \\
(E2 \times A1)-(E1 \times A2) \times 100 \\
(E3 \times N2)-(E4 \times N1)
\end{align*} \]

E1: Molar extinction coefficient (E) of oxidized Alamar Blue at 560 nm = 80586
E2: Molar extinction coefficient of oxidized Alamarblue at 595 nm = 117216
E3: Molar extinction coefficient of reduced Alamarblue at 560 nm = 155677
E4: Molar extinction coefficient of reduced Alamarblue at 595 nm = 14652
A1 = absorbance of test wells at 560 nm
A2 = absorbance of test wells at 595 nm
N1 = absorbance of negative control (Alamar Blue in media without T-cells) at 560 nm
N2 = absorbance of negative control at 595 nm
3.3.2 MTT Assay

For the tetrazolium-dye (MTT) assay, confluent monolayers of primary cells were detached with trypsin and plated out at $2 \times 10^4$ cells/well in 96-well flat-bottom plates. After a 24 hour pre-incubation period, dilutions of test samples were added to appropriate wells and plates incubated for 48hr at 37°C in a humidified incubator with 5% CO$_2$. Untreated cells were used as control. After 48 hour incubation, the medium was removed and wells washed with phosphate buffered saline (PBS). A 100 μl MTT solution (0.5 mg/mL) (Sigma) in PBS was added to each well and the plate further incubated for 3 hour at 37°C. MTT was then removed and 100 μl DMSO added to dissolve MTT crystals. The plate was shaken for 30 minutes and absorbance measured at 540nm on a multi-well plate reader. The percentage of proliferating cells was calculated as the ratio of the absorbance readings for the treated cells to that of untreated wells.

3.4 In vitro model of inflammatory epidermis.

NHEK cells were cultured to 80% of confluence in DMEM F12 medium supplemented with RM++ growth factor cocktail as described in section 3.1.2. DMEM F12 supplemented with RM++ was used only for cell maintenance. For this study, complete Epilife medium with HKGS containing final concentration of 60 μM calcium chloride, 0.2% (v/v) bovine pituitary extract (BPE), 5 μg/mL bovine insulin, 0.18 μg/mL hydrocortisone, 5 μg/mL bovine transferring and 0.2 ng/mL human epidermal growth factor, was used. This medium contains specific and necessary growth factors for undefined NHEK cell. $1 \times 10^5$ cells/well NHEK cells were seeded into 24 well plates in Epilife medium.
supplemented with Human Keratinocyte Growth Supplement (HKGS) and cultured for 24 hours. The cells were then starved for another 24 hours in Epilife medium without HKGS. The purpose of cell starvation is to retard the progression of cell proliferation and production of growth factors by the cells. After the 24-hour-starvation, the cells were stimulated with, or without, cytokine cocktail containing recombinant IL-17A, OSM, TNFα, IL-22, and IL-1α at 10 ng/mL each and incubated for 6 hours. It is anticipated that after 6 hours incubation, gene transcription necessary for secretion of the related cytokines has taken place (225). After the 6-hour stimulation period, the cells were treated either with F6, 6S or with GD and incubated for another 48 hours and supernatants tested for the level of IL-8 and IL-20. The level of IL-8 and IL-20 was determined using human IL-8 Tissue Culture kit (Mesoscale) and IL-20 ELISA kit (R & D Systems) respectively.

3.4.1 Measurement of IL-8 level.

The level of IL-8 in culture supernatants was determined using an MSD® 96-Well MULTI-SPOT kit. The protocol was carried out according to the manufacturer’s (MSD®) instructions. The principle of the assay is a sandwich immunoassay where a capture antibody is pre-coated onto a single spot in each well of a MULTI-SPOT plate. The level of IL-8 in the test supernatant is then quantified using a cytokine-specific detection antibody conjugated with MSD SULFO-TAG™ reagent. IL-8 standard was diluted in DMEM (medium used for the samples) from a stock concentration of 1 μg/mL. The standard was diluted to give a range of concentrations of 10,000, 2,500, 625, 256, 39, 9.8 and 2.4 pg/mL. Test supernatants from the non-treated and treated NHEK cells
were tested in undiluted forms. Briefly, 25 μl of supernatant (or recombinant human IL-8 standards) were incubated for 2 hours on a pre-designed human IL-8 96-well microtiter plate bearing an array of capture antibody with specificity to IL-8. After washing unbound supernatant, the detection antibody (at 1 μg/mL) conjugated to the ruthenium-based MSD Sulfo-TAG™ reagent was added. The plate was then washed with PBST. The MSD Read Buffer T (2x) was added and results read on a Sector Imager (MSD). IL-8 concentrations were determined with Softmax Pro Version 4.6 software, using a curve fit model (log-log or four-parameter log-logit) as suggested by the manufacturer.

3.4.2 Measurement of IL-20 level.

The level of IL-20 in culture supernatants was measured using Quantikine® ELISA assay kit (R&D Biosystems). The assay was carried out according to the manufacturer's instructions. The assay is, again, based on a sandwich enzyme immunoassay principle whereby plates pre-coated with monoclonal antibody specific for IL-20 is used to bind IL-20 present in test samples and bound IL-20 revealed with a conjugated detection antibody. IL-20 standards were prepared by diluting a stock solution of 40 ng/mL in Calibrator Diluent RDSP into 4000 pg/mL which serves as the high standard concentration. This standard was further diluted 1:5 serial dilution in Calibrator Diluent RDSP until the concentration of 62.5 pg/mL. The diluents served as zero concentration (0 pg/mL). Samples or supernatants collected from treated NHEK cells were tested without dilution. One hundred μl of diluent RD1W were added to each well of the 96-well assay plate followed by 100 μl of the standards, control or test samples and plate incubated for 2 hours at room temperature (RT). The
mixture was then aspirated and washed 4 times with ~400μl wash buffer using a squirt bottle. After the last wash, the plate was blotted dry on clean paper towel. Two hundred μl of enzyme-linked polyclonal antibody specific for IL-20 were added to each well and incubated for a further 2 hours at RT. The plate was washed again for 4 times as before to remove any unbound conjugate. Two hundred μl of substrate solution was added and plate was incubated for 30 minutes at RT in the dark. Finally, 50 μl of stop solution was added and colour change from blue to yellow was measured. The optical density of each well was determined at 450 nm and corrected at 560 nm using multi-well plate reader (TECAN). The data was plotted and analysed using Transform Linear regression of GraphPad Prism to determine the concentration of IL-20 in the supernatants.

3.5 Statistical analysis

Data analysed using GraphPad Prism software (GraphPad Prism, San Diego, California). Means and standard deviation were used to describe normally distributed data. The significance of differences between groups was assessed using One-way ANOVA (Tukey’s test). Difference of p<0.05 is considered significant.
4 Results

4.1 The effect of ZOR test samples on HaCaT cell proliferation.

During the early stages of the experiments, the anti-proliferative effects of F5, F6, F7 and F10 on HaCaT-cells at concentrations of 10-1000 μg/mL was assessed. The anti-proliferative effect of the fractions on HaCaT was assessed after incubation for 24, 48 and 72hr. The fractions were evaluated and ranked based on their potency in inhibiting HaCaT proliferation.

The results showed that F5, F6 and F7 reduced proliferation in a time- and dose-dependent manner (Figures 6.2a-c). For instance, at 50 μg/mL, F6 inhibited HaCaT proliferation by 23.4 ± 11.2% after 24 hours, 27.1 ± 10.6% after 48 hours and by 50.2 ± 2.5% after 72 hours. All three fractions showed comparable inhibitory activity at the concentrations of more than 50 μg/mL. However, F10 did not have a significant inhibitory effect on proliferation at the lower concentrations of 10 and 50 μg/mL. Because the fractions demonstrated similar anti-proliferative effects at 50 μg/mL, the comparative activity and potency of the fractions could be best distinguished at the lower concentrations of 10 μg/mL. Figure 6.2 shows that after all incubation periods and at 10 μg/mL, F6 was the strongest inhibitor of HaCaT proliferation. F6 at a concentration of 10 μg/mL inhibited HaCaT proliferation by 20.7 ± 12.0% after 24 hours, 21.2 ± 8.7% after 48 hours and 32.2 ± 8.8% after 72 hours. Although, at higher than 10 μg/mL concentrations F5 and F7 had similar inhibitory effect to F6, the latter remained the most consistent and, apparently, potent inhibitor of HaCaT proliferation. F10, in contrast, had the lowest anti-proliferative effects inhibitory.
Figure 6.2: The anti-proliferative effects of HB02 fractions on HaCaT cells. The HB02 fractions F5, F6, F7 and F10 were tested for their efficiency on inhibiting HaCaT-cell proliferation at the indicated concentrations (X-axis) after incubating the cells with the fractions for: a) 24hr; b) 48hr; and c) 72hr. Treated and non-treated cells were stained with AlamarBlue solution after 24, 48 and 72hr, and incubated for 4hr at 37ºC. Absorbances were measured at 560nm and 595nm (reference) using multiwell plate reader (TECAN) and proliferation calculated according to the formula described in section 3.1.2 of the methods. Data presented in mean ± SD of % cell proliferation.
4.2 The effect of HB02, F6, 6S and GD on NHEK cell proliferation

The anti-proliferation study using HaCaT-cells showed that F6 was the most potent fraction at inhibiting keratinocyte cell line proliferation. To further verify the anti-proliferative effect of F6 and also assess which of its two major components 6S and GD contributed mostly to anti-proliferation their effects on normal human epidermal keratinocytes (NHEK) at a narrow concentration range of 5-100 μg/mL and after incubation for 48 hours were tested. The inhibitory effects of HB02, F6, 6S and GD were tested in comparison with Ciclosporin A (CsA) at a concentration range of 2-20000 ng/mL. CsA was chosen as a positive control based on its potent inhibitory effect on keratinocytes proliferation and chemotactic activity as reported (226, 227) and for its use in treating psoriasis (27, 28, 71, 228).

Figure 6.3 showed the effect of HB02, F6, 6S and GD on NHEK cell proliferation after 48 hours incubation. Figure 6.3a showed that at the lowest concentration of 5 μg/mL, HB02, F6 and GD in fact enhanced cell proliferation. GD, in particular, increased cell proliferation by approximately 62%, followed by HB02 by 35% and F6 by 8%. 6S, on the other hand, reduced proliferation by 14% relative to untreated cells. However, at higher concentrations, 15 μg/mL and onwards, F6 and 6S dramatically inhibited NHEK proliferation by more than 80%. In comparison to F6, 6S showed a robust anti-proliferative effect by 94% at 15 μg/mL and by 100% at 50 μg/mL. In comparison, F6 inhibited proliferation by 70% (Figure 6.3a) at 15 μg/mL and 88% at 50 μg/mL. HB02 and particularly, GD were less effective in inhibiting NHEK proliferation.
Overall the results showed that 6S, F6, HB02 and GD at concentrations of 7.7±3.6 μg/mL, 14.6±7.3 μg/mL, 32.8±16.3 μg/mL and 42.2±14.7 μg/mL, respectively, inhibited 50% of NHEK proliferation. 6S was the most efficacious anti-proliferative or with highest cytotoxicity effect, followed by F6, HB02 and GD. It was observed that CsA was unable to inhibit more than 50% proliferation at concentrations lower than 20000 ng/mL (20 μg/mL) (Figure 6.3 b).
Figure 6.3: The anti-proliferative effects of HB02, F6, 6S and GD on NHEK cells. NHEK cells were obtained from foreskin of three healthy individual (A-page, SFK1 and SFK2). The figures show cell proliferation after 48 hours incubation with: a) HB02, F6, 6S and GD at a range of concentrations 5-100 μg/mL; b) Ciclosporin A (CsA) at 2-2000 ng/mL. NHEK cell proliferation in the presence of test samples is presented in comparison with the proliferation in the absence of the samples as indicated by the dotted line and considered as 100%. After 48 hours, proliferation of treated and non-treated cells was determined using MTT assay as described in method 3.3.2. Percentage proliferation was calculated as the ratio of proliferation obtained for the treated cells to untreated cells. Data was presented as the mean ± SD based on three independent experiments (n=3).
4.3 The effect of ZOR on inflammatory epidermis using an *in vitro* model.

The previous sections have assessed the effect of ZOR extract, fractions and isolated compounds on various general inflammatory pathways of psoriatic pathology. Within the context of the effect of ZOR on psoriasis it was important to study the direct effects of the ZOR derivatives on a model as directly relevant to psoriasis as possible. For this aim to be achieved, an *in vitro* model of inflammatory epidermis modified from a protocol by Guilloteau and colleagues (43) was established. The protocol is based on using NHEK cells and stimulated with a cocktail of pro-inflammatory cytokines. NHEK were obtained from the foreskin of three healthy individuals (A-page, SFK1 and SFK2) and stimulated with a combination of IL-17A, OSM, TNFα, IL-22, and IL-1α at 10 ng/mL. Once established, the stimulated NHEK cell cultures were treated with HB02, F6, 6S and GD at concentrations of 5, 20 and 50 μg/mL and the effects of the test compounds on morphology of the NHEK cells and cytokine production were examined. The test concentrations of HB02, F6, 6S and GD were chosen based on the effective concentration in inhibiting NHEK cell proliferation from the previous sections. CsA was tested at the concentration of 20 ng/mL.
4.3.1 Changes of the NHEK cell phenotype.

Studies of NHEK phenotype changes and of proliferation were based on the appearance of NHEK cells under the microscope. Stimulated cells are presented as polygonal-shaped cells with distinctive nuclei (Figures 6.4a-d). Figures 6.5 and 6.6 depict SFK1 cells which were stimulated with the inflammatory cocktail of cytokines (CT) and then treated with HB02, F6, 6S and GD at 20 and 50 μg/mL, respectively. Figure 6.7 depicts A-page cells which were CT-stimulated and treated with HB02, F6, 6S and GD at 5 μg/mL.
(a) Control

(b) Stimulated with cytokines cocktail (CT)

(c) CT + CsA (20 ng/mL)

**Figure 6.4: The morphology of NHEK cells following stimulation.** NHEK cells were obtained from individual SFK. The cells microscopic pictures depict the cells were either: (a) unstimulated and untreated (Control); (b) stimulated with CT (containing 10 ng/mL of each IL-17A, OSM, TNFα, IL-22, and IL-1α); and (c) CT-stimulated and treated with Ciclosporin A (CsA) at 20 ng/mL (positive control). The shown morphology is representative of three independent experiments. Prior to treatment, NHEK cells were seeded in 24 well plates in Epilife medium with HKGS for 24 hours then starved for another 24 hours in Epilife without HKGS. After starvation, the cells were stimulated with CT and incubated for 6hr before treatment with HB02, F6, 6S, GD or CsA. Changes in cell morphology were monitored at 40x magnification under microscope and captured using JVC TK-C1360B digital colour video camera.
The results showed that the NHEK cells from individual SFK1 which were stimulated with CT and then treated with HB02, F6, 6S and GD at 50 μg/mL shrank and became less confluent (Figure 6.5) compared with control (compare Figure 6.4a with 6.4b). The phenotype changes were similar to the appearance of cells undergoing apoptosis. Visually, apoptotic bodies are characterised by a condensed and ‘invaginated’ cell membrane. The number of cells in was reduced by treatment with HB02, F6, 6S and GD at 50 μg/mL compared with untreated cells, cells stimulated with the CT, and CT-stimulated and treated with CsA (Figure 6.4c). This may correlate to the strong anti-proliferative or cytotoxicity effect of these samples, particularly F6 and 6S at this concentration on unstimulated NHEK (Figure 6.3a).

Even at the concentration of 20 μg/mL, similar phenotype changes were observed and reduction in cell proliferation was also observed after treatment with HB02, F6 and 6S (Figure 6.6) and to a lesser extent with GD. F6 and 6S had more potent effects compared with HB02 and GD. Treatment with F6 and 6S at 20 and 50 μg/mL resulted in a dramatic reduction of NHEK proliferation. GD, however, did not appear to affect cell morphology at 20 μg/mL. The morphology and cell density were comparable to cells which were only stimulated with CT (Figure 6.4b). These results, therefore, confirm that GD was less affective in regulating keratinocytes proliferation compared with HB02, F6 and 6S at this concentration. This corresponds to the least anti-proliferative or cytotoxicity effect of GD on unstimulated NHEK (Figure 6.3)
Figure 6.5: Phenotype changes in NHEK cells treated with selected ZOR samples (HB02, F6, 6S and GD) at 50 μg/mL. Changes in NHEK cell phenotype were assessed microscopically. The cells were stimulated with the cytokines cocktail (CT) and treated with either: (a) HB02; (b) F6; (c) 6S; or (d) GD at 50 μg/mL for 48 hours. Significant phenotypic changes are noted in the cells indicating that the Selected ZOR compositions inhibited proliferation at 50 μg/mL and may also result in apoptosis. Cell morphology was assessed at 40x magnification and captured using JVC TK-C1360B digital colour video camera.
(a) CT + HB02 (20 μg/mL)  (b) CT+ F6 (20 μg/mL)

(c) CT + 6S (20 μg/mL)  (d) CT + GD (20 μg/mL)

Figure 6.6: Phenotype modulation of NHEK cells treated with selected ZOR samples (HB02, F6, 6S and GD) at 20 μg/mL. Changes in the NHEK cells were assessed as described in the legend to Figure 6.5 after stimulation with the CT and treatment with (a) HB02 (b) F6 (c) 6S and (d) GD at 20 μg/mL for 48 hours. The figure indicates that F6 and 6S and, to a lesser extent, HB02, reduced cell proliferation whereas GD did not neither did the latter affect morphology of the cells.
Figure 6.7 shows the effect of HB02, F6, 6S and GD at the lower concentration of 5 μg/mL on the NHEK cells (from donor A-page). The figure shows no significant changes in the morphology of the cells in the treated cells compared with untreated cells. Although, it was noted that the cells were less ‘polygonal’ than the untreated cells. (Figure 6.4b). These results indicate that treatment of the NHEK cells with the ZOR test samples at 5 μg/mL is unlikely to influence proliferation of the keratinocytes significantly. However, it is necessary to point out that this presumption is based on one dimensional analysis and hence may not be unequivocal evidence for lack of effect on cell differentiation at this concentration.

To further explore the potential of ZOR to influence keratinocyte biology and contribution to inflammation, the effect of HB02, F6, 6S and GD on the production of IL-20 by the activated NHEK was studied. IL-20 is known to have a role in inducing keratinocyte hyperproliferation and contribute to inflammation in psoriasis.
Figure 6.7: Lack of phenotype changes in NHEK cells treated with selected ZOR samples, HB02, F6, 6S and GD, at 5 μg/mL. Treatment of CT-stimulated NHEK cells with (a) HB02; (b) F6; (c) 6S and (d) GD at 5 μg/mL for 48 hours had no noticeable effects on the morphology of the cells.
4.3.2 The effect of ZOR on the production of IL-8 and IL-20 by activated keratinocytes.

IL-8 and IL-20 are important cytokines involved in inflammation and keratinocyte hyperproliferation associated with psoriasis (221, 229). Subsequent to exploring the effect of the most relevant ZOR constituent/s on NHEK morphology and proliferation, the effect of HB02, F6, 6S and GD on the production of IL-8 and IL-20 was examined. The production of both cytokines was determined in the supernatant of cells stimulated with CT and treated with HB02, F6, 6S and GD at the concentration of 5 and 20 μg/mL.

4.3.3 The effect of HB02, F6, 6S and GD on IL-20 production by keratinocytes.

IL-20 has been shown to be involved in keratinocyte hyperproliferation and the alteration of terminal differentiation process in psoriatic skin (221, 229). By assessing the effect of selected ZOR composition (HB02, F6, 6S and GD) on IL-20 production, it would be possible to confirm their potential therapeutic effects on keratinocytes proliferation and differentiation.

The experiments showed that the test samples at the lowest concentration of 5 μg/mL weakly inhibited IL-20 production (<4% inhibition) (Figure 6.8). This observation is consistent with the lack of effect of the ZOR samples on the morphology of the activated NHEK cells (Figure 6.7). However, when HB02, F6, 6S and GD were tested at 20μg/mL they significantly down-regulated IL-20 production (90.8±6.0%, 99.9±0.3%, 99.4±0.5% and 88.9±7.0%, respectively).
The inhibitory effects were significantly higher than seen with CsA at 20 ng/mL (p<0.01).

These data are, again, consistent with the potent anti-proliferative effects of HB02, F6 and 6S on the NHEK cells (Figure 6.3a and Figure 6.6). However, despite this concordance in the effect of the test compounds on IL-20 production and on proliferation and morphology, it remains to be established whether these effects are connected at the molecular level. This would require further detailed investigation which was beyond these studies, due to time limitations.
Figure 6.8: The effect of HB02, F6, 6S and GD on IL-20 production by activated keratinocytes. The samples were assessed for their inhibitory effects on IL-20 production by NHEK cells stimulated with the cytokine cocktail (CT) containing IL-17A, OSM, TNFα, IL-22, and IL-1α, each at 10 ng/mL. HB02, F6, 6S and GD were tested at two concentrations 5 and 20 μg/mL and the results analysed using One-way ANOVA (for comparison of the effect of the samples to CsA (20 ng/mL). HB02, F6, 6S and GD all significantly inhibited IL-20 production when tested at of 20 μg/mL compared with Ciclosporin A (CsA) at 20 ng/mL (p<0.001). Values are presented as the mean ± SD on three independent experiments.

4.3.4 The effect of HB02, F6, 6S and GD on IL-8 production by keratinocytes. 6S down-regulates IL-8 secretion.

IL-8 is a key chemoattractant cytokines in inflammatory responses and is known to be involved in psoriasis. In order to assess the therapeutic efficacy of ZOR in treating psoriasis, the effect of HB02, F6, 6S and GD on IL-8 by the activated NHEK cells, was studied. As in studying the effect of the test compounds on
IL-20 production, their effects on IL-8 production at both 5 and 20 μg/mL, were tested.

Figure 6.9 shows that relative to the control, all test samples increased rather than decreased IL-8 production at 5 μg/mL of concentration. F6 and 6S in particular significantly enhanced IL-8 production by the activated NHEK cells. This increase, however, was inconsistent as depicted by high SEM values.

At the higher concentration of 20 μg/mL, HB02, F6 and GD increased IL-8 expression. However, 6S reduced IL-8 production by 68.0 ± 17.0% relative to the control. This inhibition was statistically significant when compared with CsA at 20 ng/mL (p<0.05).
Figure 6.9: The effect of HB02, F6, 6S and GD on IL-8 production by activated keratinocytes. The effect of HB02, F6, 6S and GD on IL-8 production by NHEK was studied by ELISA. The NHEK cells were stimulated with the cytokine cocktail (CT) then cultured either with or without HB02, F6, 6S, GD or CsA at the indicated concentrations. One-way ANOVA (Tukey’s test) analysis was used to compare the effect of the test samples relative to that obtained with the control (CT). No significant differences were observed for the samples at 5 and 20 μg/mL relative to control (CT) or CsA (20 ng/mL) except for 6S at the concentration of 20 μg/mL (p<0.01). Values are presented as the mean ± SE on three independent experiments.
Table 6.1: Summary of the effects of HB02, F6, 6S and GD on NHEK proliferation and cytokine production.

<table>
<thead>
<tr>
<th>Samples</th>
<th>% change in proliferation of unstimulated NHEK cells</th>
<th>Phenotypic changes in stimulated NHEK</th>
<th>% change in IL-20 production</th>
<th>% change in IL-8 production</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB02</td>
<td>16.7±32.5 (-)</td>
<td>Yes</td>
<td>90.8±6.0 (-)</td>
<td>61.7±101.7 (+)</td>
</tr>
<tr>
<td>F6</td>
<td>98.3±8.2 (-)</td>
<td>Yes</td>
<td>99.9±0.3 (-)</td>
<td>46.6±112.0 (+)</td>
</tr>
<tr>
<td>6S</td>
<td>103.5±5.5 (-)</td>
<td>Yes</td>
<td>99.4±0.5 (-)</td>
<td>67.6±17.0 (-)</td>
</tr>
<tr>
<td>GD</td>
<td>23.9±31.7 (-)</td>
<td>No</td>
<td>88.9±7.0 (-)</td>
<td>26.7±62.6 (+)</td>
</tr>
</tbody>
</table>

(-) indicates inhibition/reduction while (+) indicates increase/enhancement.

Table 6.1 summarises the overall effects of HB02, F6, 6S and GD at 20 μg/mL on NHEK cells that have been reported in this chapter. The anti-proliferative effects were tested using unstimulated NHEK cells while the effect on morphology and IL-8 and IL-20 production was assessed on CT-stimulated NHEK cells. All samples at this concentration reduced proliferation of un-stimulated NHEK cells and reduced IL-20 production by CT-stimulated NHEK. It is interesting to note that only 6S reduced IL-20 and IL-8 production in NHEK and also reduced cell proliferation. Furthermore, 6S had a superior effect on IL-8 and IL-20 production compared with CsA. However, given that CsA was tested at a concentration of 1000 times lower than 6S (Figure 6.8 and 6.9), it could, if tested at 20 μg/mL, promote equal or better effect than 6S. This hypothesis, however, need further studies. In the aspect of cytotoxicity, it is worth-noting that HB02 and GD which were less toxic toward normal unstimulated cell, showed potent inhibitory effect on IL-2 production by CT-stimulated cells. Hence, this issue needs further studies.
5 Discussion

5.1 HB02, F6 and 6S induce apoptosis-like morphology in keratinocytes activated with a cocktail of pro-inflammatory cytokines.

The experiments described in this chapter show that HB02, F6, and 6S when used at high concentrations (≥20μg/mL) inhibited NHEK cell proliferation (Figure 6.3), and caused NHEK morphological changes similar to cells undergoing apoptosis (Figure 6.6) and reduced IL-20 production in activated NHEK (Figure 6.8). 6S, in addition to causing all these changes in activated NHEK cells, also reduced IL-8 production (Figure 6.9).

Changes in the morphology of stimulated NHEK cells after treatment with 20 and 50 μg/mL of HB02, F6, 6S and GD suggest that cells may be undergoing apoptosis with structures resembling apoptotic bodies in the treated cells (Figure 6.5 and 6.6). Thus, there is the appearance of condensed cytoplasms, dismantled cytoskeleton, invaginated cell membranes and rounding-up cells, which are some of the characteristics of apoptotic cells (230, 231). HB02 induced less apoptotic morphology than F6 and 6S. Cells treated with GD, however, did not display apoptotic features at the lower concentration of 20 μg/mL, but rather the cells resembled healthy, elongated and proliferating cells.

Generally, apoptosis and terminal differentiation in the skin cells are defined as cell death, however, based on cell nomenclature, both can be distinguished by different molecular and metabolic pathway (230). Apoptosis is an accelerated
process of cell death that begins with induced or involuntary disruption of cell proliferation at the basal layer that caused cessation of organelle functions. Consequently, this leads to organelles’ degradation, by which cell membranes are invaginated, and the cytoskeleton dismantled, attracting macrophage to phagocytose the cells. Terminal differentiation of the cells, on the other hand, is a constitutive process of skin renewal. Cell proliferation begins at the basal layer, followed by stages of differentiation in the suprabasal and granular layer. Finally, this resorts to terminal differentiation, or cornification that forms the corneocytes of stratum corneum. Unlike apoptotic cells, the cornecytes, or dead skin cells are sloughed instead of being phagocytosed (230).

Based on visual observation of the cells, as sampled in Figure 6.6, it is possible to propose that HB02, F6 and 6S induce apoptosis in stimulated NHEK cells. This appears to be indirectly supported by the potent anti-proliferative effect of these 3 test samples on unstimulated NHEK. However, this was not demonstrated for GD. Nevertheless, the fact that GD strongly reduced IL-20 production at 20 μg/mL is intriguing. Of further interest is that GD demonstrated anti-proliferative effects on unstimulated NHEK but seemed not to affect the survival and differentiation of stimulated cells. These observations may suggest that GD could help maintain skin homeostasis if used therapeutically. In contrast, the robust effects of HB02, F6 and 6S had on NHEK cell proliferation, may be beneficial for therapeutic effects on hyperproliferating cells in psoriatic lesions. In order to determine whether any of the test samples induce apoptosis or cause cornification of keratinocytes it would be necessary to study the expression of proliferation- and differentiation-associated proteins as well as
related metabolic changes. Such experiments were not possible due to time constraints.

5.2 6S modulates IL-20 and IL-8 production in CT activated keratinocytes.

Experiments carried out to assess the effect of the ZOR derivatives tested in this chapter revealed that HB02, F6, 6S and GD had paradoxical effects on the production of two key cytokines. Thus, whereas HB02, F6, 6S and GD all inhibited IL-20 production to varying degrees, only 6S had suppressive effects on IL-8 production. For these experiments, CsA was used as a positive control based on the fact that CsA has been shown to influence the expression of genes associated with inflammation. Indeed, CsA has been used in the treatment of psoriatic patients and it has been shown to influence signalling pathways mediated by a number of cytokines including IL-1, IL-12, IL-23, IL-20, IL-17, IL-22, CCL20, TNFα, IFNα and CXCL9 (232).

The results also showed that GD and 6S displayed rather different mechanisms of action in influencing cell proliferation and inflammation in the in vitro model of inflammatory epidermis. At 20 μg/mL, the activity of both compounds was different in that 6S reduced the production of both IL-8 and IL-20, whereas GD enhanced the production of IL-8.

Interestingly, the study showed that CsA at 20 ng/mL did not reduce IL-8 production despite its effect on IL-20. This observation is in agreement with previous studies showing that CsA did not inhibit IL-8 production by cytokine-
stimulated keratinocytes (233). This was attributed due to the fact that the effect of CsA on keratinocytes cytokine production is indirect and reflect the effect of CsA on the immune system (233). This may suggest that evaluating the effect of CsA, as well as HB02, F6 and GD on IL-8 production in cytokine-stimulated keratinocytes is insufficient to provide a conclusive view on how these could influence psoriatic inflammation. Thus, an inclusive approach involving the effect of these test samples on other immune cells including T-cell, B cells and macrophages would be necessary. Nevertheless, the approach used in these studies has provided relevant information that could be used to construct a tentative outline for how these test samples could modulate inflammation relevant to psoriasis. In this respect, the ability of 6S to reduce IL-8 production by activated keratinocytes is intriguing and suggest that 6S could also modulate the production of other chemotactic factors associated with the induction of IL-8 such as anti-microbial peptides, as suggested by Guillteau and colleagues (43).
6 Conclusions

The experiments described in this chapter have shown that 6S is highly effective in modulating keratinocyte proliferation as well as inhibiting the production IL-20 and IL-8 by cytokine-activated keratinocytes. These activities were significantly more effective than seen for HB02, F6 and GD. The ability of 6S to inhibit cell proliferation may partly be due to its potent effect on IL-20 expression. However, whether this could result in apoptosis of the cells or promote the terminal differentiation of keratinocytes is to be established. These observations appear to suggest that ZOR derived compounds could have therapeutic benefits in treating psoriasis provided that potential side effects are adequately assessed. However, the safe application of ZOR derived compounds, most likely including 6S, would require further testing in vitro and in vivo using relevant animal models.
CHAPTER 7

General Discussion
1 Summary of the findings

Psoriasis is an autoimmune skin disease triggered by a combination of genetics and environmental factors leading to the induction of various inflammatory pathways. Numerous therapeutic approaches are used to treat patients with psoriasis ranging from the use of UV light for treatment, to a range of pharmaceutical drugs to biologics. Thus far, no specific treatment that embarks consistent efficacy in all patients has been discovered. This is due to variability in environmental triggers and the nature of immune effector mechanisms triggered in different patients subgroups or individual patients (234, 235). The aim of this thesis was to identify potential mechanisms of action of extract and compounds from a ginger species known as Halia barbara or Z. officinale Roscoe var. rubrum (ZOR), known to have therapeutic benefits in inflammatory diseases. This study encompasses a detailed assessment of ZOR extracts and derived compounds on activated macrophages, leukocytes and keratinocytes, all of which are known to be key players in psoriasis as well as most autoimmune diseases. Bearing in mind the complexity of the cytokines network in psoriasis, it is hypothesised that the outcomes of this study will provide a framework of potential mechanisms of action of ZOR against inflammatory cascades in psoriasis. Furthermore, it was hoped that the study could identify active compounds in ZOR that exert modulating effects on key cytokines involved in inflammatory cascades in most chronic conditions.
1.1 The effect of ZOR on macrophages.

The first stage of this study involved investigating the anti-inflammatory effects of ZOR extracts, fractions and isolated compounds on activated macrophages. This was carried out by assessing the inhibitory effects of these ZOR samples on the production of a number of key pro-inflammatory mediators produced by LPS-activated macrophages that include NO, PGE$_2$, TNFα, IL-12/IL-23p40 and IL-23p19 (Table 7.1). There is good evidence from different investigators for the involvement of all of these pro-inflammatory mediators in the pathogenesis of psoriasis. The mediators are transcribed when the relevant transcription factors are activated by LPS interaction with a multitude of receptors (TLR4), coreceptors (CD14, MD-2) and messenger proteins and signaling molecules (LBP, IRAK, TRAF) in macrophages (236). These interactions activate a number of signalling pathways such as NF-κB, ERK, JNK and p38 MAP kinase resulting in production of cytokines, which, however, beyond the scope of this thesis.

In the preliminary experiments, it was substantiated that ZOR extracts potently inhibited NO and PGE$_2$ production by activated macrophages (the RAW264.7 cell line). In this respect, the results showed that the chloroform extract from ZOR; HB02 was the most potent among extracts obtained using different solvent, in inhibiting both NO and PGE$_2$ production when added to macrophages before LPS stimulation. Furthermore, the fractions (F5, F6, F7 and F10) extracted from HB02 were more efficacious than L-NAME in inhibiting NO production. In addition, these fractions were comparable to dexamethasone and indomethacin in their ability to inhibit PGE$_2$ production. These findings are
in agreement with previous studies on the inhibitory effects of red ginger on the production of NO and PGE$_2$ (147). These results also highlighted the relationship between the NO and COX pathways (140, 141, 145).

To elaborate on how ZOR affected the NO pathway, the effect of HB02 and its fractions on iNOS mRNA and protein levels when added to macrophages at two time points: before and after LPS stimulation of the RAW264.7 cell line, was determined. HB02 and its fractions reduced iNOS mRNA levels both when added before and after LPS stimulation. Among the fractions, fraction 6 (F6) and F7 showed the strongest effects on reducing iNOS mRNA levels when added to macrophage before and after LPS stimulation. Culture of the macrophages with F7 caused a dramatic reduction in iNOS protein levels that was significantly more pronounced compared with the other fractions. However, F7 was also cytotoxic to macrophages at high concentrations (≥50µg/mL). F6, in contrast, was less effective than F7 but had not cytotoxic effects on the RAW264.7 cell line. F6 suppressed iNOS mRNA and protein levels at high concentration while sustaining cell viability. Based on this observation, F6 was identified as the potential fraction for further characterisation.

These studies were followed by series of purification processes that resulted in the identification of the main components of F6. Extensive characterisation of the components revealed that these were 6-shogaol (6S) and 1-dehydro-6-gingerdione (GD). Both compounds are derivatives of gingerol, a phenolic compound commonly present in fresh ginger (97), wherein both 6S and GD are the dehydrated form of gingerol. Referring to them as gingerol-related compounds, both exhibit ‘druggable’ structures with the presence of hydroxyl
and carbonyl moieties on their side chains (Figure 2.17). These structures are among the characteristics of a drug-like compound as proposed by Lipinski’s Rules of five (133).

Based on these findings, the contribution of these two compounds to the biological effects of F6 on iNOS mRNA and protein levels was investigated. 6S showed similar effects to F6 in reducing iNOS mRNA level. In contrast, GD showed a tendency towards reducing the enzymatic activity of iNOS. Hence, the effect of F6 on iNOS mRNA levels may be attributed to 6S rather than GD. Interestingly, however, neither compound had a significant effect on iNOS protein level or NO production when added after macrophage activation. F7, in contrast, had a profound inhibitory effect on iNOS protein level but limited effects on NO production. Suffice is to say, these results suggest that HB02 contains compounds that preferably act in a prophylactic manner before inflammation initiated by activated macrophage.

The effect of HB02, F6 and 6S on TNFα production was unexpected that all enhanced TNFα production both when added before or after LPS stimulation. This observation is inconsistent with other studies in which common ginger extracts were shown to suppress TNFα production by LPS-stimulated macrophages (217) and synoviocytes (148). According to Frondoza et al (2004), a ginger extract containing hydroxyl-methoxy-phenyl compounds suppress NF-κB activation and induce IκB-α, resulting in the inhibition of TNFα production and inactivation of COX-2 in synoviocytes. However, it remains unclear to which extent this disagreement between the effects of ZOR and
common ginger is due to variation in the compositions of the extracts and to differences in the experimental protocols.

There is currently no data on how HB02, F6 and 6S enhance TNFα production by LPS-stimulated RAW264.7 cells. However, it is possible that 6S, or other as yet unidentified compounds, could be activating a pathway that leads to TNFα production. In this respect, there is evidence that the production of TNFα in LPS-stimulated macrophages is influenced by the activation of ERK, JNK (236) and MAPK/p38 (237). There is also evidence that the process is facilitated by specific receptor-associated proteins such as TNFα receptor-associated factor-6 (TRAF6) and IL-1 receptor-associated kinase (IRAK) (236).

Paradoxically, the results showed that GD reduced TNFα mRNA levels when added to the cells before LPS stimulation. This finding could relate to the ability of this compound to reduce TNFα gene transcription by suppressing TLR4 signalling, or by activating IRAK. Further, there is evidence that activation of IRAK-M and AKT, that function in negative feedback, repress TLR4 signaling and modulate TNFα release (238). Nonetheless, this hypothesis needs to be further studied. However, it is unclear why F6 did not show similar inhibitory effects on reducing TNFα mRNA whilst GD did. One hypothesis is that the effect of GD might be masked by a bystander effect from other compounds present in F6.

Alternatively, F5 and F7 may affect posttranscriptional events that regulate TNFα mRNA levels. Modulation at posttranscriptional level may involve various complex events such maintaining RNA stability, translation initiation, transcript
distribution and regulation of 3’ ARE (AU-rich element) of TNFα mRNA (236, 239). This observation, remain to be clarified by future study. Bearing in mind the pleiotropic effects of TNFα in promoting and inhibiting inflammation, it is noteworthy that the differential effects of components of ZOR on TNFα expression might embody the multi-targeting ability of ZOR components before, during and after transcription of TNFα.

Experiments to assess the effects of ZOR on the IL-12/IL-23 axis showed that the ZOR samples strongly suppressed IL-12p40 and IL-23p19 mRNA levels when added to the macrophages before LPS stimulation. In addition, HB02, F6 and 6S, but not GD, also inhibited IL-12p40 production when added to the macrophages after LPS stimulation. This observation could be relevant to psoriasis since IL-12 and IL-23 in key cytokines in the differentiation Th1 and Th17 cells, respectively (25, 160, 240). These findings, therefore, suggest that HB02, F6 and 6S could modulate the generation of Th1 and Th17 T-cell functions. These findings are important since IL-23 is established as one of the prime inflammatory mediators in psoriasis (169, 170) and targeting p40 subunit has been used for the treatment of psoriasis (34). These results could, thus, suggest that ZOR and, particularly F6 and its compounds, could strongly modulate macrophage responses during inflammation.

1.2 The effect of ZOR on leukocyte migration and T-lymphocyte activation.

A key feature of chronic diseases is the continuous migration of immune cells such macrophages and polymorphs (PMN) into sites of disease under the
influence of a chemotactic environment resulting in inflammation. Therefore, the next phase involved examining the effect of HB02, F6, 6S and GD on PMN adhesion and migration using TNFα-stimulated human endothelial cells (HUVEC) \textit{in vitro}. Examination of PMN adhesion and migration under the microscope showed that all tested ZOR samples reduced adhesion and migration at low concentrations. Concurrent flow cytometric analyses for the expression of adhesion molecules showed that treatment with the ZOR samples enhanced L-selectin (CD62L) shedding and CD11b expression by activated PMNs. The increase in CD11b expression and L-selectin shedding was unexpected and may suggest that the effect of ZOR on PMNs is complex but that it ultimately leads to reduced cell migration to site of inflammation.

Results of testing HB02, F6, 6S and GD for their effect on PMN migration showed that 6S had the most potent inhibiting effects on PMN adhesion but that GD had little effects. Instead, treatment of PMNs with GD promoted margination of PMNs on endothelial cells but inhibited adhesion and migration through endothelial cells. These results suggest that 6S and GD have different, but possibly, complimentary modes of action on PMN migration.

In addition to the effects previously shown for the tested ZOR samples in modulating iNOS and IL-12/IL-23 by activated macrophages, these results highlight their modulatory effects on the response of PMNs to inflammation. This property could be important since PMN activation is linked to the balance of interaction between iNOS, IL-12/IL-23 and PGE$_2$ (161, 241). Therefore, although these observations are based on limited assay systems (macrophages
and PMNs), it is sufficient to indicate that the tested ZOR samples possess important anti-inflammatory properties.

In light of these observations, the effects of 6S and GD on the activation of helper CD4\(^+\) and cytotoxic CD8\(^+\) T-lymphocytes were studied. Primary T-lymphocytes were obtained from the spleen of healthy 8-12 week old mice. The effect of 6S and GD on inflammatory responses of isolated T-lymphocytes was studied following activation of the cells with antibodies to mouse CD3 and CD28. The results revealed that neither 6S nor GD had significant effects on the activation of mouse CD4\(^+\) T-lymphocytes. In contrast, the two compounds showed inhibitory effects on the activation of CD8\(^+\) T-lymphocytes. This was indicated by the reduction in the level of expression of the activation markers CD25 and CD69 on CD8\(^+\) T-lymphocytes. Based on these rather unexpected findings, the effect of 6S and GD on the level of TNF\(\alpha\) and IL-17 produced by this T-lymphocyte subset was studied. The results showed that 6S reduced the production of both cytokines by CD8\(^+\) T-lymphocytes. These results, thus, further highlight the potential of 6S in regulating activation of CD8\(^+\) T-lymphocytes. However, further studies would be required to verify these results and determine the potential relevance to treatment for patients with psoriasis.

1.3 The effect of ZOR on keratinocytes.

In view of the diverse and, at times, paradoxical anti-inflammatory effects observed for HB02, F6, 6S and GD on macrophages, PMNs and T-lymphocytes it was necessary to also assess their effects on keratinocytes. Abnormally
activated keratinocytes play a key role in sustaining the inflammatory response in psoriasis through proliferation and the production of pro-inflammatory cytokines and chemokines (219). Therefore, the effect of the ZOR samples on keratinocytes in an *in vitro* model of inflammatory epidermis was studied. The model has been published and has been shown to closely resemble cellular and molecular profiles of psoriatic skins (43). In this model, human primary keratinocytes are stimulated with IL-17, IL-22, TNFα, IL-1β and OSM (a member of IL-6 family), all established key cytokines in psoriasis pathogenesis (42, 43, 218, 220, 242). The effect of the ZOR samples on keratinocyte proliferation and cytokine/chemokine production in this system were then investigated. Cytokines studied included IL-20, which is known to promote cell hyperproliferation in psoriasis (224) and IL-8 that plays a important role in inflammatory responses, particularly as a chemoattractant (243). IL-20 is produced exclusively by keratinocytes in response to Th22 and Th17-related cytokines (221) and is expressed on the entire layer of the epidermal keratinocytes in patients with psoriasis (224). IL-8 production by keratinocytes is triggered by the synergistic effect of TNFα and IL-17 (218).

The results revealed that 6S had a profound effect on inhibiting IL-20 and IL-8 production by activated keratinocytes. This effect was significantly more pronounced compared with the effect seen for HB02, F6 and GD. The effect observed for 6S was also higher than seen for the positive control CsA. In view of the reported increase in IL-20 production and its role in keratinocyte proliferation (224), it is possible that the ability of 6S to inhibit keratinocyte proliferation may partly due to its potent inhibitory effect on IL-20 production.
It was previously suggested that IL-20 triggers CD8$^+$ T-cell to induce keratinocyte growth factor (KGF) production that facilitates cell hyperproliferation in psoriatic skins (224 405). It was, thus, interesting to note that 6S was also shown to inhibit murine CD8$^+$ T-lymphocytes activation and reduce TNFα and IL-17 by this T-lymphocyte subset. These results, therefore suggest that 6S could have therapeutic benefits in treating cutaneous inflammations associated with IL-20 production and CD8$^+$ T-lymphocytes activation. This beneficial therapeutic effect would further be bolstered by the ability of 6S to strongly inhibit IL-8 production. A previous study proposed that IL-8 production is primarily localised to the suprabasal layer of the psoriatic epidermis, suggestive of the role IL-8 plays in keratinocyte differentiation rather than proliferation (244).

6S also promoted changes in the phenotype of activated keratinocytes suggestive of inducing apoptosis in these cells. This effect was also noted for HB02 and F6 and, thus, highlights the potential of ZOR in psoriasis. GD, in contrast, did not affect the phenotype of the keratinocytes nor had an effect on IL-8 production despite its effect on IL-20 production.

2 Conclusions

Results of experiments detailed in this thesis have shown that ZOR extract, fractions and two identified compounds have modulatory effects on a range of pro-inflammatory pathways including the production of cytokines, cell migration and proliferation. Interestingly, however, the study also revealed that the two identified and fully characterised from one of the most relevant ZOR fractions,
6S and GD displayed differential effects on activated macrophage, PMNs, T-lymphocytes and keratinocytes (Table 7.1). Overall, the data presented provide a detailed insight into the multi-target effects of 6S and its modulatory effects on inflammatory responses mediated by immune cells and by keratinocytes. Furthermore, the similarities between the biological effects revealed for 6S and, the rather unrefined HB02 and F6, highlights the influence of 6S on the activity of the HB02 and, by inference, ZOR. It was interesting to note, however, that in contrast to 6S, GD had limited anti-inflammatory effects with its key notable effect in reducing the level of TNFα mRNA. Interestingly, 6S was unable to reduce the level of TNFα mRNA. In terms of function-structural relationships, it is tempting to speculate that these differences might be due to structural differences between 6S and GD. The two compounds are differentiated by the presence of an enolic-hydroxyl moiety at C-5 on the side chain of GD which is absent in 6S. Instead, at the same position in 6S, there is a double bond. These observations raise issues as to whether it would be preferable to use the compounds individually or in combination. However, further detailed studies are required as these questions were beyond the scope of this study.

Overall, the study provides a new overview of potential mechanisms of action of ZOR in modulating inflammatory cascades, potentially relevant to psoriasis. However, further studies exploring the effect of ZOR on the interaction between immune cells and keratinocytes in an in vivo model system of psoriasis would be required before the potential therapeutic efficacy of ZOR in psoriasis could be substantiated.
3 Potential implication for the use of ZOR in a therapeutic strategy for psoriasis.

Although extensive studies on the molecular and cellular pathogenic mechanisms involved in psoriasis have been carried out, the etiology and key pathogenic mechanism(s) that sustains the disease remains in debate. Nevertheless, knowledge and understanding key pathogenic aspects of the disease has led to emergence of broader therapeutic approaches for treatment. This study has examined the potential of naturally-occurring active compounds in *Z. officinale* Roscoe var. *rubrum* (ZOR) to be used as anti-psoriatic agents. Thus, the study shows that active compounds isolated from ZOR; 6-Shogaol (6S) and 1-dehydro-6-gingerdione (GD) have the ability to regulate important cytokine networks and inflammatory pathways relevant to psoriasis.

Overall, both compounds, albeit at different doses, possess complimentary effects in regulating the production of NO and PGE$_2$, reduce the level of *IL-12p40* and *IL-23p19* mRNA, inhibit PMN migration, reduce the activation of CD8$^+$ T-cells and inhibit IL-20 production by keratinocytes. The evidence provided in the study, thus, suggests that the compounds could work synergistically to modulate a range of inflammatory pathways in psoriatic plaques. Thus, further work that arises from this study is to identify the mechanistic interaction of both compounds in modulating the inflammatory events mentioned above.
The study has also revealed a number of interesting mechanisms through which 6S and GD could influence inflammation. Thus, the results have suggested that the suppressive effects of the two compounds on NO and PGE\(_2\) production may indirectly reduce \textit{IL-12/IL-23} mRNA expression by macrophages. The reduction in IL-12 and IL-23 levels, in turn, will reduce the activation and differentiation of Th1 and Th17 which have been shown to be important for the development of psoriasis. Further, the ability of the two compounds to inhibit PMN migration indicates that the compounds would have strong anti-inflammatory effects during the initiation of inflammation. Furthermore, since both compounds preferably affected CD8\(^+\) T-cells as compared with CD4\(^+\) T-cells, it is likely that the compounds would modulate inflammation at the cutaneous level. Because CD8\(^+\) T-cells are mainly found in the epidermal layer in psoriatic skin (30), this may suggest that the two compounds may suppress the inflammatory response locally. Thus, this may be beneficial for cutaneous therapeutic treatment on psoriatic lesion. In addition, reduction in IL-17 production by CD8\(^+\) T-cell, although marginal, suggests that the compound have the potential to regulate Tc17, or IL-17 producing CD8\(^+\) T-cells. Based on studies conducted elsewhere, the role of Tc17 resembles that of Th17 cells (245, 246). However, this hypothesis needs to be directly examined in experiments in which this T-cells subset is directly studied.

In addition to the inhibitory effects of 6S and GD on immune cells, the two compounds also modulated IL-20 production by keratinocytes. IL-20 is a member of the IL-10 family of cytokines and was recently shown to be involved in inflammation and bone resorption in patients with inflammatory conditions (247). In addition to the relevance of this observation to the pathogenesis of
psoriasis, the data suggest that the compounds could have therapeutic benefits in psoriatic arthritis which is associated with joint damage and bone loss. However, further studies will be required to test this possibility.
Table 7.1: Summary of the effects of ZOR’s extract (HB02), fractions (F5-10) and compounds (6S and GD) on inflammatory responses produced by activated macrophages, PMNs, T-lymphocytes and keratinocytes.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Effects on cytokines expression by activated macrophages (at mRNA level)</th>
<th>iNOS protein expression</th>
<th>NO production</th>
<th>PMN recruitment and migration (treated with samples at 3.3 μg/mL)</th>
<th>T-cell activation</th>
<th>Keratinocytes (treated with samples at 20 μg/mL)</th>
<th>IL-6 expression</th>
<th>IL-20 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pretreatment with samples at 20 μg/mL before LPS stimulation (Before)</td>
<td>Treatment with samples at 20 μg/mL after LPS stimulation (After)</td>
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References


REFERENCES


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APPENDICES
### Table 1: Selected plants for preliminary anti-inflammatory screening

<table>
<thead>
<tr>
<th>Plants</th>
<th>Anti-inflammatory properties</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mangifera indica</strong>&lt;br&gt;Common name: Mango, Mangga</td>
<td>VIMANG®, a Cuban standard aqueous extract from the stem bark of <em>Mangifera indica</em> consisting of 10% Mangiferin demonstrated good antioxidant and anti-inflammatory activities. The extract was shown to have the ability to inhibit both cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) pathways by inhibiting arachidonic acid metabolites such as PGs and LTs in animal models (248). It also reduced serum levels of TNFα and human synovial secretory phospholipase A2 (PLA₂) (248). sPLA₂ plays a significant role in initiating the production of arachidonic acid from cell membrane phospholipids. Thus, inhibiting sPLA₂ could lead to the inhibition of both COX and LOX pathways. Therefore, the extract might have the potential to possess inhibitory effects on the pathogenesis of psoriasis.</td>
</tr>
<tr>
<td><strong>Ipomoea batatas</strong>&lt;br&gt;Common name: Sweet potato, Ubi biru</td>
<td>It is a type of sweet potato with blue colour rhizome/root content. Upon fermentation, it produces polyphenolate which is mainly a caffeoylquinic acid derivative with potent antioxidant effects. This derivative exhibits radical scavenging activity, and has inhibitory effects on mutagenesis, cancer, diabetes, bacterial and viral infections, inflammation, and also inhibits melanin (249).</td>
</tr>
<tr>
<td><strong>Crinum asiaticum</strong>&lt;br&gt;Common Name: Crinum lily, Tembaga suasa</td>
<td><em>Crinum asiaticum</em> leaves are usually used by Malay traditional medicine practitioners for the treatment of fractured bones. The leaves are wrapped around fractured areas to reduce swelling. The active constituents isolated from <em>Crinum asiaticum</em> L var. japonicum include: crinamine, lycorine, norgalanthamine and epinorgalanthamine. Crinum was found to inhibit hypoxia inducible factor-1 (HIF-1) which functions in activating the transcription of genes involved in angiogenesis, cell survival and glucose metabolism in cancerous cells (250).</td>
</tr>
</tbody>
</table>
**Pandanus amaryllifolius**

Common name: Screwpine, Pandan

*Pandanus amaryllifolius* is a pandanus species with scented leaves widely found in Southeast Asia. Studies have been carried out to identify the active compounds within the root and leaves. The aroma from the leaves is due to the presence of 2-acetyl-1-pyroline. Several alkaloids with pyrrole-derived structures have been identified in the leaves using acid-base extraction. These alkaloids are pandanamine and pandamerilactones. Although the active constituents of the plant from different locations might vary, the majority of the alkaloids have at least one \(\alpha,\beta\)-unsaturated \(\gamma\)-lactone ring derived from pandanamine (251). Subsequently, a lectin designated Pandanin was identified using saline extraction of fresh leaves. It is an unglycosylated protein with a molecular mass of 8.0 kDa with 50-60% homology to those of mannose-specific lectin. Pandanin exhibits hemagglutinating activity against rabbit erythrocytes and anti-viral activities towards human viruses such as herpes simplex virus type-1 (HSV-1) and influenza virus (HIN1) (252).

**Zingiber officinale Roscoe var. rubrum**

Common name: Ginger Sp., Halia Bara

*Zingiber officinale* Roscoe var. *rubrum* originated from Zingiberaceae family is native to Southeast Asia. Traditionally, in Malaysia, it is used as one of the ingredients in postnatal treatment of wound healing. To date, the plant has not been studied for its anti-inflammatory activity, although many other species of Zingiberaceae family are known to possess such activities. The major active constituent of the plant is geraniol with 28.4% recovery in the essential oil of *Zingiber officinale* Roscoe var. *rubrum* (253). The major compound of the Zingiberaceae family, Zingiberene is present at ∼3-17%.

**Artocarpus heterophyllus**

Common name: Jackfruit, Nangka

*Artocarpus heterophyllus* is an edible fruit widely distribution in South and Southeast Asia, New Guinea and Southern Pacific. Studies were carried out to identify the active constituents of the plant in the leaves, bark and the fruit of jackfruit. Several flavonoids have been identified and isolated and shown to possess good antioxidant and anti
inflammatory activities. The most potent is artocarpanone which exhibits significant effects on NO production and iNOS protein expression in macrophages, RAW 264.7 cells (254). The inhibitory effect of artocarpone on NO production in LPS-activated macrophage line RAW was due to the suppression of iNOS protein expression. A phenolic compound derived from the fruit extract, artocarpesin was also found to have the potential to suppress NO and PGE$_2$ production by down regulating the expression of iNOS and COX-2 protein (255). Studies have shown that iNOS and COX-2 were found to be up regulated in psoriatic lesion (31), therefore, these plant actives might have the potential as an anti-psoriatic agent.

### Morinda citrifolia

**Common name:** Mengkudu, Noni, Indian Mulberry

Many studies have been done on the active constituents of the leaves, bark and fruit of *Morinda citrifolia* in terms of their chemical characteristics and potential bioactivity. It is a native common name of the plant in Malaysia has been used in traditional medicine to treat diabetes. The leaves taste bitter and are normally eaten as vegetable side dishes with rice. The leaves were shown to contain flavonol glycoside that has good antioxidant properties. The inhibitory activities of the extract against LOX and COX might signify its potential as an inhibitor of psoriasis pathogenesis.

### Artocarpus altillis

**Common name:** Breadfruit, Sukun

The plant is originally from Southeast Asia, has spread to pacific islands which have a tropical climate. In Malaysia and Indonesia, the plant is known as Sukun, or breadfruit due to the structure of the fruit which is spongy bread-like structure. *Artocarpus altillis* is in the family of Moraceae and Order of Rosales. It can grow to a height of 30 meters and produce up to 200 fruits per year. The fruits have a high content of carbohydrates and are eaten as delicacies in form of chips. The leaves are usually used as animal feed. In Indonesia, the leaves are used for the treatment of liver cirrhosis, hypertension and diabetes. Various studies have shown that flavonoids as well as triterpenes that are extracted from the
leaves possess good antioxidant, anti-platelet aggregation, cytotoxicity and anti-inflammatory activities. Yu Wang et al. (2007) elucidated that the geranyl flavonoid of *Artocarpus altilis* has potential as an anti-cancer agent.

<table>
<thead>
<tr>
<th><strong>Piper sarmentosum</strong></th>
<th>Originated in Asia; China, India and Southeast Asia, <em>Piper sarmentosum</em> is from the family of Piperaceae. It is used as a food additive or flavouring and in folk medicine. Within the region of the Malay and Indonesian archipelago, the roots and leaves of the plant are used to treat toothache, fungus dermatitis, coughing, asthma and pleurisy. Previous studies have shown that the plant has larvicide and adulticide activity on mosquitoes (256) as well as antimalarial activity (257). Studies indicate that a compound from flavonoid group Narigenin is extractable from the leaves and that this possesses good antioxidant and superoxide scavenging activity (258). These findings, therefore, suggest that the plant has an inhibitory effect on many inflammatory pathways.</th>
</tr>
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<tbody>
<tr>
<td><strong>Tinospora crispa</strong></td>
<td><em>Tinospora crispa</em> is a wild-type plant species widely distributed in the primary rainforest throughout Philippines, Indonesia, Malaysia, Thailand and Vietnam, in tropical and subtropical regions of India and some parts of the Far East. It is the origin of the family of Menispermaceae. The plant is usually used in the Malay traditional medicine for the treatment of diabetes and hypertension. In the Philippines, the plant is used to treat chronic rheumatism and malarial fever. In India, The plant is used as a tonic, antiperiodic and diuretic agent. The plant was first studied by westerners at the beginning of the 20th century and recognised that the plant contained alkaloids and bitter glucosides. More recent studies have identified the active constituents and their bioactivity properties. Basically, the bitter taste is due to the presence of alkaloids, and some alkaloids have been identified; columbine (whole plant), picroretine (leaves), tinosporidine and tinosporine. In addition, the stem was</td>
</tr>
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</table>
found to contain glucosides such as berberine, triterpenes, cycloecalenol, cycloecalenone and flavonoids; flavone O-glycoside and others. Studies have confirmed that *Tinospora crispa* has antidiabetic (259), antioxidant (260) and anti-inflammatory (261) properties.

| **Archidendron bubalinum** | *Archidendron bubalinum* originated from the Leguminosae family and is found in Southeast Asia. It is a small to medium-sized plant which grows to a fairly large tree of up to 42 m tall. The seeds' features are ellipsoid, flattened, without pleurogram and possess a pungent smell and bitter taste. The seeds are used as food additives and flavouring in Malay food, but could also be eaten raw. Traditionally, the seeds are used as a diuretic and also as a medicine for diabetes. To date, no study has been carried out to identify the active medicinal compound, or compounds in the seeds as well as the bioactivity of such compound(s). |
| **Marantha arundinacea** | *Marantha arundinacea* is a herbal plant about 0.5-1 meter tall with starchy rhizomes. It is a beet species from the Marantaceae family and is traditionally used as disinfectant and antidote to poisons. There is very little scientific information on the plant |
Table 2: Levels of evidence, evaluating quality of studies on drugs.

<table>
<thead>
<tr>
<th>Level of Evidence (LOE)</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Highest level with randomised, controlled clinical and cohort studies</td>
</tr>
<tr>
<td>B</td>
<td>Conclusive retrospective, or analytical cohort studies, research results, case control studies from level A</td>
</tr>
<tr>
<td>C</td>
<td>Case report or follow up studies from B</td>
</tr>
<tr>
<td>D</td>
<td>Expert opinion without scientific background, pure laboratory research or mechanism of action.</td>
</tr>
</tbody>
</table>

Figure 1: $^1$H NMR spectrum of 6-shogaol dissolved in chloroform-D (CDCl$_3$), 500 MHz.
Figure 2: The $^{13}$C NMR spectrum of 6-shogaol dissolved in CDCl$_3$, 125 MHz.
Figure 3: NMR DEPT-135 spectrum of 6-shogaol dissolved in CDCl$_3$, 125 MHz.
Figure 4: COSY 2D-NMR spectrum of 6-shogaol dissolved in CDCl$_3$. 
Figure 5: HMQC 2D-NMR spectrum of 6-shogaol dissolved in CDCl$_3$. 
Figure 6: HMBC 2D-NMR spectrum of 6-shogaol dissolved in CDCl$_3$. 
Figure 7: Infrared spectrum of 6-shogaol.
Figure 8: UV Spectrum of 6-shogaol.
Figure 9: $^1$H NMR spectrum of 1-dehydro-6-gingerdione dissolved in CDCl$_3$, 500 MHz.
Figure 10: $^1$H NMR spectrum of 1-dehydro-6-gingerdione dissolved in solvent mixture of CDCl$_3$ and methanol-D$_4$, 500 MHz
Figure 11: The $^{13}$C NMR spectrum of 1-dehydro-6-gingerdione dissolved in CDCl$_3$, 125 Mhz.
Figure 12: NMR DEPT-135 of 1-dehydro-6-gingerdione dissolved in CDCl$_3$, 125 MHz
Figure 13: COSY 2D-NMR spectrum of 1-dehydro-6-gingerdione dissolved in CDCl$_3$. 
Figure 14: HMQC 2D-NMR spectrum of 1-dehydro-6-gingerdione dissolved in CDCl₃.
Figure 15: HMBC 2D-NMR of 1-dehydro-6-gingerdione dissolved in CDCl₃
Figure 16: Infrared spectrum for 1-dehydro-6-gingerdione.
Figure 17: UV spectrum of 1-dehydro-6-gingerdione
Figure 18: Accurate mass spectrum of 1-dehydro-6-gingerdione