# THE ROLE OF GELAM HONEY IN MODULATING THE IMMUNE RESPONSE OF SEPSIS IN ANIMAL MODEL

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## SYNOPSIS

Persistent systemic inflammatory response syndrome is a serious health condition that may lead to multiple organ dysfunction, organ failure, and ultimately death. It leads to both acute inflammation, caused by either infective (microbes or lipopolysaccharide [LPS]) or non-infective (chemicals) sources, and sepsis, an infection caused by a lethal dose of LPS (endotoxemia). These conditions have similar inflammatory mediators such as cytokines, nitric oxide (NO), high-mobility group box-1 (HMGB1), and heme oxygenase-1 (HO-1), suggesting that they may result from similar pathogenic mechanisms. Previous studies have investigated the applications of natural products in targeting these inflammatory mediators. Honey, for example, is used to treat inflammation and heal wounds. Gelam honey is most commonly in Malaysia. The floral source of Gelam honey is *Melaleuca cajuputi* Powell, traditional *Melaleuca cajuputi* Powell has been used to treat many diseases and it has medicinal antiseptic, antibacterial, anti-inflammatory, and anodyne properties. However, it is currently unknown whether Gelam honey has a protective effect against systemic inflammatory response during acute inflammation and sepsis. We first investigated the effects of honey, honey methanol extract (HME), and honey ethyl acetate extract (HEAE) on acute inflammation, using animal models. These products inhibited edema and pain, in correlation with their potent inhibitory activities against NO and prostaglandin E2 (PGE$_2$) in all models. Phenolic compounds have been implicated in these inhibitory activities. We also evaluated the anti-inflammatory activity of Gelam honey extracts using High-Performance Liquid Chromatography (HPLC) and liquid chromatography–mass spectrometry (LC-MS). Subsequently, HME and HEAE were tested in vitro for their effect on NO production in stimulated macrophages, as well as for their effects on tumor necrosis

factor-α (TNF-α) cytotoxicity in L929 cells. These extracts protected cells against TNF cytotoxicity and inhibited NO production, with HEAE exhibiting greater activity. Moreover, we investigated the effect of the intravenous injection of honey in rats with LPS-induced endotoxemia. We found that cytokines (TNF-α, IL-1β, and IL-10), HMGB1, and NO levels decreased, and HO-1 levels increased significantly in the honey-treated groups. We also found that Gelam honey protects organs from lethal doses of LPS, as evidenced by improved blood parameters, reduced neutrophil infiltration, and decreased myeloperoxidase activity, as well as reduced mortality in honey-treated groups compared with untreated groups. We also examined the ability of Gelam honey to scavenge peroxynitrite during immune responses mounted by the murine macrophage cell line RAW 264.7. Significantly, improved viability of LPS/IFN-γ-treated RAW 264.7 cells and significant inhibition of NO production were observed, similar to those observed with an inhibitor of inducible NOS. In addition, Gelam honey inhibited peroxynitrite production from the synthetic substrate SIN-1 as well as peroxynitrite synthesis in LPS-treated rats (endotoxemia). Thus, by suppressing the production of cytotoxic molecules such as NO and peroxynitrite, honey may attenuate the inflammatory responses that lead to cell damage and, potentially, to cell death. The results therefore suggest that honey has therapeutic uses for a wide range of inflammatory disorders.

# SINOPSIS

Sindrom tindak balas keradangan sistemik berterusan merupakan keadaan kesihatan yang serius yang boleh menyebabkan disfungsi organ berganda, kegagalan organ, dan akhirnya kematian. Ia boleh membawa kepada kedua-dua keradangan akut, yang disebabkan oleh sama ada sumber berjangkit (mikrob atau lipopolysaccharide yang [LPS]) atau tidak berjangkit (kimia) ,sepsis, dan jangkitan yang disebabkan oleh dos maut LPS (endotoxemia). Syarat-syarat ini mempunyai radang mediator yang sama seperti sitokin, nitrik oksida (NO), kumpulan kotak tinggi mobiliti-1 (HMGB1), dan heme oxygenase-1 (HO-1), mencadangkan bahawa mereka mungkin diakibatkan oleh mekanisma patogenic yang sama. Kajian sebelumnya telah menyiasat aplikasi produk semulajadi dalam mensasarkan mediator radang. Madu, contohnya, digunakan untuk merawat keradangan dan menyembuhkan luka. Madu Gelam yang paling biasa digunakan di Malaysia (sumber bunga:. tradisional Melaleuca cajuputi Powell, telah digunakan untuk merawat pelbagai jenis penyakit dan ia mempunyai ubat antiseptik, antibakteria, anti-radang, dan hartanah anodyne. Walau bagaimanapun, buat masa ini tidak diketahui sama ada produk semula jadi ini (Gelam madu) mempunyai kesan perlindungan terhadap tindak balas radang sistemik semasa keradangan akut dan sepsis. Kami mula menyiasat kesan madu, madu ekstrak metanol (HME), dan ekstrak madu etil asetat (HEAE) pada keradangan akut, menggunakan model haiwan. Produk-produk ini menghalang bengkak dan kesakitan, dan ini berhubungan dengan aktiviti perencatan mujarab mereka terhadap NO dan PGE2 dalam semua model. Sebatian fenolik telah terbabit dalam aktiviti perencatan. Kami juga menilai aktiviti anti-radang ekstrak madu Gelam menggunakan HPLC dan LC -MS. Selepas itu, HME dan HEAE telah diuji secara in vitro untuk kesan mereka ke atas pengeluaran NO dalam makrofaj dirangsang, serta kesannya terhadap nekrosis tumor faktor-α (TNF-α) sitotoksiti

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dalam sel-sel L929 . Ekstrak ini melindungi sel daripada sitotoksiti TNF dan menghalang pengeluaran NO, dengan HEAE ia mempamerkan aktiviti yang lebih besar. Lebih-lebih lagi, kami juga menyiasat kesan suntikan intravena madu dalam tikus dengan endotoxemia akibat LPS. Kami mendapati bahawa tahap cytokine (TNF-α, IL-1β, dan IL-10) , HMGB1, dan NO menurun, dan tahap HO-1 meningkat dengan ketara dalam kumpulan yang dirawat dengan madu . Kami juga mendapati bahawa madu Gelam melindungi organ daripada dos maut LPS, seperti yang dibuktikan oleh parameter darah yang lebih baik, penyusupan neutrophil dikurangkan, dan myeloperoxidase menurun aktivitinya, serta kematian dikurangkan dalam kumpulan yang dirawat dengan madu berbanding dengan kumpulan-kumpulan yang tidak dirawat. Kami juga mengkaji keupayaan madu Gelam untuk mencari peroxynitrite semasa tindakbalas imun yang dipasang oleh garis sel macrophage murine yang RAW 264,7 daya maju Ketara bertambah baik LPS / IFN-γ dirawat RAW 264,7 sel-sel dan perencatan dengan pengeluaran NO yang ketara diperhatikan, sama seperti yang diperhatikan dengan perencat NOS inducible. Di samping itu, madu Gelam menghalang pengeluaran peroxynitrite dari sintetik substrat SIN-1 serta sintesis peroxynitrite dalam tikus yang dirawat dengan LPS (endotoxemia). Oleh itu, dengan pengeluaran pembenteras molekul sitotoksik seperti NO dan peroxynitrite, madu boleh melemahkan tindak balas keradangan yang menyebabkan kerosakan sel dan berpotensi menyebabkan kematian sel. Satu lagi komponen aktif madu, caffeic asid phenethyl ester, mempamerkan ciri-ciri antioksidan, antimitogenic, anticarcinogenic, aktiviti anti-radang, dan immunomodulateri. Apabila diuji dalam LPS / IFN-γ-sel dirawat 264,7 RAW dan-tikus yang dirawat di LPS, keputusan yang diperolehi adalah serupa dengan apa yang diperolehi dengan madu Gelam, dan ini menyediakan bukti untuk potensi terapeutik yang serupa.

# ACKNOWLEDGEMENTS

# TABLE OF CONTENTS

# LIST OF SYMBOLS AND ABBREVIATIONS

| Abbreviations | Description |
|---|---|
| 1400W | 1400W Dihydrochloride (iNOS inhibitor) |
| ALP | Alkaline phosphatase |
| ANOVA | Analysis of variance |
| ATCC | American type culture collection |
| BALF | Broncho-alveolar lavage fluid |
| CAPE | Caffeic Acid Phenethyl Ester |
| CLP | Caecal ligation and puncture |
| COX | Cyclooxygenase |
| CO2 | Carbon dioxide |
| CRP | C reactive protein |
| DHR-123 | Dihydrorhodamine 123 |
| DMEM | Dulbecco modified Eagle medium |
| DMSO | Dimethyl sulfoxide |
| EC50 | Median maximal effective concentrations |
| ELISA | Enzyme-linked immunosorbent assay |
| ESI-MS | Electrospray ionization mass spectrometry |
| FBS | Fetal bovine serum |
| HDAC2 | Histone deacetylase |
| HEAE | Honey ethyl acetate extract |
| HME | Honey methanol extract |
| HMGB1 | High-mobility group box-1 |
| HO-1 | Heme oxygenase-1 |
| HPLC | High-performance liquid chromatography |
| ICAM1 | Intracellular adhesion molecule 1 |
| ICAM-1 | Intracellular adhesion molecule-1 |
| IFN-γ | Interferon-γ |
| IKKs | I kappa B kinase |
| IL | Interleukin |
| iNOS | Inducible nitric oxide synthase |
| INDO | Indomethacin |
| IRAK4 | Interleukin-1 receptor associated kinase 4 |
| LC-MS | Liquid chromatography–mass spectrometry |
| LPS | Lipopolysaccharide |

| | |
|---|---|
| LTA | Lipoteichoic acid |
| MODS | Multiple organ dysfunction syndrome |
| MPO | Myeloperoxidase |
| MRSA | Methicilline Resistant *Staphylococcus aureus* |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| MW | Molecular weight |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NO | Nitric oxide |
| ONOO | Peroxynitrite |
| PAF | Platelet activating factor |
| PAMPs | Pathogen associated molecular patterns |
| PBS | Phosphate buffered saline |
| $PGE_2$ | prostaglandin E2 |
| PMN | Polymorphonuclear leukocytes |
| PRRs | Pattern recognition receptors |
| ROS | Reactive oxygen species |
| SIN-1 | 3-morpholinosydnonimine |
| SIRS | Systemic inflammatory responsesyndrome |
| TAB1 | TAK1-binding protein 1 |
| TAB2 | TAK2-binding protein 2 |
| TLRs | Toll-like receptors |
| TNF-α | Tumor necrosis factor alpha |
| TRAF6 | Tumor necrosis factor-associated factor 6 |

# LIST OF PUBLICATIONS

1. Kassim M, Achoui M, Mansor M, Yusoff KM: **The inhibitory effects of Gelam honey and its extracts on nitric oxide and prostaglandin E(2) in inflammatory tissues.***Fitoterapia* 2010, **81:**1196-1201.

2. Kassim M, Achoui M, Mustafa MR, Mohd MA, Yusoff KM: **Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity.***Nutr Res* 2010, **30:**650-659.

3. Kassim M, Yusoff KM, Ong GSY, Sekaran SD, Yusof MYM, Mansor M: **Gelam honey inhibits lipopolysaccharide-induced endotoxemia in rats through the induction of heme oxygenase-1 and the inhibition of cytokines, nitric oxide, and high-mobility group protein B1.***Fitoterapia* 2012, ***In press***.

4. Kassim M, Mansor M, Al-Abd N, Yusoff KM: **Gelam Honey Has a Protective Effect against Lipopolysaccharide (LPS)-Induced Organ Failure.***International Journal of Molecular Sciences* 2012, **13:**6370-6381.

5. Kassim M, Mansor M, Suhaimib A, Ong GSY, Yusoff KM: **Gelam honey scavenges peroxynitrite during the immune response.** *International Journal of Molecular Sciences* 2012, **accepted**.

# CHAPTER 1.     INTRODUCTION

## 1.1.  General overview

Sepsis is defined as a type of systemic inflammatory response syndrome (SIRS) caused by microbial infection (Kibe *et al*., 2011; Matsuda N, 2006). Sepsis has systemic effects, including hemodynamic and cardiovascular instability, decreased ejection fraction, and reduced systemic vascular resistance, which are indistinguishable from the signs of acute inflammation (Wilson *et al*., 1998; Yoon, 2012). Inflammation is an immune response that occurs in the tissue. It is a nonspecific reaction to any cell-injuring agent, either physical (such as heat or cold), chemical (such as a concentrated acid or alkali or any other caustic chemical), or microbiological.  It has a rich history intimately linked to war wounds and infections (Goris, 1996; Seth *et al*., 2012). Although the complex pathophysiology of acute and chronic inflammation is gradually becoming better understood, in modern hospitals, acute inflammation continues to be a main threat to patient health. Local inflammation is tightly regulated by the immune and nervous systems, combating invading pathogens and removing injured cells. If the local inflammatory response fails to contain the insults, systemic inflammation may occur. In general, SIRS is an entire normal response to injury or infection. It may lead to excessive leukocyte activation, multiple organ dysfunction syndromes (MODSs), organ failure, and ultimately death. At the late irreversible stage, even the removal of initial local inflammatory stimulus may have no effect on the progression of organ failure and mortality (Fernandes *et al*., 2012; Latifi SQ, 2002; Milam *et al*., 2010). Moreover, acute inflammation and sepsis share many inflammatory mediators (Kuper *et al*., 2012; MacGarvey *et al*., 2012; Seija *et al*., 2012; Setoguchi *et al*., 2012). Neutrophil infiltration and activation is a hallmark of SIRS. Neutrophils play an important role in

host defense by releasing many proteolytic enzymes and producing free radicals such as reactive oxygen species (ROS) to degrade internalized pathogens. However, excessive production of these lytic factors by overwhelming numbers of activated neutrophils may correlate with host tissue damage and organ failure during sepsis. The incidence of sepsis and the number of sepsis-related deaths are increasing; previous studies have confirmed that there are more than 750,000 cases of severe sepsis per year in the United States, resulting in an annual national hospital cost of \$16.7 billion (Angus *et al*., 2001). Therefore, new immune-modulating therapeutic approaches for sepsis are needed to control neutrophil migration and infiltration during sepsis; reduce systemic cytokines, NO, and HMBG1; induce HO-1 activation and thus increase the survival rate.

The aim of the present study is to validate the hypothesis that modulation of immune response during sepsis with natural products such as honey can protect against systemic inflammatory response, using animal models of inflammation and sepsis. Moreover, our study also aims to reveal some underlying activities of these natural products in vitro and in vivo through monitoring their effects on cytokines, HMGB1, NO, HO-1, myeloperoxidase, and on peroxynitrite scavenging, as well as on systemic inflammatory response during sepsis development and, consequently, on the survival rate of animals.

This study looked into the extraction of Gelam honey and evaluation of the anti-inflammatory activity of these extracts. Honey extracts were analyzed using liquid chromatography-mass spectrometry (LC-MS) to identify phenolic compounds and then the extracts were also tested for their effects on tumor necrosis factor alpha (TNF) cytotoxicity in L929 cells and nitric oxide in RAW 264.7. The major phenolics in the extracts were ellagic, gallic, and ferulic acids, myricetin, chlorogenic acid, and caffeic acid. Other compounds found in lower concentrations were hesperetin, *p*-coumaric acid, chrysin, quercetin, luteolin and kaempferol. Honey and its extracts inhibited NO during

immune response and protection from TNF cytotoxicity. Rats' paws induced with *carrageenan* in the non-immune inflammatory and nociceptive model, and lipopolysaccharide (LPS) in the immune inflammatory model inhibited edema and pain in inflammatory tissues as well as showing potent inhibitory activities against NO and $PGE_2$ in both models. The decrease in edema and pain correlates with the inhibition of NO and $PGE_2$. Phenolic compounds have been implicated in the inhibitory activities. However, local immune response is not like systemic inflammatory response syndrome such as sepsis so we have to confirm if Gelam honey can protect from sepsis. We investigated the effect of the intravenous injection of honey in rats with LPS-induced sepsis. The results showed that after 4 h of treatment, honey reduced cytokine (tumor necrosis factor-α, interleukins 1β, and 10) and NO levels and increased heme oxygenase-1 levels. After 24 h, a decrease in cytokines and NO and an increase in HO-1 were seen in all groups, whereas a reduction in HMGB1 occurred only in the honey-treated groups as well as Honey-treated rats which showed reduced mortality after sepsis compared with untreated rats. We then investigated the role of Gelam honey against sepsis-induced organ failure. Treatment with honey showed protective effects on organs through the improvement of organ blood parameters, reduced infiltration of neutrophils, and decreased myeloperoxidase activity. Honey-treated rabbits also showed reduced mortality after sepsis compared with untreated rabbits. Honey may have a therapeutic effect in protecting organs during sepsis. During immune response and sepsis cell produces a high toxic and oxidant molecules that lead to cell death and tissues damage. Peroxynitrite is a short-lived oxidant and a potent inducer of cell death. It induces lipid, protein and DNA damage and the potential biological targets of peroxynitrite include membrane as well as cytosolic and nuclear receptors. Conditions in which the reaction products of peroxynitrite have been detected and in which

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pharmacological inhibition of its formation or its decomposition have been shown to be of benefit include sepsis, vascular diseases, ischaemia–reperfusion injury, inflammation, pain and neurodegeneration, therefore we further showed the ability of Gelam honey to scavenge peroxynitrite during sepsis and immune responses mounted in the murine macrophage cell line RAW 264.7 when stimulated with lipopolysaccharide/interferon-γ (LPS/IFN-γ) and in LPS-treated rats and also significantly improved the viability of LPS/IFN-γ–treated RAW 264.7 cells and inhibited nitric oxide production—similar to the effects observed with an inhibitor of inducible nitric oxide synthase. Furthermore, honey inhibited peroxynitrite production from the synthetic substrate 3-morpholinosydnonimine (SIN-1) and prevented the peroxynitrite-mediated conversion of *dihydrorhodamine 123* to its fluorescent oxidation product rhodamine 123. Honey inhibited peroxynitrite synthesis in LPS-treated rats and thus, honey may attenuate inflammatory responses that lead to cell damage and death, suggesting its therapeutic uses for the pharmacological strategies to attenuate the toxic effects of peroxynitrite in immune response and sepsis.

**CHAPTER 2.    LITERATURE REVIEW**

**2.1.    Sepsis**

The word "sepsis" is derived from the Latin origin *sepsios*, meaning musty; it is a condition that is not accepted passively by living organisms. The discovery of microbes in 1674 by Antony van Leeuwenhoek was the main event that helped elucidate the origin of sepsis. The determination of the body's systemic responses to defend itself against microbial pathogens and their toxins took several hundred years of rigorous studies (Hoffmann *et al*., 1999). Sepsis is a systemic inflammatory process caused by infection. It develops from SIRS, and severe sepsis then leads to MODS. An epidemiological study has reported that more than 750,000 patients develop sepsis annually in the United States alone (Angus *et al.*, 2001), with an estimated cost per year for treatment of $18 billion. The mortality rate of sepsis high, i.e., from 30% to 50% (Riedemann *et al*., 2003). It affects the whole body, with the most common sites of infection being the lungs, abdominal cavity, urinary tract, and primary infections of the bloodstream (Agnello *et al*., 2002). Invading pathogens, including gram-negative and gram-positive bacteria, viruses, parasites, and fungi, may be the initial cause of sepsis. The immune response starts in the innate immune system, which employs special receptors to recognize the highly conserved components of these pathogens, i.e., pathogen-associated molecular patterns (PAMPs). The Toll-like receptors (TLRs) are the most important of these receptors. They consist of many members, most of which are expressed in human neutrophils (Hayashi, 2003). TLR4 acts as the central receptors for LPS, whereas TLR2 acts as the main receptor for gram-positive cell wall components, yeast cell wall zymosan, and mycobacterial cell wall components (Hildebrand *et al*., 2012; Underhill, 1999). After the TLRs are activated by their ligands, MyD88 is associated with TLR complex as the adaptor molecule, which leads to the

formation of the TNF-associated factor 6 (TRAF6)/IL-1 receptor associated kinase 4 (IRAK4)/IRAK-1 complex (Ye, 2002). This complex interacts with another complex comprising transforming growth factor-beta-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), and TAB2 (Shibuya, 1996; Takaesu *et al*., 2000). TAK1 is subsequently activated in the cytoplasm, leading to the activation of I kappa B kinase kinases (IKKs) (Shibuya, 1996). IKK activation leads to phosphorylation and degradation of I kappa B, translocation of NF-κB into the nucleus, and upregulation of inflammatory cytokines. TLRs activate many cells such as neutrophils, macrophages, and epithelial and endothelial cells to produce inflammatory mediators including cytokines (TNF-α, IL-1β, etc.) and NO. The expression of adhesion molecules on endothelial cells is induced either directly by TLR activation or indirectly through the proinflammatory cytokines TNF-αand IL-1 (Parker *et al*., 2005) . These events promote neutrophil migration to the site of inflammation during sepsis.

## 2.2.    Immune response to sepsis

Neutrophils have a key regulatory role in vascular inflammation. The hallmark of MODS or acute respiratory distress syndrome (ARDS) is infiltration of neutrophils into the microvasculature of the organ involved, such as the lung (Phillipson & Kubes, 2011; Shah *et al*., 2010). Sepsis causes endothelial injury and neutrophil infiltration into tissues, leading to local injury, disturbed capillary blood flow and enhanced microvascular permeability, disseminated intravascular coagulation, circulatory collapse, hypoxia and, ultimately, multiple organ failure (Cohen, 2002). Sepsis-induced acute lung injury (ALI) remains a major clinical problem with significant morbidity and mortality (Razavi *et al*., 2004). A pathological hallmark of acute lung injury is subsequent tissue infiltration of neutrophils and pulmonary microvascular sequestration (Kindt *et al*., 1991; Tate & Repine, 1983). Enhanced pulmonary neutrophil

sequestration and infiltration during sepsis changes the neutrophil profile by increasing neutrophil surface expression and activating cell-cell adhesion molecules, and enhancing the release of soluble mediators, production of cytokines, and generation of reactive oxygen species, NO, and ONOO⁻ (Brown *et al*., 1995; Goode & Webster, 1993; Novelli, 1997; Skoutelis *et al*., 2000). Neutrophils express adhesion molecules that enable them to adhere to the microvascular endothelium and sequester in vital organs. Various proinflammatory mediators released during the sepsis response are supposed to increase and activate the expression of adhesion molecules on the surface of both neutrophils and endothelial cells, such as intracellular adhesion molecule-1 (ICAM-1) and β1-integrins CD11/CD18 (Alves-Filho *et al*., 2006). The toxicity of PMNs stems not only from their adhesive and migratory properties but also from their ability to generate ROS. The mechanism of ROS generation is assumed to involve nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) stimulation of neutrophils with TNF-α, IL-1β, and LPS, which results in the assembly of the NADPH complex and the production of the superoxide radical (AW, 2006). NADPH is a membrane-bound enzyme that catalyzes the formation of superoxide anion from molecular oxygen. Superoxide is subsequently converted to hypochlorous acid and hydrogen peroxide, which are both unstable and highly reactive oxygen species. ROS produced by neutrophils are capable of direct DNA damage, lipid peroxidation, and interference with actin metabolism, leading to changes in cellular function and organ damage. NADPH inhibition by apocyanin has been shown to decrease lung injury in guinea pigs (Deng *et al*., 2012; Grommes & Soehnlein, 2011). The NO synthase (NOS) pathway is another pathway that may also be involved in the generation of free radicals by neutrophils. NO knockout mice have been shown to have decreased lung injury when injected with LPS. Phenolic compounds reduce sepsis-induced remote organ injury, at

least in part, through their ability to balance oxidant-antioxidant status, to inhibit neutrophil infiltration and to regulate the release of inflammatory mediators (Kolgazi *et al*., 2006). Cells of the monocyte/macrophage family are the basis of the innate immune system; they play a vital role in the immune response against microorganisms. Monocytes derived from bone marrow circulate in the blood for less than 48 hours and enter tissues to differentiate into macrophages. Macrophages perform different biological activities depending on the type of the tissue microenvironment. Macrophages have a longer life span and more heterogeneous functions than monocytes (Wu *et al*., 2005). Although the role of an innate immune reaction is to eradicate the invading microorganisms, a hyperinflammatory monocyte system contributes to the etiology and progression of SIRS. Macrophages are critical in recognizing LPS, lipoteichoic acid (LTA), or other bacterial components through various pattern recognition cellular receptors such as the Toll receptors. The interaction between macrophages and pathogens results in the beginning of inflammatory mediators and coagulation cascades. These pathways produce many soluble mediators that function in autocrine or paracrine loops and further activate the proinflammatory cascades (Panda *et al*., 2012). Macrophages can release a series of inflammatory mediators, including IL-1, IL-6, TNF-α, platelet activating factor (PAF), eicosanoids, NO, and ROS, when stimulated with bacterial products (Bergenfelz *et al*., 2012; Kleiman *et al*., 2012; Risco *et al*., 2012; Vandevyver *et al*., 2012). They are also a source of intracellular superoxide and hydrogen peroxide ($H_2O_2$), responsible for killing phagocytosed microorganisms. During sepsis, macrophages exist in an overactive state and can release some inflammatory mediators and ROS that contribute to tissue damage (Van Amersfoort *et al*., 2003) . Further, activated macrophages increase their expression of CD40 and TNF-α receptors, and secrete TNF-α. This autocrine stimulus synergizes with interferon-γ

(IFN-γ) secreted by T helper 1 (TH1) cells to increase the antimicrobial action of the macrophage, in particular by inducing the production of NO and ROS (Van Amersfoort *et al.*, 2003). Previous studies showed that natural product such as flavonoids, phenolic acid and polyphonic inhibit the activation of macrophage through NF-κB activity or DNA binding, IKK, COX2, iNOS and cytokines inhibition (Cho *et al.*, 2003; Jeong & Jeong, 2010; Joo *et al.*, 2012; Kanters *et al.*, 2003; Sgambato *et al.*, 2001).

TNF-α is produced by monocytes and macrophages after inflammation; it is a pivotal cytokine in the pathogenesis of inflammatory diseases. It is the major early mediator of sepsis and has been reported to have role in the development of MODS in sepsis (Mira *et al.*, 1999; Qiu *et al.*, 2011). Two hours after exposure to LPS, TNF-α will be in its highest level in serum where it can activate many cell types; initiate the proinflammatory cascade with up-regulation of adhesion molecules on neutrophils; and stimulate the release of IL-1, IL-6, eicosanoids, and PAF, among others (Agnello *et al.*, 2002), thus initiating inflammatory cell migration into tissues. TNF-α upregulates phospholipase A2, cyclooxygenase, and NOS (Bhatia & Moochhala, 2004) . It is a potent activator of NF-κB. and TNF-α antibodies and soluble TNF-α receptors have been used in humans . Furthermore, it has been confirmed that neutralized monoclonal antibodies against TNF-α can diminish the production of some inflammatory mediators and other proinflammatory cytokines (Brennan FM, 1989). Reactive oxygen species (ROS) play a critical role in mediating TNF-α-induced cytotoxicity (Kunnumakkara *et al.*, 2008). It was shown that such cytotoxicity can be blocked by specific free radical scavengers (Goossens *et al.*, 1995). Previous research has reported that Malaysian honey has free radical scavenging activity (Aljadi & Kamaruddin, 2004). Therefore, it is believed that the free radical scavenging capacity of flavonoids identified in the honey extracts may play a role in protecting cells from this cytotoxicity (Middleton et al.,

2000). In fact, Habtemariam (Habtemariam, 2000)  reported that phenolics, such as caffeic acid, effectively inhibit TNF-induced cytotoxicity in L929 cells. It also inhibits TNF in vivo and reducing the mortality in sepsis (Fidan et al., 2007).

IL-1β is a proinflammatory cytokine produced by many cell types, such as monocytes/macrophages, fibroblasts, and endothelial cells, and is involved in inflammatory and immunological processes (Dinarello, 2011b) . It has a vital role in endotoxemia and also contributes to the development of severe sepsis and MODS, and induces the production of other cytokines and inflammatory mediators involved in sepsis (CA, 2000; Dinarello, 2011a; Yoza & McCall, 2011). Further, an IL-1 receptor antagonist (IL-1ra) has been characterized to be structurally related to IL-1, and in vitro has been demonstrated to protect against endotoxin-induced lung injury in rats and to improve survival rate after lethal endotoxemia in mice (Dinarello, 2011a; Tsuchiya et al., 2012). There are some  natural product such as ethanol extract of propolis , mushroom and  Epigallocatechin-3-gallate, a Green Tea-Derived Polyphenol, ellagic acid  have been shown a potent inhibiting of IL-1 β (Blonska *et al*., 2004; Jedinak *et al*., 2011; Wheeler *et al*., 2004; Yu *et al*., 2007).

IL-6 is a pro- and anti-inflammatory cytokine. It is produced by macrophages/monocytes, endothelial cells, and smooth muscle cells, and its expression increases in response to endotoxin, TNF-α, and IL-1β (Agnello *et al.*, 2002). IL-6 is an important cytokine that is up-regulated during sepsis (Gouel-Cheron *et al*., 2012; Kruttgen & Rose-John, 2012). It has been demonstrated to increase in post-burn injuries and after surgery (Nijsten *et al*., 1991) . It mediates the synthesis of acute phase proteins, including C-reactive protein (CRP) (Castell JV, 1989). Serum IL-6 level is a biomarker that can be used in identifying patients at risk of progression to sepsis, severe sepsis, septic shock, and MODS early in the infective and inflammatory processes (Qiu

*et al*., 2012). Natural product such as green tea , propolis ,some phenolic compounds from plant such as (-)-epigallocatechin-3-gallate, chrysin, quercetin and resveratrol have shown a potent inhibition of IL-6 and protect from sepsis (Cernada *et al*., 2012; Lin *et al*., 2010; Liu *et al*., 2005; Woo *et al*., 2005; Zhong *et al*., 1999).

IL-10 is anti-inflammatory cytokine produced mainly by monocytes/macrophages and, to a lesser degree, by lymphocytes. It plays an important role in sepsis (Howard, 1993; Standiford, 1995). It has been identified as a vital modulator of the lethal excessive production of inflammatory cytokines. It inhibits the in vitro synthesis of IL-1α, IL-1β, TNF-α, and IL-6 (de Vries, 1995; Fiorentino *et al*., 1991). It is an important mediator of endotoxemia-induced immunosuppression, and is a critical mediator of macrophage and leukocyte deactivation during LPS desensitization and endotoxemia (Pils *et al*., 2010; Steinhauser *et al*., 1999; Urbonas *et al*., 2012). Administration of recombinant IL-10 has been shown to significantly protect animals from sepsis (Gerard, 1993; Hickey *et al*., 1998; Standiford, 1995). IL-10 is a potent suppressor of macrophage production of important activating and/or chemotactic cytokines (Cassatella, 1993; Kasama, 1994; Rolph, 1992). Furthermore, in vitro, IL-10 cytokine directly inhibits neutrophil and macrophage bactericidal activity and phagocytosis (Laichalk, 1996; Ocuin *et al*., 2011; Oswald, 1992). Both in humans and in animal models, the period that follows septic events has been shown to result in the preferential expression of anti-inflammatory cytokines, particularly IL-10 (O'Sullivan, 1995). IL-10 administration in animal models of SIRS has been shown to protect from organ injury; no animal studies are available to date (Mittal *et al*., 2010) . HMGB1 is a ubiquitous nuclear factor released by activated immune cells such as neutrophils and macrophages; it mediates organ damage in severe sepsis and is released by necrotic cells (Wang, 1999; Yang, 2004). HMGB1 has been implicated in some diseases such as

ischemia/reperfusion injury of the lung and liver (Wang H, 1999). It is capable of stimulating the release of proinflammatory cytokines, such as TNF, and upregulating endothelial adhesion (Andersson *et al*., 2000). It also contributes to activation of NADPH oxidase via a TLR4 signaling pathway in PMNs (Fan *et al*., 2007) . HMGB1 is a necessary and sufficient mediator of organ damage in severe sepsis (Chavan *et al*., 2012). It has been shown that administration of HMGB1 to experimental animals causes epithelial cell dysfunction and lethal organ damage (Wang, 1999; Yang, 2004). In contrast, administration of anti-HMGB1 antibodies prevents epithelial dysfunction and organ damage during sepsis in mice (Chavan *et al.*, 2012). Previous studies showed that polyphenolic compounds (-)-epigallocatechin-3-gallate  from green tea, oleanolic acid inhibited HMGB and protect from sepsis (Kawahara *et al*., 2009; Li *et al*., 2007).

Hemeoxygenase (HO) is an enzyme that catalyzes the degradation of heme to produce biliverdin, iron, and carbon monoxide. HO consists of constitutive and inducible isozymes (HO-1, HO-2) (Ryter *et al*., 2006). HO-1, which is released during endotoxemia, is a protein associated with oxidative stress protection. HO-1 is regulated by apoptotic stimuli, including heme, TNF-α, and apoptotic compounds. It has antiapoptotic, antioxidant, and immunomodulatory functions (MacGarvey *et al.*, 2012; Ryter *et al.*, 2006). Upregulation of HO-1 inhibits the release of cytokines, HMGB1, and NO, which may have a protective effect against the effects of endotoxemia in rats. HO-1 protects against severe inflammation, and its upregulation has been shown to inhibit the release of cytokines (e.g., TNF-α and IL-1β), HMGB1, and NO, which may have a protective effect against endotoxemia in animals (Bortscher *et al*., 2012; Yu & Yao, 2008). HO-1 also inhibits NF-κB, thereby modulating cytokine release and inhibiting inducible NOS, with a subsequent decrease in NO (Bellezza *et al*., 2012; Brouard *et al*., 2002; Carchman *et al*., 2011). Flavonoids were shown to induce HO-1

gene expression (Scapagnini *et al*., 2002). Similarly, potent HO-1-inducing abilities were identified in other natural products (such as (-)-epigallocatechin-3-gallate (EGCG)); moreover, these natural products include immunomodulators of LPS-induced HMGB1 release, and their administration increases the survival of HO-1-deficient mice (Li *et al.*, 2007; Takamiya *et al*., 2009).

Prostaglandin is a very important mediator of all types of inflammation. It is synthesized by the enzyme cyclooxygenase (COX) which is stimulated in the inflammatory phase by pro-inflammatory mediators, such as cytokines, LPS and carrageenan. Previous studies have shown that COX-2 is responsible for increased prostaglandin production in inflamed tissue (DeWitt, 1991). Previous studies have shown that phenolic compounds inhibited COX-2 such as phenolic acid and flavonoids (Yang *et al*., 2009). Honey has been proven to have a potent activity against gastritis and stomach ulcers (Kandil  *et al*., 1987b). Specific inhibition of COX-2 expression at the transcriptional level is a potent mechanism in the treatment of inflammatory disease (O'Banion *et al*., 1992). It is possible that honey and its extracts are selective inhibitors of COX-2 because honey has no side effects on the gastrointestinal system. In relation with the above results, the inhibition of $PGE_2$ by honey extracts is more pronounced. Ellagic acid has an inhibitory effect on $PGE_2$ release from monocytes and other phenolic compounds in such as quercetin, chrysin and luteolin which have been demonstrated to have  inhibitory effects on interleukin, 1β, and cyclooxygenase-2 (COX-2) expression, prostaglandin E2 ($PGE_2$) synthesis and NF-κB (Gutierrez-Venegas *et al*., 2007; Romier *et al*., 2008). Nitric oxide (NO) is a pleiotropic mediator of inflammation and was first discovered as a factor released from endothelial cells that causes vasodilatation by relaxing vascular smooth muscle (Furchgott, 1980). It has a short half-life (5–10 seconds), is derived from L-arginine, and is rapidly converted to

nitrate and nitrite after being secreted (Boveris A, 2002). NO is synthesized by the NOS family of enzymes, which includes the calcium-independent, inducible isoforms and the calcium-dependent, constitutive (cNOS) isoforms (Wang *et al*., 2012). It plays a controversial role in sepsis and septic shock. It has vasodilatory effects in sepsis (Landry & Oliver, 2001) ; has pro- and anti-inflammatory as well as antioxidant and oxidant properties (Enkhbaatar *et al*., 2009; Wink *et al*., 2001); and acts as a "vital poison" in the immune and inflammatory network (Dugas *et al*., 1995) . NO also causes increased leukocyte adhesion in the liver and lungs, as well as inhibits acute-phase protein production. The late phase of hypotension, cellular suffocation, apoptosis, vasoplegia, lactic acidosis, and multi-organ failure in endotoxic shock or severe sepsis is implicated with the levels of NO and its derivatives such as (ONOO-) (C, 2003). LPS was found to increase endothelial NO release, further suggesting that NO may be responsible for induction of hypotension in severe sepsis. NO is therefore a key contributory mediator of tissue damage in SIRS (Ishikawa *et al*., 2012; Iskit & Guc, 2003). An understanding of the biological, pharmacological, and pathological activity of NO during inflammatory diseases and sepsis has provided a new strategy for identifying therapeutic targets in the management of sepsis (Anderson, 2012). Nitric oxide (NO) is known to be an important mediator of inflammation (Misko *et al*., 1993). Inducible nitric oxide synthase (iNOS) is the enzyme responsible for NO production in the inflammatory response. Aminoguanidine, a highly selective inhibitor of iNOS (Romier *et al.*, 2008), totally inhibited NO production in activated macrophages at 1mM. Similarly, HME and HEAE dose-dependently inhibited the production of NO without affecting the viability of RAW 264.7 cells. Some flavonoids, including hesperetin, and naringin; induce HO-1 and can inhibit LPS-induced NO production. Moreover, genistein, kaempferol, quercetin, and daidzein inhibit the activation of the signal

transducer and activator of transcription 1 (STAT-1), another important transcription factor for iNOS (Hamalainen *et al*., 2007). Additionally, quercetin, caffeic acid, chrysin, ellagic acid and various polyphenolic compounds are known for their down-regulation of NF-κB (Del Boccio & Rotilio, 2004), this in turn reduces biosynthesis of iNOS, and ultimately inhibits the production of nitric oxide. Most of the phenolic compounds mentioned above were identified in this study; it can therefore be assumed that the inhibition of nitric oxide production by the honey extracts was due to these compounds. Although the concentrations of the phenolics identified were higher in HME, the in vitro anti-inflammatory activity seemed to be better for HEAE. It was reported that ethyl acetate extracts will contain a higher concentration of bioactive compounds; an example being the anti-inflammatory compound caffeic acid phenethyl ester (CAPE) (Del Boccio & Rotilio, 2004; Habtemariam, 2000; Song *et al*., 2002). This supports the observation that HEAE showed better activity. Peroxynitrite ($ONOO^-$) is produced by the reaction of superoxide and nitric oxide; it is a short-lived and highly oxidant molecule. Its decomposition to nitrate and nitrite is intimately coupled with the redox biochemistry of this species. It is a very active molecule and targets all parts of the cell from the membrane to the nucleus. Peroxynitrite levels increase during inflammation, which leads to the inactivation of interleukins and decrease in inducible NOS activity (Freels, 2002; Lanone, 2002; Zouki, 2001). Also, peroxynitrite enhances nuclear factor-κB (NFκB)-mediated proinflammatory activity (Seija *et al.*, 2012; Shacka, 2006). It is a potent inducer of cell death through different pathways such as MLK/p38/JNK, and stimulates the release of apoptosis-inducing factor (AIF) from the mitochondria, which subsequently triggers DNA fragmentation processes (Hao *et al*., 2011; Shacka, 2006; Zhang, 2002). Some physiological inhibitors of peroxynitrite, such as uric acid, have been used in many studies and showed valuable effects in animal models of

inflammation, sepsis and reperfusion injury (Kooy, 1994; Lanone, 2002; Nin *et al*., 2011; Robinson, 2004; Scott, 2002, 2005; Seija *et al.*, 2012; Soriano *et al*., 2011; Whiteman, 2002). Recent studies show that peroxynitrite stimulates the release of the mitochondrial apoptosis-inducing factor, which subsequently triggers DNA fragmentation (Zhang *et al*., 2002), release of mitochondrial pro-apoptotic factors, and cytochrome *c*-dependent apoptosis in the cytosol through peroxynitrite-dependent oxidation of the mitochondrial permeability transition pore. The key role of peroxynitrite in promoting mitochondrial dysfunction is clearly exemplified in experimental sepsis, in which peroxynitrite production results in the inhibition of mitochondrial respiration in the diaphragm in a process associated with mitochondrial protein nitration. The latter is prevented by NO synthase inhibitors and Mn-porphyrin therapy (Nin *et al*., 2004). Peroxynitrite-induced activation of the MLK/p38/JNK pathway also plays a crucial role in apoptosis (Dubuisson *et al*., 2004; Rhee *et al*., 2005; Trujillo *et al*., 2004). Earlier studies showed that inhibit NO production, and thus peroxynitrite formation, thereby reducing the effects of these cytotoxic compounds both *in vitro* and *in vivo*. Moreover, scavengers of peroxynitrite are known to be protective against tissue damage (Szabo *et al*., 2007). Some scavengers of peroxynitrite, such as uric acid, ebselen, mercaptoalkylguanidines, *N*-acetylcysteine, and dihydrolipoic acid, and some chemicals that work as decomposition catalysts of peroxynitrite, such as metalloporphyrins of iron and manganese, can attenuate the toxic effects of peroxynitrite*in vitro* and *in vivo* (Daiber *et al*., 2000; Hooper *et al*., 1998; Klotz & Sies, 2003; Lancel *et al*., 2004; Noiri *et al*., 2001; Ploner *et al*., 2001; Scott *et al*., 2005; Spitsin *et al*., 2000; Szabo, 2003; Szabo *et al*., 1997). These compounds can reduce 3-nitrotyrosine immunoreactivity in various pathophysiological conditions and have beneficial effects in animal models of inflammation, sepsis, and reperfusion injury

(Beckman, 1996; Briviba *et al*., 1996; Brodsky *et al*., 2004; Crow, 2000, 2006; Nin *et al*., 2004; Noiri *et al*., 2001; Tabuchi *et al*., 1995; Wang *et al*., 2003). Many phenolic compounds such as gallicacid, caffeic acid, kaempferol, ferulic acid, *p*-coumaric, and quercetin have been shown to inhibit peroxynitrite. Monohydroxylated phenolic compounds, such as ferulic acid and *p*-coumaric acid, act as peroxynitrite scavengers by nitration. On the other hand, compounds with a catechol moiety, such as caffeic acid and chlorogenic acid, reduce peroxynitrite by electron donation (Klotz & Sies, 2003; Pannala *et al*., 1998).

## 2.3.    Multiple Organ Dysfunction Syndromes (MODSs)

MODSs arise because of uncontrolled immune-inflammatory response and is characterized by generalized and persistant inflammation. This process results in cellular damage at the level of the endothelium, decreasing the endothelial permeability to fluid, proteins, and cells, as well as to gut bacteria. Cytokine and coagulation cascades are promoted, resulting in microvascular thrombosis and infiltration of organs with various leukocytes. Cellular death occurs in local and regional environments secondary to hypoxia, causing further release of toxic oxygen species and inflammatory mediators (Emura & Usuda, 2010; Luo *et al*., 2009). Thus, both hemorrhagic as well as septic shock have been recognized as the leading causes of MODS. In the 1980s, however, MODS was thought to arise secondarily to an overwhelming infectious process. Approximately 90% of multiple organ failure MOF in these patients was due to a septic source (Emura & Usuda, 2010). To date, several studies have concluded that MODS after a trauma represents the bridge from mild organ dysfunction to death; however, the process itself begins as early as within 24 hours of the original insult. Coagulopathy ensues, as well as hyperglycemia, dysfunction of the gut, and adrenal insufficiency, followed by CNS dysfunction and eventually death (de Montmollin &

Annane, 2011). Septic shock is the main cause of MODS in intensive care units, and the intensity of MODS is correlated directly to mortality (Carrillo Esper *et al.*, 2011; de Montmollin & Annane, 2011). Former studies showed that phenolic compounds such as Caffeic acid phenethyl ester and thymoquinone protect organs from sepsis-induced injury (Alkharfy *et al.*, 2011; Fidan *et al.*, 2007).

## 2.4. Animal models of inflammation and sepsis

Many animal models are used to study acute and chronic inflammation. In acute inflammation, the most common models are paw and ear edema induced with various chemicals such as carrageenan (Kumar & Kuttan, 2009; Murta & Ferrari, 2012; Nikota & Stampfli, 2012; Ogino *et al.*, 2009; Tweedie *et al.*, 2012), acetic acid (Whittle, 1964), xylene (Kou *et al.*, 2005), phorbolmyristate acetate (Griswold *et al.*, 1998), formalin (Wheeler-Aceto *et al.*, 1990), and oxazolone (Beaulieu *et al.*, 2007); some enzymes and fatty acids; histamine (Amann *et al.*, 1995) and arachidonic acid (Romay *et al.*, 1998); and components of bacterial cell wall such as LPS. In chronic inflammation, the most commonly used model is cotton pellet-induced granuloma (Shahavi & Desai, 2008). Sepsis models can be divided into exogenous administration of a toxin (such as LPS or endotoxins), a viable pathogen such as bacteria, or by alteration of the animal's endogenous protective barrier (inducing colonic permeability, allowing bacterial translocation) (Buras *et al.*, 2005; Li *et al.*, 2011; Orman *et al.*, 2011, 2012; Sadowitz *et al.*, 2011). LPS is a major constituent of gram-negative bacterial cell walls. It typically consists of lipid A and O-antigen. LPS is found both on the surface of phagocytic cells and as soluble proteins in the bloodstream. It is scavenged by neutrophils and macrophages through complex mechanisms and is transported by LPS-binding protein as well as membrane-bound or soluble CD14, enabling its interaction with TLRs on the phagocyte membrane. CD14 is a receptor of LPS in mice and in humans. The prototype

model for innate immune responses is leukocyte activation by LPS. Gram-negative bacterial infection accounts for approximately 60% of cases of sepsis in humans , and it causes sepsis in animal models (Brudecki *et al*., 2012). LPS has an important role in the pathogenesis of sepsis, and it is a highly stable and toxic molecule. It can activate numerous cells of the immune system to produce proinflammatory cytokines (e.g., TNF-$\alpha$, IL-1, IL-6, IL-8, IL-12, and IFN) and ROS. Understanding the physiological effects of LPS on the cellular and molecular levels holds the key to understanding the responses of innate immunity (Merrill *et al*., 2011; Yamanishi *et al*., 2012). Cecal ligation and puncture (CLP) belongs to the endogenous category and is considered the best standard for sepsis research (Parker & Watkins, 2001). The CLP model was designed to simulate ruptured appendicitis or perforated diverticulitis in humans (Jung *et al*., 2012). The technique involves midline laparotomy, exteriorization of the cecum, ligation of the cecum distal to the ileocecal valve, and puncture of the ligated cecum. This procedure generates bowel perforations with leakage of fecal contents into the peritoneum, which establishes an infection with mixed bacterial flora and provides an inflammatory source of necrotic tissue. The CLP technique has achieved popularity because of its ease of use, general reproducibility, and similarity to human disease progression. Most markedly, the CLP model recreates the hemodynamic and metabolic phases of human sepsis (Dejager *et al*., 2011). Moreover, apoptosis of selected cell types and host immune responses seem to mimic the course of human disease, adding further clinical validity to this model (Ayala & Chaudry, 1996; Hotchkiss & Karl, 2003). One comparison study has shown that LPS causes a rapid induction of cytokines, followed by an early decline in mice, whereas CLP induces a slower sustained increase of cytokines in both the plasma and peritoneum, which mimic the responses in sepsis patients (Dejager *et al.*, 2011).

## 2.5.    Natural product

Throughout human history, herbal medicine has formed the basis of folk remedies for various inflammatory ailments. The use of willow bark extract to reduce pain and fever was documented by a Greek physician (Hippocrates) in the 5th century BC, and the subsequent discovery of salicylic acid as its pain/fever-relief active component gave rise to the first synthetic anti-inflammatory drug, aspirin, and the birth of the pharmaceutical industry (Yang & Landau, 2000). Honey is a sweet and viscous fluid produced by bees from floral nectars. It includes over 400 different chemical compounds, more than 95% of which mainly consist of sugars and water. Its major components are carbohydrates (glucose and fructose, as well as disaccharides such as maltose and sucrose, and trisaccharides such as melezitose (Lazaridou *et al*., 2004). Proteins, enzymes, organic acids, mineral salts, vitamins, phenolic acids, flavonoids, free amino acids, and volatile compounds constitute the minor components of honey (Alissandrakis, 2005). The moisture content in honey usually amounts to 20%, reaching, in some exceptional cases, almost 23% in heather honey. The characteristics of the moisture content depend on the water activity in the honey. In liquid honeys, water activity reaches values from aw = 0.53 for 15.8% of water content to aw = 0.69 for 22.20% of water content (Sanz S., 1994).

Honey is alluded to in the ancient Sumerian and Babylonian cuneiform writings, the Hittite code, the sacred writings of India (Vedas), and in the ancient writings of the ancient Egyptians, Chinese, Greeks, and Romans (Jackson, 1995). Muslim physicians use honey to heal a variety of human diseases. In the Qur'an and many Prophetic narrations, honey is described as a universal healing agent. **"And your Lord inspired the bee, saying: 'Take you habitations in the mountains and in the trees and in what they erect. Then, eat of all fruits and follow the ways of your Lord made easy**

**for you.' There comes forth from their bellies a drink of varying color wherein is healing for mankind. Verily, in this is indeed a sign for people who think." (Al-Qur'an 16:68-69)**.The Muslim traditionalist Abu Sa'id al-Khudri related that a man came to the Prophet (peace and the blessings of Allah upon him) and said: "My brother has some abdominal trouble." The Prophet (peace and the blessings of Allah upon him) said to him "Let him drink honey." The man returned to the Prophet (peace and the blessings of Allah upon him) and said, "O Messenger of Allah! I let him drink honey but it caused him more pain." The Prophet (peace and the blessings of Allah upon him) said to him, "Go and let him drink honey!" The man went and let his brother drink honey, then returned  back and said, "O Messenger of Allah, it did not cause him except more pains." The Prophet (peace and the blessings of Allah upon him) then said, "Allah has said the truth, but your brother's abdomen has told a lie. Let him drink honey." So he made him drink honey and he was healed (Compiled by Bukhari (a).Narrated by Ibn 'Abbas). The Prophet (peace and the blessings of Allah upon him) said, "Healing is in three things: cupping, a gulp of honey or cauterization, (branding with fire) but I forbid my followers to use cauterization (branding with fire)." (Bukhari (b), Muslim). It is also reported that the Prophet (peace and the blessings of Allah upon him) said, "Make use of the two remedies: honey and the Qur'an".

Honey is a naturally sweet viscous fluid produced by bees from floral nectar. To date, more than 400 different chemical compounds have been identified in many varieties of honey (Lazaridou *et al.*, 2004), including proteins, enzymes, organic acids, mineral salts, vitamins, phenolic acids, flavonoids, free amino acids, fatty acids and small quantities of volatile compounds (Gheldof *et al.*, 2003; Weston & Brocklebank, 1999). Carbohydrates are the major components in honey, which are mainly monosaccharides such as glucose (30.31%) and fructose (38.38%). The next

major components are the disaccharides such as sucrose, maltose, turanose, isomaltose, and maltulose. In addition, honey also contains oligosaccharides (Rizelio *et al*., 2012; Sereia *et al*., 2011; White, 1980). Many organic acids have been discovered in honey including formic, butyric, gluconic, acetic, citric, maleic, oxalic, lactic, succinic, and pyroglutamic acids (White, 1978a), in addition to glycolic, pyruvic, α-ketoglutaric, 2- or 3-phosphoglyceric, and tartaric acids; glucose-6-phosphate; and α- or β-glycerophosphate. Many amino acids are also present in honey, proline being the most dominant (Daniele *et al*., 2012; White, 1978b). Some antibiotic peptides (e.g., lysozymes, apidaecin, and abaecin) were found in the body fluid of bees that had been injured and infected by bacteria, showing that honey has a nonperoxide antibacterial activity (Casteels, 1993; Fujiwara, 1990; Hultmark, 1996; Weston, 2000). Enzymes are another important constituent of honey; they play important roles in the production of honey from the nectar of the plant. Additionally, enzymes are heat sensitive and their activities are decreased during storage, which can be used as indicators of the freshness of honey (Crane, 1979). The enzymes in honey are known to have antioxidant properties. Glucose oxidase is a highly specific enzyme, the nonenzymatic hydrolysis of which using molecular oxygen spontaneously releases hydrogen peroxide from gluconic acid (Chaplin, 1990; Uhlig, 1998). Invertase (α-glucosidase) is added to the nectar by the bees during the process of harvesting and ripening of honey. Amylase (α- and β) (diastase) break down starch. Catalase breaks down hydrogen peroxide, which is responsible for antimicrobial activity. Moreover, acid phosphatase has been detected in nectar, but the activity of this enzyme in honey is not yet known. Honey contains several essential vitamins, particularly B, C, and E (Crane, 1979). It also contains several minerals including calcium, chlorine, potassium, sulfur, sodium, phosphorus, magnesium, silica, iron, manganese, and copper (White, 1980). The interest in phenolic

compounds in honey has only recently increased due to their potential roles in biological activity and as biochemical markers for authenticating the geographical (Martos, 2000b; Tomás-Barberán, 1993a) or botanical origins of honeys (Andrade, 1997b, 1997a; Tomás-Barberán, 1989, 2001) or both (Anklam, 1998; Anklam, 2001; Martos, 2000b, 1997). Many groups of phenolic compounds, such as flavonoids, phenolic acid, and other polyphenolic compounds, are found in honey. Flavonoids are a group of phenolic compounds that are relatively lipophilic and act as antioxidants (Shahidi, 1992). Flavonoids in honey can originate from nectar, propolis, or pollen (Ferreres, 1992). In general, the flavonoid concentration in honey is approximately 20 mg/kg (Ferreres, 1994c). The concentration of flavonoid in honey is much lower than in propolis (Bogdanov, 1989; Ferreres, 1991). More than 30 flavonoids have been identified in honey, such as chrysin, quercetin, luteolin, kaempferol, and apigenin (Boudourova-Krasteva, 1997). Many flavonoids are used to treat diseases such as inflammation and cancer (Jang *et al*., 1997). Phenolic acids act as potent antioxidants. Over 70 other phenolics have been identified from honey and propolis (Andrade, 1997b, 1997a; Bankova, 1987; Joerg, 1996; Joerg, 1992, 1993; Sabatier, 1992b; Tazawa, 1999). The most common phenolic acids found in honeys are benzoic acids and cinnamic acids, and their esters such as caffeic acid and its ester caffeic acid phenyl ester (Sabatier, 1992a). Like flavonoids, many phenolic compounds are also used to treat diseases such as inflammation and cancer (Jang *et al.*, 1997). The antimicrobial activity of honey has been proven by many studies. The active factors identified concerning this antimicrobial activity include hydrogen peroxide, phenolic compounds, and other chemical compounds, which are responsible for the acidity and osmolality of honey (White, 1963). Hydrogen peroxide was the first and the major antimicrobial factor discovered in honey. It has been shown to confer good protection against some harmful

microorganisms (White, 1963). The Malaysian Gelam honey, for example, has been reported to have antimicrobial activities (Aljadi A. M. , 2003). Besides its many beneficial properties as discussed above, the viscosity of honey provides protection in the affected area, allows absorption of edema fluids, and accelerates the formation of new granulation tissues (Bulman, 1953). Honey is a popular wound healing dressing owing to its anti-inflammatory, antioxidant, and antimicrobial activities. It is also known to reduce pain, is nontoxic, is self-sterile, and is nutritive. Honey has been shown to promote wound healing in ulcer, gangrene, and skin graft (Postmes, 1997; S.E, 1993). Malaysian honey has been proven to stimulate fibroblast cells, activate epithelization, and accelerate the healing process (Aljadi A. M. , 2003). Infections of the intestinal tract are extremely common. It affects humans of all ages worldwide. Honey has a bactericidal activity against many enteropathogenic organisms, including *Shigella* and *Salmonella* species and *E. coli* (Jeddar, 1985). Previous studies showed that a 20% solution of honey inhibited *Helicobacter pylori* in vitro. In contrast, *H. pylori* exhibited resistance to other antimicrobial agents (AT, 1991). Honey has been used to treat patients with gastritis, duodenitis, and duodenal ulcers. After recovery following treatment, it was found that the hemoglobin levels of most patients had increased and that fecal blood loss had decreased (Salem, 1982). Previous animal and clinical studies have also shown that honey reduces the secretion of gastric acid. Additionally, gastric ulcers have been successfully treated by the use of honey as a dietary supplement (Kandil *et al*., 1987a). Topical application of honey has been reported to reduce inflammation (Subrahmanyam, 1998), edema, and exudation (Subrahmanyam, 1996). Honey has also been shown to reduce the number of inflammatory cells infiltrating the wound area (Postmes, 1997), and has been demonstrated to show anti-inflammatory activity . Honey antioxidant activity is one of the most important properties of honey

due to the diversity of its components that can act as antioxidants (Tonks *et al*., 2003). The antioxidant sources and, consequently, the activity of these antioxidants depend on the flora of honey (Gheldof, 2002b). The sources of antioxidant in honey are phenolic compounds (e.g., flavonoids, phenolic acid, and polyphenolic acid), vitamins (e.g., vitamin C and E), enzymes (e.g., glucose oxidase, catalase), organic acid, carotenoid-like substances, and Maillard reaction products (Frankel, 1998; Gheldof, 2002a; Gheldof, 2002c). Many studies have implicated antioxidant activity with protection from many diseases such as cancer and cardiovascular, autoimmune, and inflammatory diseases (Briviba *et al.*, 1996; Brodsky *et al.*, 2004; Grimble, 1994; Nuttall *et al*., 1999; Taylor *et al*., 1994).

# CHAPTER 3.  RESULTS

Results are presented as compilation of all papers.

## 3.1  Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity

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# Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity

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## Abstract

Natural honey has been used in traditional medicine of different cultures throughout the world. This study looked into the extraction of Malaysian honey and the evaluation of the anti-inflammatory activity of these extracts. It was hypothesized that honey extracts contain varying amounts of phenolic compounds and that they possess different in vitro anti-inflammatory activities. Honey extracts were analyzed using liquid chromatography–mass spectrometry to identify and compare phenolic compounds, whereas high-performance liquid chromatography was used for their quantification. Subsequently, honey methanol extract (HME) and honey ethyl acetate extract (HEAE) were tested in vitro for their effect on nitric oxide production in stimulated macrophages. The extracts were also tested for their effects on tumor necrosis factor–α (TNF) cytotoxicity in L929 cells. The major phenolics in the extracts were ellagic, gallic, and ferulic acids; myricetin; chlorogenic acid; and caffeic acid. Other compounds found in lower concentrations were hesperetin, $p$-coumaric acid, chrysin, quercetin, luteolin, and kaempferol. Ellagic acid was the most abundant of the phenolic compounds recorded, with mean concentrations of 3295.83 and 626.74 $\mu$g/100 g of honey in HME and HEAE, respectively. The median maximal effective concentrations for in vitro nitric oxide inhibition by HEAE and HME were calculated to be 37.5 and 271.7 $\mu$g/mL, respectively. The median maximal effective concentrations for protection from TNF cytotoxicity by HEAE and HME were 168.1 and 235.4 $\mu$g/mL, respectively. In conclusion, HEAE exhibited greater activity in vitro, whereas HME contained a higher concentration of phenolic compounds per 100 g of honey.
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## 1. Introduction

Honey, which is consumed worldwide, is increasingly being used as a substitute for granulated sugar [1]. In

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addition to its sweetening properties and lower glycemic load [2]; honey is an important natural source of antioxidants and has potential therapeutic value in the treatment of heart disease, cancer, cataracts, and several inflammatory diseases [3]. The therapeutic actions of honey include antioxidant capacity and antimicrobial properties, as well as wound-healing and anti-inflammatory activities [4,5].

Of particular interest in this study is honey's anti-inflammatory activity. Inflammation is a nonspecific response of mammalian tissues to a variety of hostile agents [6]. There are many mediators of inflammation, examples of which are some cytokines and nitric oxide (NO). Tumor necrosis factor–$\alpha$ (TNF-$\alpha$) is a pleiotropic cytokine that induces a wide range of biological effects, including production of inflammatory cytokines, cell proliferation, differentiation, and death [7]. Nitric oxide is known to be an important mediator of acute and chronic inflammation [8]. Although the anti-inflammatory activity of honey has been studied previously [9], this is the first time, to the best of our knowledge, that the effects of Malaysian honey extracts on TNF activity and NO inhibition have been evaluated in vitro. Natural products present in our daily diet were revealed to be potential chemopreventive compounds due to their ability to adjust and modify cellular responses to abnormal proinflammatory stimuli [10]. In fact, flavonoids, such as those found in many plants and honey, show various cytoprotective effects [11,12]. It is hoped that, via empirical evidence of its benefits, more individuals will turn to incorporating honey, a highly nutritious and healing food, into their daily diet as a prophylaxis for inflammation.

There are various components in honey; and its antioxidant activity can be attributed to the following elements: flavonoids, phenolic acids, ascorbic acid, catalase, peroxidase, carotenoids, and products of the Maillard reaction [13]. The quantity of these components varies widely according to the floral and geographical origin of the honey [14]. Phenolic compounds are one of the important groups of compounds that occur in plants. These compounds are reported to exhibit anticarcinogenic, anti-inflammatory, antiatherogenic, antithrombotic, immune-modulating, and analgesic activities, among others, and exert these functions as antioxidants [15-17].

Because of the presence of various phenolic compounds in honey [18], it is hypothesized that different extraction methods and solvents will yield extracts containing differing phenolic concentrations. Furthermore, because honey has been used for various therapeutic purposes, we also propose that the extracts will exhibit anti-inflammatory effects in vitro. In addition, as it has been demonstrated that the phenolic content of honey correlates with various biological activities [3,19], it is then assumed that the extracts and their differing phenolic contents will have distinctive anti-inflammatory activities. The objectives of this study were to identify and quantify phenolic compounds in honey, most of which are bioactive flavonoids, subsequent to the extraction of honey with 2 different solvents, namely,

methanol and ethyl acetate. Liquid chromatography–mass spectrometry (LC-MS) and high-performance liquid chromatography (HPLC) were used for the identification and quantification of these compounds, respectively. Honey methanol extract (HME) and honey ethyl acetate extract (HEAE) were tested on 2 in vitro models of inflammation with the specific aim of evaluating the extracts' ability both to inhibit NO production by stimulated macrophages and to protect L929 cells from TNF-mediated cytotoxicity.

## 2. Methods and materials

### 2.1. Materials

Fresh Malaysian honey (Gelam, collected by *Apis mellifera*; Brix value = 21%) was obtained from the National Apiary, Department of Agriculture, Parit Botak, Johor; Malaysia. The physical characteristics of honey were a smooth, amber liquid appearance with a strong penetrating odor and a solubility of 99.9% in warm water. All chemicals and reagents used were of analytical grade.

### 2.2. Extraction of phenolic compounds from honey by XAD-2 resin

The honey extract was prepared as described in previous studies [14,20] with some modifications. Liquefied honey (100 g) was thoroughly mixed with acidified deionized water (500 mL), adjusted with concentrated hydrochloric acid to pH 2 for 60 minutes (with no heating), until completely dissolved. The resulting solution was filtered by vacuum suction to remove particles. The filtrate was mixed with 150 g of clean, swelled XAD-2 resin and stirred slowly with a magnetic stirrer for 60 minutes. The XAD-2 resin/honey solution slurry was poured into a glass column (42 × 3.2 cm); and the resin was washed at a rate of 10 mL/min with acidified water (300 mL, pH 2), followed by rinsing with deionized water (500 mL at 10 mL/min) to remove all sugars and other polar constituents of honey.

The phenolic compounds adsorbed onto the column were eluted with methanol (1000 mL, adjusted to pH 7). The extract was concentrated to dryness on a rotary evaporator at 40°C under reduced pressure. The extract was divided into 2 portions: one was redissolved in 1 mL methanol (HPLC grade) and filtered through a 0.45-$\mu$m membrane filter before HPLC analysis, whereas the other was redissolved in deionized water (5 mL) and extracted with ethyl acetate (5 mL × 3) instead of diethyl ether [14]. It can be presumed that ethyl acetate can extract more flavonoids and other phenolic compounds than diethyl ether, as the former is a more polar solvent [14]. Methanol and honey ethyl acetate extracts and standard phenolic compounds prepared at a concentration of 100 $\mu$g/mL were evaporated to dryness by flushing with nitrogen while being warmed on a hot plate. The dried residues were redissolved in 1 mL methanol. The solutions were filtered through a 0.45-$\mu$m membrane filter before LC-MS analysis.

### 2.3. HPLC analysis

Twenty microliters of each sample was injected into the HPLC machine. The phenolic compounds were detected using UV absorption spectra monitored at 290 and 340 nm; the majority of honey flavonoids and phenolic acids demonstrate their UV absorption maximum at these 2 wavelengths [14]. The column used was a reversed-phase C18 column, Agilent ZORBAX Eclipse XDC18 ($3 \times 250$ mm; particle size, 5 $\mu$m) (Agilent Technologies, Santa Clara, Calif). The mobile phase constituted of 0.25% formic acid and 2% methanol in water (solvent A) and methanol (solvent B) at a constant solvent flow rate of 1 mL/min. The following gradient was used according to the previously mentioned method with minor modifications: there was an isocratic flow through the column with 10% solvent (B) and 90% solvent (A) for 15 minutes, before increasing to 40%, 45%, 60%, 80%, and 90% at 20, 30, 50, 52, and 60 minutes, respectively. Isocratic elution followed with 90% methanol (B) from the 60th to 65th minute. Finally, the gradient was changed to 10% methanol from the 65th to 68th minute; and the composition was held until the 73rd minute. A calibration curve of caffeic acid at 290 nm was used to calculate phenolic acids concentrations, whereas calibration curves of quercetin and ellagic acid at 340 nm were used for flavonoids and other polyphenolics, respectively. This is because the different phenolic compounds are absorbed better at these wavelengths [14]. The calibration curves of the standards were used to determine the concentrations of the phenolic compounds in the extracts [21].

### 2.4. LC-MS condition

Analyses of phenolic compounds by LC–electrospray ionization (ESI)–MS were carried out using a Thermo Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan Co, San Jose, CA) equipped with an electrospray interface. Liquid chromatography separation was performed on a reversed-phase Zorbax SB-C18 column ($250 \times 4.6$ mm; particle size, 5 $\mu$m; Agilent Technologies) at 25°C. The conditions of LC-MS were the same as HPLC, although solvent A was replaced with 1% acetic acid in water in the mobile phase. The UV detector was set to an absorbance wavelength of 280 to 340 nm. The ESI parameters were as follows (optimized depending on compounds): nebulizer, 30 psi; dry gas (nitrogen) flow, 10 $\mu$L/min; and dry gas temperature, 325°C. The ion trap mass spectrometer was operated in negative and positive ion modes with a scanning range of $m/z$ 50 to 800.

### 2.5. Activity of honey extracts in vitro

#### 2.5.1. Cell culture

Murine fibrosarcoma cell line L929 was purchased from American Type Culture Collection (Manassas, VA). Murine macrophage cell line RAW264.7 was obtained from the Department of Biotechnology, University Putra Malaysia. Cells were maintained in high glucose Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum and no

antibiotics, undergoing passage every 2 to 3 days with standard aseptic techniques. Cells from 70% to 90% confluent flasks with greater than 90% viability were seeded in 96-well culture plates by dispensing 100 $\mu$L per well. Cell density was $1 \times 10^5$ (L929) or $1 \times 10^6$ (RAW264.7) cells per milliliter of culture medium. The plates were incubated for 24 hours (L929) or 2 hours (RAW264.7) at 37°C, after which they were treated with honey extracts and a combination of agents, as detailed in "Section 2.5.2."

#### 2.5.2. Cell viability and cytotoxicity

In both assays, cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as described by Mosmann [22] with some modifications. Following 24-hour incubation of the cells with the extracts and controls, culture medium was replaced with 100 $\mu$L fresh DMEM and 20 $\mu$L of 5 mg/mL MTT and incubated for 1 hour. Subsequently, the cell medium was aspirated; and 100 $\mu$L of 100% dimethyl sulfoxide was added to all wells to dissolve the insoluble purple formazan product into a colored solution, the absorbance of which was measured at a wavelength of 570 nm using a microplate reader (Hidex Chameleon, Turku, Finland). The optical density (OD) of the samples was compared with that of the negative control to obtain the percentage viability, as follows: cell viability (%) = [(OD$_{570}$ (sample)/OD$_{570}$ (negative control)) $\times$ 100].

#### 2.5.3. TNF-α cytotoxicity assays

To measure the ability of the extracts to protect against TNF-α–induced cytotoxicity, 2 methods were used as described in previous studies [23-25] with some modifications. In the first method, L929 cells seeded in 96-well plates were pretreated with various concentrations of the honey extracts (50-250 $\mu$g/mL) and actinomycin D (1 $\mu$g/mL) for 30 minutes. Tumor necrosis factor–α was added to the treated wells at a final concentration of 1 ng/mL [23]. The same method was applied for the second assay, but excluded actinomycin D [25,26]. Cells treated with anti–TNF-α were used as a positive control in both assays. The plates were incubated for another 24 hours, after which viability was assessed by microscope examination and the MTT colorimetric assay. The viability of cells in treated wells was compared with that of the dimethyl sulfoxide–treated negative control.

#### 2.5.4. NO inhibition assay

Tests were prepared as described in previous studies [27,28] with some changes. Murine macrophage RAW264.7 cells were seeded in 96-well plates with a cell density of $5 \times 10^4$ cells per well and incubated for 2 hours. The cells were stimulated with interferon-γ (IFN-γ) and lipopolysaccharide (LPS) with final concentrations of 200 U/mL and 10 $\mu$g/mL, respectively, in DMEM without phenol red. Stimulated cells were treated either with the honey extracts at different concentrations (0, 25, 50, 75, and 100 $\mu$g/mL) or with the inducible nitric oxide synthase (iNOS) inhibitor aminoguanidine at 1 mmol/L as a positive control; untreated cells were used as negative controls. The final

volume per well was 100 $\mu$L. The plates were then incubated for 16 to 20 hours at 37°C, 5% $CO_2$.

Following incubation, NO inhibition was assessed by quantifying nitrite ($NO_2^-$) released in the culture medium via the Griess reaction [29]. Fifty microliters of cell supernatant from treated and untreated wells was mixed with an equal volume of the Griess reagent. The resulting color was measured at 550 nm with a microplate reader (Tecan Sunrise, Grödig, Austria). The absorbance values were compared with a standard sodium nitrite curve and converted to corresponding nitrite concentrations (in micromoles per liter). The percentages of NO inhibition by the extracts were calculated as follows:

$$\% \text{ Inhibition} = 100 \times \left[ \left( \left[ NO_2^- \right] \text{control} - \left[ NO_2^- \right] \text{sample} \right) \right.$$
$$\left. / \left[ NO_2^- \right] \text{control} \right]$$

### 2.6. Statistical analyses

The values represent the mean ± standard deviation of 5 replicates for HPLC and LC-MS analyses of the honey extracts. On the other hand, data were collected from 3 independent experiments for in vitro assays. Data were expressed as the mean ± standard deviation. Data were analyzed using either unpaired $t$ test or 1-way analysis of variance followed by Tukey multiple comparison tests, as indicated. Graph Pad Prism (version 4; GraphPad Software Inc., La Jolla, Calif) statistical software was used for the analysis, and $P$ value < .05 was considered statistically significant. Post hoc power analysis was conducted using the software Primer of Biostatistics (version 6.0; McGraw-Hill, New York, NY); the range of power of the tests conducted was 0.8 to 0.95, where $\alpha = .05$.

The median maximal effective concentrations ($EC_{50}$) for the inhibition of NO production in RAW264.7 cells and inhibition of TNF cytotoxicity in L929 by the honey extracts were calculated using sigmoidal dose-response (variable slope) equation under nonlinear regression (curve fit) with Graph Pad Prism 4.

### 3. Results

One hundred grams of liquefied fresh Malaysian honey *Apis mellifera* yielded 52 ± 0.17 and 10 ± 0.13 mg of methanol and ethyl acetate extracts, respectively. The yield was significantly different for the 2 extracts when compared with unpaired $t$ test ($P < .001$). The yield ratio for HEAE to HME was ca 1:5 for every 100 g of honey.

### 3.1. Identification and quantification of phenolic compounds in Malaysian honey by HPLC and LC-MS

Compared with the methanolic extract, a lower recovery of gallic acid and ellagic acid was observed in HEAE in chromatograms recorded at 290 and 340 nm. Fig. 1 shows the UV absorption chromatogram of Malaysian honey at 290 nm, following isolation by XAD-2 then extraction with ethyl acetate. Standard compounds eluted from XAD-2 resin showed the following recovery ranges: 18% to 45% for phenolic acids, except for gallic acid that had a recovery of 3%. The flavonoids had a recovery of 35% to 90%, and the polyphenol ellagic acid had a recovery of 4%. The concentrations of phenolic compounds in Malaysian honey calculated from peak areas of the compounds found in both HME and HEAE are summarized in Table 1.

Ellagic acid recorded the highest concentration among the phenolic compounds in Malaysian honey in both extraction methods, with a total of 3295.83 $\mu$g/100 g of honey in XAD-
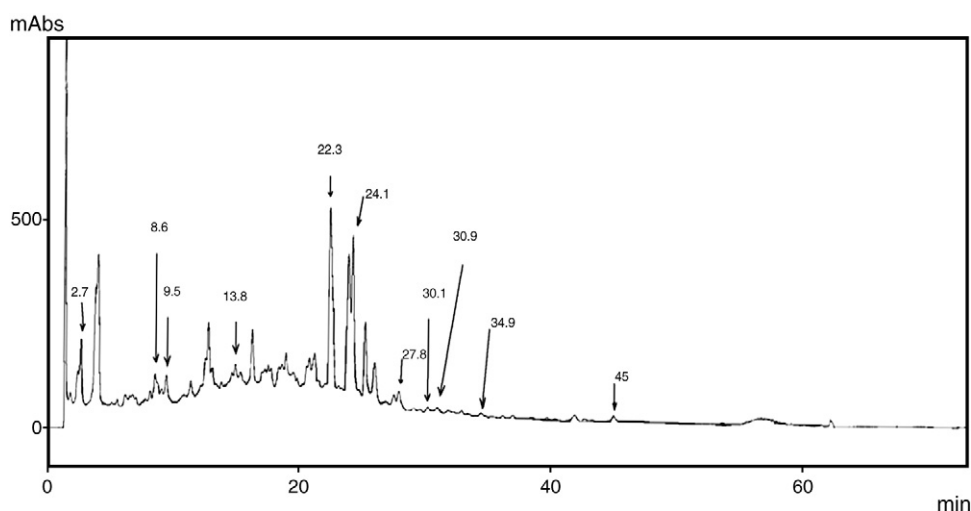


Fig. 1. Absorption chromatogram at 290 nm of honey phenolic acids and flavonoids detected in Malaysian honey using HPLC: 2.7 minutes = gallic acid, 8.6 minutes = chlorogenic acid, 9.5 minutes = caffeic acid, 13.8 minutes = *p*-coumaric acid, 16.3 minutes = ferulic acid, 22.3 minutes = ellagic acid, 24.1 minutes = myricetin, 27.8 minutes = quercetin, 30.1 minutes = hesperetin, 30.9 minutes = luteolin, 34.9 minutes = kaempferol, 45 minutes = chrysin.

Table 1
Concentrations of phenolic compounds detected in Malaysian honey using HPLC (each value represents the mean ± SD)

| Retention time (min) | µg/100 g honey at 290/340 nm ethyl acetate extract | µg/100 g honey at 290/340 nm methanol extract | Compounds |
|---|---|---|---|
| 2.7 | 35.37 ± 2.8 | 341.01 ± 28 | Gallic acid |
| 8.6 | 34.84 ± 3.1 | 153.65 ± 12.5 | Chlorogenic acid |
| 9.5 | 37.36 ± 2.9 | 158.39 ± 17.4 | Caffeic acid |
| 13.8 | 39.90 ± 3.7 | 80.68 ± 7.6 | p-Coumaric acid |
| 16.3 | 78.99 ± 9.3 | 239.08 ± 19.4 | Ferulic acid |
| 22.3 | 626.74 ± 56.2 | 3295.83 ± 38.6 | Ellagic acid |
| 24.1 | 93.01 ± 12.3 | 223.57 ± 27.1 | Myricetin |
| 27.8 | 22.63 ± 1.9 | 66.50 ± 7.2 | Quercetin |
| 30.1 | 46.5 ± 5.6 | 109.27 ± 13.5 | Hesperetin |
| 30.9 | 8.60 ± 1.1 | 33.61 ± 4.2 | Luteolin |
| 34.9 | 10.71 ± 1.3 | 16.12 ± 1.9 | Kaempferol |
| 45 | 8.75 ± 0.9 | 69.01 ± 8.7 | Chrysin |

The HEAE and HME were analyzed with HPLC with the UV detector set at 290/340 nm. The unknown concentrations of the phenolic compounds in the honey extracts were derived by calculating the peak area from the calibration curves of the standards used. Values represent mean concentration ± standard deviation of 5 replicates (HME: n = 5, HEAE: n = 5).

2 without ethyl acetate extraction and 626.7 µg/100 g in XAD-2 with ethyl acetate extraction. Liquid chromatography–MS was used for the identification of some phenolic compounds. Fig. 2 depicts the peak of chrysin detected in Malaysian honey using negative ESI-MS. Summarized in Table 2 are the mass spectra, UV spectra, and fragments of the identified compounds using positive and negative

ionization. Some compounds did not ionize under the conditions used for analysis. In addition, as displayed in Table 2, negative ESI-MS was more useful for identifying compounds in the extracts than positive ESI-MS.

Liquid chromatography–MS analysis for the identification of the phenolic constituents in honey extracts demonstrated the presence of phenolic compounds in free form (aglycones), derivative, as well as conjugated forms (sugar moieties). As presented in Table 2, the peaks at 8.39 and 11.53 minutes were both identified as ferulic acid (molecular weight [MW] = 194) [M − H] −193 $m/z$ at 8.39 minutes and [M − H − H$_2$O] 175 $m/z$ at 11.53 minutes after water loss. The peak at 23.51 minutes was identified as ellagitannin (MW = 802) [M + H] +803 $m/z$, which is in agreement with a previous study [30].

Some phenolic compounds appeared as a sugar moiety, such as ellagic 3-O-glucoside, rhamnosyl naringenin, and quercetin-3-O-glucoside. Hesperetin, ellagic acid, and quercetin have identical MWs of 302 g/mol; however, it has been reported that the MS$^n$ fragmentation pattern can be used to distinguish between these compounds. Further ionization produced major fragments at $m/z$ 271, 255, 179, and 151, which demonstrated the presence of quercetin as an aglycone, but not ellagic acid [31], whereas further fragments of hesperetin produced major ions at $m/z$ 286, 188, and 164 [32]. Besides the fragmentation patterns, retention time and UV spectra are also very important to differentiate between hesperetin, quercetin, and ellagic acid. Moreover, some compounds were identified using both positive and negative ionization such as elenolic acid
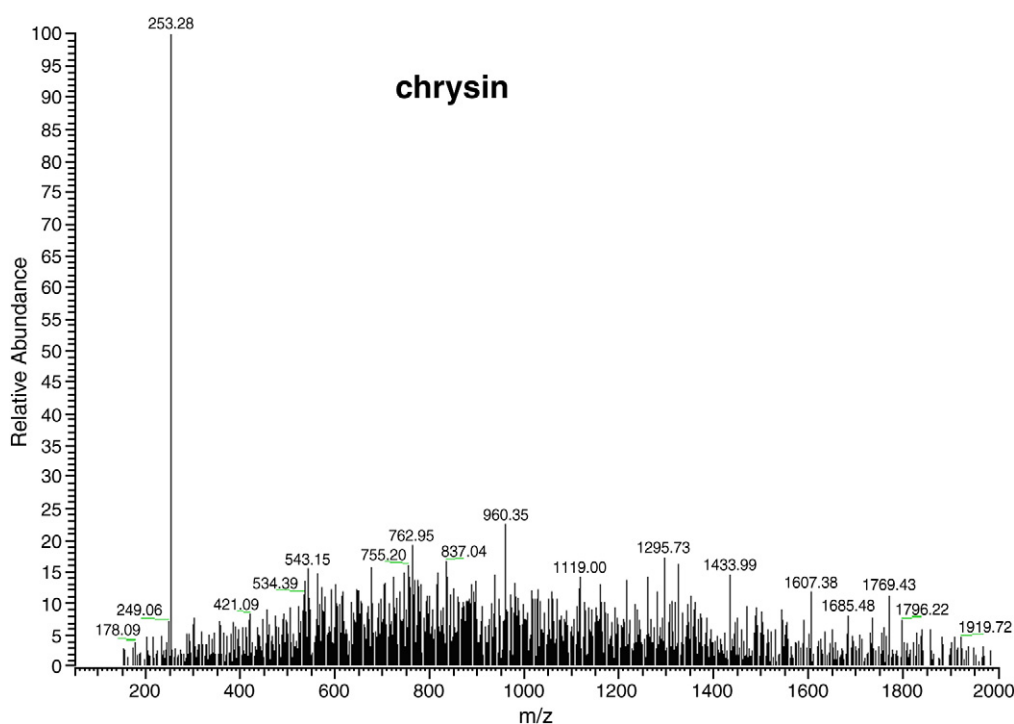


Fig. 2. Electrospray ionization–mass spectra negative ionization for chrysin. MW = 254.242 g/mol, ESI-MS [M − H] = 253.28.

Table 2
Phenolic compounds identified in Malaysian honey using LC-MS

| Retention time (min) | MW | MS | ESI-MS [M ± H] | UV band (nm) | Fragment ions | Compound names |
|---|---|---|---|---|---|---|
| 8.39 | 194 | 175 | − | 296, 324 | | Ferulic acid |
| 11.53 | 194 | 193 | − | 296, 324 | 175 | Ferulic acid |
| 21.35 | 299 | 298 | − | | 242, 172, 158 | Kaempferide |
| 21.66 | 464 | 463 | − | | 361, 303, 199, 172 | Ellagic-glucoside |
| 22.64 | 242 | 243 | + | 235, 260, 350, 385 | | Elenolic acid |
| 22.43 | 242 | 241 | − | 235, 260, 350, 385 | | Elenolic acid |
| 23.51 | 801 | 803 | + | 235, 285 | 161, 261, 303 | Ellagitannin |
| 23.64 | 302 | 301 | − | | 242, 199, 155 | Quercetin |
| 24.63 | 302 | 303 | + | 235, 280, 235 | 263, 203 | Ellagic acid |
| 27.23 | 302 | 303 | + | 270,290 | 249, 188 | Hesperetin |
| 29.35 | 299 | 298 | − | | 172, 158 | Kaempferide |
| 29.39 | 299 | 300 | + | | 172, 158 | Kaempferide |
| 33.26 | 254 | 253 | − | 260, 295, 320, 335 | 178 | Chrysin |
| 38.09 | | 229 | + | — | 195, 183 | Unknown |
| 55.22 | 462 | 463 | + | | 303, 271, 165 | Quercetin-3-*O*-glucoside |
| 59.95 | 418 | 272 | + | | 273, 204 | Rhamnosyl naringenin |

The phenolic compounds above were identified in Malaysian honey using LC-MS. This was achieved by comparing the mass spectrometric data with standards and literature data. Both positive and negative ionizations were used to detect the MS and fragment ions. Data shown are from a single experiment and are representative of 3 experiments.

[M + H] 243 $m/z$ and [M − H] 241$m/z$, and kaempferide [M + H] 300 $m/z$ and [M − H] 298 $m/z$.

### 3.2 Viability and cytotoxicity

#### 3.2.1. Effect of honey extracts on L929 and RAW264.7 cells viability

The effect of the extracts on the viability of cells is important to distinguish between their toxic and therapeutic effects. This is especially important in the NO assay to indicate that the reduction of NO release is due to the inhibition of inflammatory pathways rather than cell death, which will also alter the concentration of NO. As can be seen in Fig. 3, the honey extracts caused no significant cytotoxicity at the tested concentrations of (1-250 $\mu g$/mL) in L929 cells and (3.125-100 $\mu g$/mL) in RAW264.7 cells. However, although the differences were not statistically significant ($P > .05$), HEAE seemed to cause a mild toxicity in L929 cells.
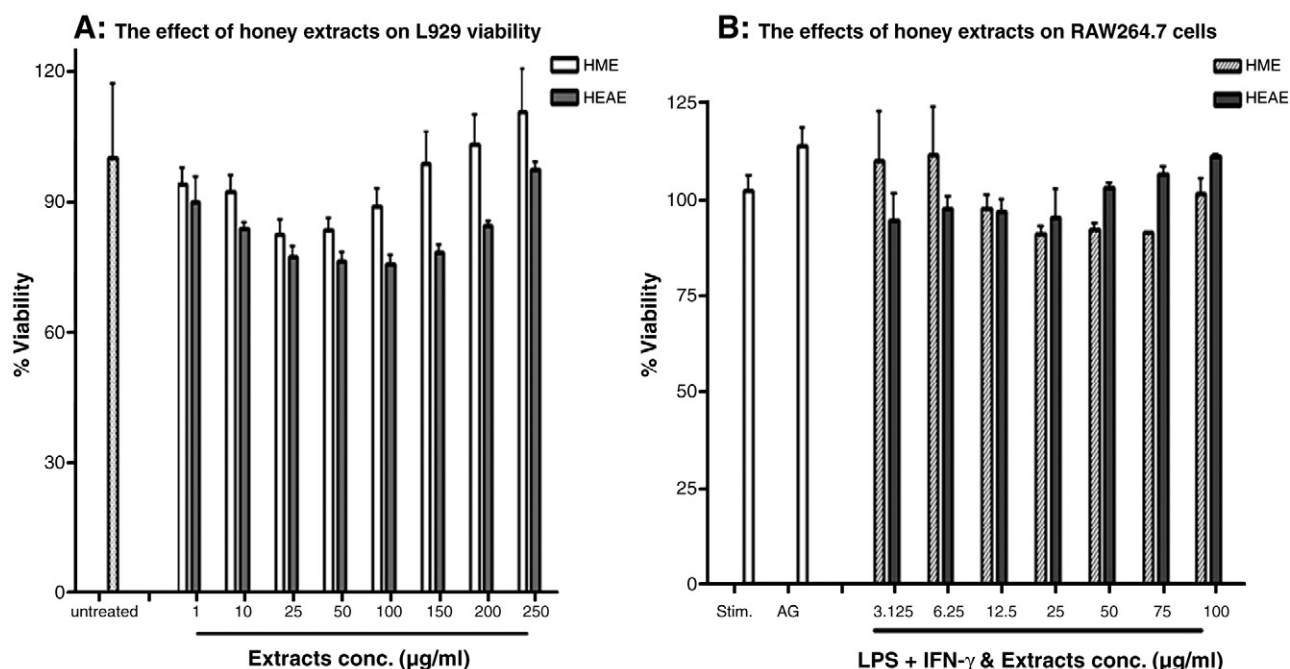


Fig. 3. The honey extracts did not cause significant toxicity to L929 cells at the tested doses ($P > .05$ when compared with cells in DMEM alone) (A). The extracts did not affect the viability of RAW264.7 cells (B) at the tested doses ($P > .05$ when compared with stimulated cells in media only. Data shown are means ± SD of 3 independent observations. Stim indicates cells stimulated with LPS + IFN-γ; AG, aminoguanidine.

**The protective activity of honey extracts on TNF-induced cytotoxicity in L929 cells**
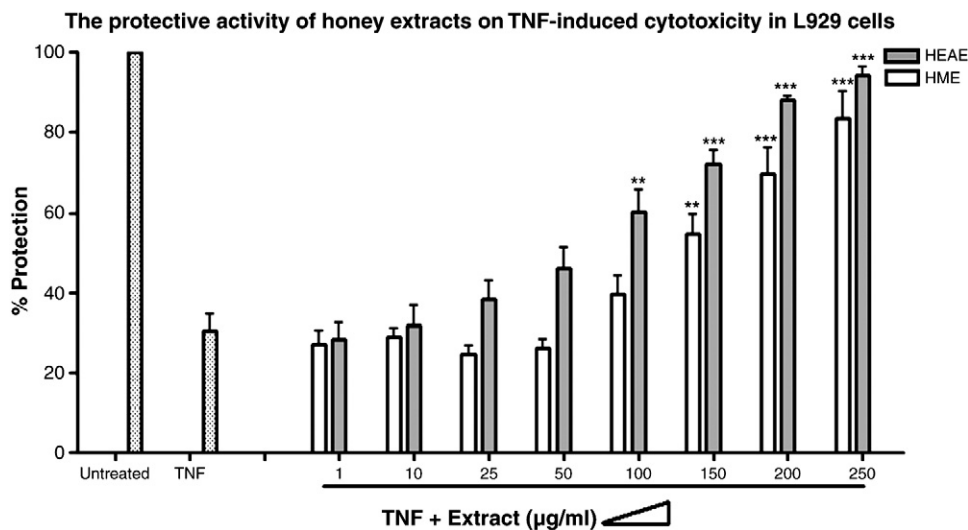


Fig. 4. Treatment of L929 cells with TNF (1 ng/mL) led to 70% cytotoxicity; this was reversed significantly and dose-dependently with the honey extracts. Data shown are means ± SD of 3 independent observations (***$P < .001$ and **$P < .005$ when compared with cells treated with TNF alone).

### 3.2.2. Effect of honey extracts on TNF-α cytotoxicity

*3.2.2.1. Effect of honey extracts on L929 cells treated with TNF and actinomycin D.* In this method, neither HME nor HEAE caused a significant protective effect (data not shown).

*3.2.2.2. Effect of honey extracts on L929 cells treated with TNF alone.* The cytotoxicity in cells treated with TNF-α alone was more than 70% as shown in Fig. 4. Both honey extracts appeared to significantly inhibit TNF cytotoxicity. At the highest concentration tested (250 μg/mL), HEAE and HME almost fully reversed the cytotoxic effects of TNF, with a viability of 94% and 84%, respectively. Moreover, the extracts showed dose-dependent protective effects. The calculated $EC_{50}$ for protection from TNF cytotoxicity by HEAE and HME were 168.1 and 235.4 μg/mL, respectively.

### 3.2.3. Effect of honey extracts on NO production in RAW264.7 cell induced with LPS and IFN-γ

This test was performed to assess the potential anti-inflammatory activity by evaluating the effects of honey extract on NO production in LPS- and IFN-γ–stimulated macrophages. As seen in Fig. 5 (stimulated cells), there was a 20-fold increase in NO concentration in RAW264.7 cells supernatant after 16 to 20 hours of LPS and IFN-γ stimulation. Fig. 5 depicts the inhibition of NO production in cells treated with honey extracts. The highest inhibition percentages were 80% (4.3 μmol/L of NO) and 40% (16 μmol/L) for HEAE and HME (100 μg/mL), respectively.
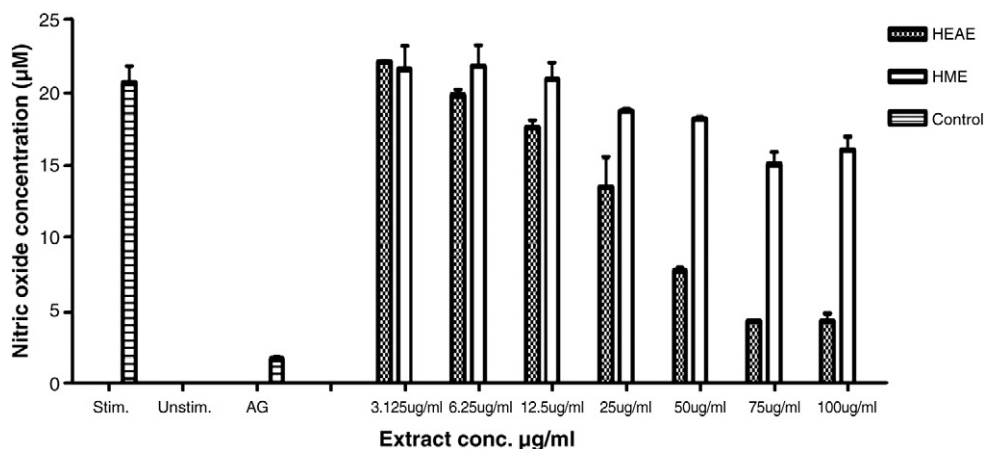


Fig. 5. Honey ethyl acetate extract dose-dependently reduced the concentration of NO produced from stimulated RAW264.7. Cells were tested at the indicated doses (3.125-100 μg/mL) of honey extracts with LPS and IFN-γ (10 μg/mL and 200 U/mL, respectively) for 16 to 20 hours. The NO concentration in the medium was measured using Griess reagent and converted to equivalent micromolar concentrations as compared with a sodium nitrite standard curve. Data shown are means ± SD of 2 independent observations. Unstim indicates cells in media alone.
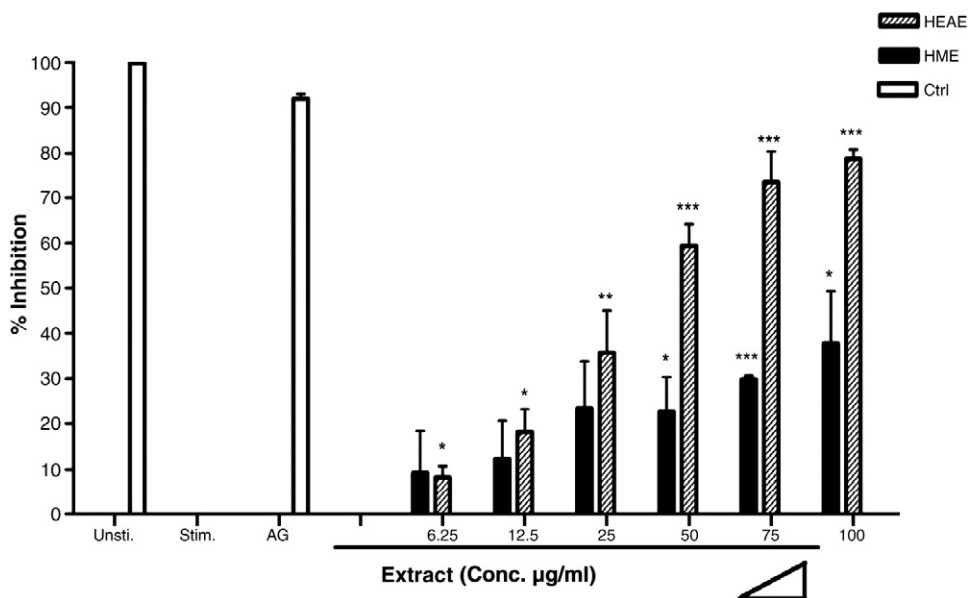
Fig. 6. Both honey extracts (HEAE and HME) significantly inhibited NO production from stimulated RAW264.7 cells when compared with stimulated cells (Stim = 0% inhibition). The protection was more profound in the ethyl acetate extract when compared with the methanol extract. All data shown are means ± SD of 3 independent observations (***$P < .001$, **$P < .005$, and *$P < .05$).

The concentration of NO was inhibited in a dose-dependent manner in the presence of honey extracts as seen in Fig. 6, although the inhibition was more profound for HEAE. The calculated $EC_{50}$ for NO inhibition by HEAE and HME were 37.5 and 271.7 $\mu$g/mL, respectively.

## 4. Discussion

The phenolic compounds in honey are bound to sugar moieties, making them more soluble in water; this could explain the poor recovery of gallic and ellagic acid in this study, which is in agreement with a previous report [33]. The poor recovery can also be attributed to weak binding of these compounds to XAD-2 resin and their strong solubility in water. Although HPLC did not provide information about some compounds and their derivatives and conjugates, the identification of some phenolic compounds and their derivatives, such as ellagic acid and ellagitannin and their conjugates, was possible with LC-MS.

Most phenolic compounds identified from the honey extracts possess antioxidant activity [12,13,19]. This in turn lead to exploration of the use of honey extracts as chemopreventive agents in diseases known to involve free radicals, such as cancer and inflammation [4]. There is increasing evidence that dietary phenolic compounds play a role in preventing cancer [34-36], a disease strongly associated with chronic inflammation [10]. The inhibition of inflammatory mediators, such as TNF and NO, which were explored in this study, is one of the important steps in controlling inflammation.

Reactive oxygen species play a critical role in mediating TNF-$\alpha$–induced cytotoxicity [37]. It was shown that such cytotoxicity can be blocked by specific free radical scavengers [38]. Our findings show that both types of the honey extracts had a dose-dependent protective effect in TNF-$\alpha$–mediated cytotoxicity. Previous research has reported that Malaysian honey has free radical scavenging activity [19]. Therefore, it is believed that the free radical scavenging capacity of flavonoids identified in the honey extracts may play a role in protecting cells from this cytotoxicity [11]. In fact, Habtemariam [39] reported that phenolics, such as caffeic acid, effectively inhibit TNF-induced cytotoxicity in L929 cells.

Another mechanism by which phenolics may protect the cells is by either inducing or acting as a substrate for cytoprotective enzymes such as heme oxygenase–1 (HO-1). Flavonoids were shown to induce HO-1 gene expression [40]. Actinomycin D, a transcription inhibitor used in this study [41], inhibits de novo protein synthesis such as HO-1 expression [42]. This could explain the reason for the protective effect of the extracts on cells treated with TNF alone compared with the absence of significant bioactivity in L929 cells treated with TNF and actinomycin D. Furthermore, the cytotoxicity mechanisms involved in treatment with TNF alone or TNF + actinomycin D were shown to be different [38]. It may be appropriate, therefore, to presume that the protection of the extracts is due to, at least in part, the induction of HO-1 and inhibition of reactive oxygen species.

Nitric oxide is known to be an important mediator of inflammation [43]. Inducible nitric oxide synthase is the enzyme responsible for NO production in the inflammatory

response. Aminoguanidine, a highly selective inhibitor of iNOS [44], totally inhibited NO production in activated macrophages at 1 mmol/L. Similarly, HME and HEAE dose-dependently inhibited the production of NO without affecting the viability of RAW264.7 cells.

Some flavonoids, including hesperetin and naringin, induce HO-1 and can inhibit LPS-induced NO production. Moreover, genistein, kaempferol, quercetin, and daidzein inhibit the activation of the signal transducer and activator of transcription 1, another important transcription factor for iNOS [45]. In addition, quercetin, caffeic acid, chrysin, ellagic acid, and various polyphenolic compounds are known for their down-regulation of nuclear factor–$\kappa$B [46]; this in turn reduces biosynthesis of iNOS and ultimately inhibits the production of NO. Most of the phenolic compounds mentioned above were identified in this study; therefore, it can be assumed that the inhibition of NO production by the honey extracts was due to these compounds.

Although the concentrations of the phenolics identified were higher in HME, the in vitro anti-inflammatory activity seemed to be better for HEAE. This could be explained by the fact that the concentrations were reported for every 100 g of honey extracted. The dry extract yield ratio of the HEAE to the HME had been 1:5 for every 100 g of honey, hence overrepresenting the concentrations of the phenolic compounds in the methanol extract. This introduced a limitation in this study, as it was not possible to compare between the extracts' phenolic content (ie, for every milligram of extract). On the other hand, it was possible to compare the extracts' in vitro activities because of adequate presentation of their concentration. It was reported that ethyl acetate extracts will contain a higher concentration of bioactive compounds, an example being the anti-inflammatory compound caffeic acid phenethyl ester [40,47,48]. This supports the observation that HEAE showed better activity.

In conclusion, we accept the hypothesis for this study because of the fact that the results of this study indicated that different extraction methods and solvents will yield different concentrations of phenolic compounds in honey. In addition, this study's findings also supported our hypothesis that Malaysian honey extracts would display varying anti-inflammatory activities in the 2 in vitro models of inflammation used. This bioactivity may be attributed, at least in part, to the phenolic compounds within the extracts. As such, this study has made a contribution to the elucidation of the potential therapeutic value of honey and its extracts in inflammatory conditions, thus highlighting the nutritional value of this food.

## References

[1] Phillips KM, Carlsen MH, Blomhoff R. Total antioxidant content of alternatives to refined sugar. J Am Diet Assoc 2009;109:64-71.

[2] Ischayek JI, Kern M. US honeys varying in glucose and fructose content elicit similar glycemic indexes. J Am Diet Assoc 2006;106: 1260-2.

[3] Al-Mamary M, Al-Meeri A, Al-Habori M. Antioxidant activities and total phenolics of different types of honey. Nutr Res 2002;22:1041-7.

[4] Basualdo C, Sgroy V, Finola MS, Marioli JM. Comparison of the antibacterial activity of honey from different provenance against bacteria usually isolated from skin wounds. Vet Microbiol 2007;124: 375-81.

[5] Postmes T, Van Den Bogaard A, Hazen M. Honey for wounds, ulcers, and skin graft preservation. Lancet 1993;341:756-7.

[6] Sobota R, Szwed M, Kasza A, Bugno M, Kordula T. Parthenolide inhibits activation of signal transducers and activators of transcription (STATs) induced by cytokines of the IL-6 family. Biochem Bioph Res Co 2000;267:329-33.

[7] Evans SW, Whicher JT. Cytokines in disease. Clin Chem 1990;36: 1269-81.

[8] Ahn KS, Noh EJ, Zhao HL, Jung SH, Kang SS, Kim YS. Inhibition of inducible nitric oxide synthase and cyclooxygenase II by *Platycodon grandiflorum* saponins via suppression of nuclear factor–kb activation in RAW 264.7 cells. Life Sci 2005;76:2315-28.

[9] Tonks AJ, Cooper RA, Jones KP, Blair S, Parton J, Tonks A. Honey stimulates inflammatory cytokine production from monocytes. Cytokine 2003;21:242-7.

[10] Kundu JK, Surh YJ. Breaking the relay in deregulated cellular signal transduction as a rationale for chemoprevention with anti-inflammatory phytochemicals. Mutat Res 2005;591:123-46.

[11] Middleton JE, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 2000;52:673-751.

[12] Erlund I. Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability,and epidemiology. Nutr Res 2004;24:851-74.

[13] Gheldof N, Wang XH, Engeseth NJ. Identification and quantification of antioxidant components of honeys from various floral sources. J Agr Food Chem 2002;50:5870-7.

[14] Martos I, Cossentini M, Ferreres F, Tomás-Barberán FA. Flavonoid composition of tunisian honeys and propolis. J Agr Food Chem 1997; 45:2824-9.

[15] Vinson JA, Hao Y, Su X, Zubik L. Phenol antioxidant quantity and quality in foods: vegetables. J Agr Food Chem 1998;46:3630-4.

[16] Nasuti C, Gabbianelli R, Falcioni G, Cantalamessa F. Antioxidative and gastroprotective activities of anti-inflammatory formulations derived from chestnut honey in rats. Nutr Res 2006;26:130-7.

[17] Viuda-Martos M, Ruiz-Navajas Y, Fernández-López J, Pérez-Álvarez JA. Functional properties of honey, propolis, and royal jelly. J Food Sci 2008;73:R117-24.

[18] Peterson J, Dwyer J. Flavonoids: dietary occurrence and biochemical activity. Nutr Res 1998;18:1995-2018.

[19] Aljadi A, Kamaruddin M. Evaluation of the phenolic contents and antioxidant capacities of two malaysian floral honeys. Food Chem 2004;85:513-8.

[20] Tomas-Barberan FA, Blazquez MA, Garcia-Viguera C, Ferreres F, Tomas-Lorente F. A comparative study of different amberlite XAD resins in flavonoid analysis. Phytochem Analysis 1992;3:178-81.

[21] Harris DC. Quantitative chemical analysis. San Francisco: W.H. Freeman; 2003.

[22] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.

[23] Kishida E, Tajiri M, Masuzawa Y. Docosahexaenoic acid enrichment can reduce L929 cell necrosis induced by tumor necrosis factor. BBA-Mol Cell Biol L 2006;1761:454-62.

[24] Culver CA, Michalowski SM, Maia RC, Laster SM. The anti-apoptotic effects of nordihydroguaiaretic acid: inhibition of cPLA2 activation during TNF-induced apoptosis arises from inhibition of calcium signaling. Life Sci 2005;77:2457-70.

[25] Uboldi AD, Savage N. The adenylate cyclase activator forskolin partially protects L929 cells against tumour necrosis factor-alpha–mediated cytotoxicity via cAMP-independent mechanism. Cytokine 2002;19:250-8.

[26] Mendoza-Milla C, Rodriguez CM, Alarcon EC, Bernal AE, Toledo-Cuevas EM, Martinez EM, Dehesa AZ. NF-kb activation but not PI3K/akt is required for dexamethasone dependent protection against TNF-a cytotoxicity in L929 cells. FEBS Lett 2005;579:3947-52.

[27] Syahida A, Israf DA, Lajis NH, Habsah S, Jasril M, Permana D, et al. Effects of compounds isolated from natural products on IFNγ/LPS-induced nitric oxide production in RAW 264.7 macrophages. Pharm Biol 2006;44:50-9.

[28] Tairaa J, Nanbub H, Uedab K. Nitric oxide–scavenging compounds in agrimonia pilosa ledeb on LPS-induced raw264.7 macrophages. Food Chem 2009;115:1221-7.

[29] Dirsch V, Stuppner H, Vollmar A. The griess assay: suitable for a bio-guided fractionation of anti-inflammatory plant extracts? Planta Med 1998;64:423-6.

[30] Zafrilla P, Ferreres F, Tomás-Barberan FA. Effect of processing and storage on the antioxidant ellagic acid derivatives and flavonoids of red raspberry (*Rubus idaeus*) jams. J Agr Food Chem 2001;49:3651-5.

[31] Hughes RJ, Croley TR, Metcalfe CD, March RE. A tandem mass spectrometric study of selected characteristic flavonoids. Int J Mass Spectrom 2001;210:371-85.

[32] De Rijke E, De Kanter F, Ariese F, Brinkman UA, Gooijer C. Liquid chromatography coupled to nuclear magnetic resonance spectroscopy for the identification of isoflavone glucoside malonates in *T. pratense L.* leaves. J Sep Sci 2004;27:1061-70.

[33] D'Arcy BR. Antioxidants in australian floral honeys identification of health enhancing nutrient components. Australia: RIRDC Publication; 2005.

[34] Azuinea MA, Bhideb SV. Adjuvant chemoprevention of experimental cancer: catechin and dietary turmeric in forestomach and oral cancer models. J Ethnopharmacol 1994;44:211-7.

[35] Aggarwal BB, Kumar A, Bharti AC. Anticancer potential of curcumin: preclinical and clinical studies. Anticancer Res 2003;23:363-98.

[36] Kunnumakkara AB, Anand P, Aggarwal BB. Curcumin inhibits proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell signaling proteins. Cancer Lett 2008;269:199-225.

[37] Delhalle S, Deregowski V, Benoit V, Merville MP, Bours V. NF-kB–dependent mnSOD expression protects adenocarcinoma cells from TNFα-induced apoptosis. Oncogene 2002;21:3917-24.

[38] Goossens V, Grooten J, De Vos K, Fiers W. Direct evidence for tumor necrosis factor–induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. P Nat Acad Sci U S A 1995;92:8115-9.

[39] Habtemariam S. Natural inhibitors of TNF-a production, secretion and function. Planta Med 2000;66:303-13.

[40] Scapagnini G, Foresti R, Calabrese V, Giuffrida Stella AM, Green CJ, Motterlini R. Caffeic acid phenethyl ester and curcumin: a novel class of heme oxygenase–1 inducers. Mol Pharmacol 2002;61:554-61.

[41] Sobell HM. Actinomycin and DNA transcription. P Nat Acad Sci USA 1985;82:5328-31.

[42] Hartfield CL, Alam J, Choi AMK. Transcriptional regulation of the heme oxygenase 1 gene by pyrrolidine dithiocarbamate. FASEB J 1998;17:1675-82.

[43] Argaman Z, Young VR, Noviski N, Castillo-Rosas L, Lu XM, Zurakowski D, et al. Arginine and nitric oxide metabolism in critically ill septic pediatric patients. Crit Care Med 2003;31:657-9.

[44] Misko TP, Moore WM, Kasten TP, Nickols GA, Corbett JA, Tilton RG, et al. Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. Eur J Pharmacol 1993;233:119-25.

[45] Romier B, Van De Walle J, During A, Larondelle Y, Schneider YJ. Modulation of signaling nuclear factor–κb activation pathway by polyphenols in human intestinal CACO-2 cells. Brit J Nutr 2008;100:542-51.

[46] Hämäläinen M, Nieminen R, Vuorela P, Heinonen M, Moilanen E. Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-kappaB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-kappaB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. Mediat Inflamm 2007.

[47] Del Boccio P. Quantitative analysis of caffeic acid phenethyl ester in crude propolis by liquid chromatography–electrospray ionization mass spectrometry. J Sep Sci 2004;27:619-23.

[48] Song YS, Park EH, Hur GM, Ryu YS, Lee YS, Lee JY, et al. Caffeic acid phenethyl ester inhibits nitric oxide synthase gene expression and enzyme activity. Cancer Lett 2002;175:53-61.

## 3.2 The inhibitory effects of gelam honey and its extracts on nitric oxide and prostaglandin E2 in inflammatory tissues

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# The inhibitory effects of Gelam honey and its extracts on nitric oxide and prostaglandin $E_2$ in inflammatory tissues

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## ARTICLE INFO

## ABSTRACT

We investigated the effects of honey and its methanol and ethyl acetate extracts on inflammation in animal models. Rats' paws were induced with carrageenan in the non-immune inflammatory and nociceptive model, and lipopolysaccharide (LPS) in the immune inflammatory model. Honey and its extracts were able to inhibit edema and pain in inflammatory tissues as well as showing potent inhibitory activities against NO and $PGE_2$ in both models. The decrease in edema and pain correlates with the inhibition of NO and $PGE_2$. Phenolic compounds have been implicated in the inhibitory activities. Honey is potentially useful in the treatment of inflammatory conditions.

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## 1. Introduction

Honey is a viscous, liquid, natural product with a complex chemical composition. It is made up of carbohydrates, free amino acids, vitamins, trace elements and phenolic compounds [1]. It possesses both antioxidant and antibacterial activities [2]. Many animal and clinical studies have investigated the activity of honey against various microorganisms. It has been shown to have a broad-spectrum antimicrobial activity on gram-negative and gram-positive bacteria [3]. It is used both in modern medicine to treat infected wounds [2] and as an important ingredient in traditional alternative therapies due to its antimicrobial and anti-inflammatory properties. Animal and clinical studies have shown that honey aids in the healing of gastric ulcers and may even accelerate the healing process compared with nonsteroidal anti-inflammatory drugs [4].

Gelam honey has been shown to stimulate fibroblast cells, activate epithelialization, and accelerate wound healing in an animal model study. It has antibacterial activity against bacteria including Methicilline Resistant *Staphylococcus aureus* (MRSA), as well as demonstrating a high antioxidant capacity and free radical scavenging activities [5]. Inflammation is an immunological and pathophysiological response of tissues. It can be caused by infectious organisms, cancer, autoimmune diseases, toxic chemical substances or physical injury and leads to the local accumulation of plasma fluid and blood cells. Pain, heat, redness, and swelling are all markers of the natural inflammatory processes. Phospholipase $A_2$ then causes arachidonic acid to be released when the integrity of a cell's membrane becomes compromised. It is then transformed into prostaglandins and thromboxanes through the action of COX. Nitric oxide (NO) is a gaseous free radical. It is highly labile with a half-life of less than 10 seconds in the presence of oxygen. NO is rapidly metabolized to nitrate and nitrite [6]. It is produced from the amino acid L-arginine by the enzymatic action of nitric oxide synthase (NOS). There are three isoforms of NOS, two of which are constitutive (cNOS: eNOS) and the other which is inducible NOS (iNOS). Co-factors for NOS include oxygen, NADPH, tetrahydrobiopterin and flavin adenine nucleotides. The activity of iNOS is stimulated during inflammation by bacterial endotoxins such as lipopolysaccharide (LPS) and cytokines such as tumor necrosis factor (TNF) and interleukins. During

inflammation, the amount of NO produced by iNOS may be 1,000-fold greater than that produced by cNOS [7].

In this study, we investigated the anti-inflammatory and anti-nociceptive activities of Gelam honey and its extracts in the inflammatory tissue of immune and non immune animal models, focusing on the inflammatory mediators, $PGE_2$ and NO.

## 2. Materials and methods

### 2.1. Materials

Fresh Malaysian honey *Apis mellifera* (Gelam) was obtained from the National Apiary, Department of Agriculture, Parit Botak, Johor, Malaysia. All chemicals and reagents used were of analytical grade.

### 2.2. Methods

#### 2.2.1. Preparation of extraction from honey by solid phase extraction (SPE)

C18 cartridges of SPE were preconditioned for neutral flavonoids and phenolics by sequentially passing 8 ml of methanol and 4 ml of deionized water adjusted to pH 7.0. For phenolic acids, cartridges were preconditioned by passing 4 ml of 0.01 M HCl instead of deionized water [8]. The honey was prepared as described by Martos [9] with certain modifications. In each condition, the honey (100 mg) was thoroughly mixed with deionized water for 30 min, 5 times, until completely dissolved. The resulting honey solution was then filtered under vacuum to remove any solid particles. This solution was divided into two parts. The first part was adjusted to pH 7.0 with diluted NaOH solution, loaded onto the neutral fractionating C18 column, and washed with 10 ml of pH 7.0 deionized water. The second part was adjusted to pH 2.0 with 2.0 M HCl, passed through the preconditioned acidic column and washed with 5 ml of 0.01 M HCl. For the methanol extract, the adsorbed fractions were eluted with 12 ml of methanol and evaporated using a rotary evaporator until dry at 40 °C with a water bath. The residues from all the above conditions were re-dissolved individually in 1 ml of methanol for HPLC and after that they were dried again. The dried methanol extract was divided into two portions. One portion was used as the methanolic fraction and the second portion was redissolved in deionized water (1 ml) and re-extracted with ethyl acetate (1 ml×3) [9]. 20 µl of each extract was then injected into the HPLC system.

#### 2.2.2. HPLC analysis

Samples of a volume of (20 µl) each were injected. The phenolic compounds were detected using UV absorption spectra and monitored at 290 nm and 340 nm. The majority of the honey flavonoids and phenolic acids show their UV absorption maximum at these two wavelengths [9]. The column used was a reversed phase C18 column, Agilent ZORBAX Eclipse XDC-18 (3×250 mm, particle size 5 µm). The mobile phases were 0.25% formic acid and 2% methanol in water (solvent A) and methanol (solvent B), at a constant solvent flow rate of 1 ml/min. The following gradient was used, according to the method devised by Martos [9], except for minor modifications: 10% methanol (B) flowed through the column isocratically with 90% solvent A for 15 min which was then was increased to 40% methanol (B) for 20 min, to 45% methanol (B) for 30 min, to 60% methanol (B) for 50 min, to 80% methanol (B) for 52 min, to 90% methanol for 60 min, and then followed by isocratic elution with 90% methanol (B) for 65 min. Finally, the gradient was changed to 10% methanol for 68 min, and this composition was held until 73 min.

#### 2.2.3. Animals

For this study, male Sprague Dawley rats with an average weight of 200-250 g were kept in individual cages under standard conditions (Temperature at $22 \pm 2$ °C 12 h light, 12 h dark), fed on Purina lab chaw and given water *ad libitum*. Five groups (n = 6) were used for each model.

#### 2.2.4. Formation and measurement of paw edema

Edema was induced by a sub-plantar injection of carrageenan or LPS into the footpad of the right hind paw of all animals in the study groups in both models. The animals were pretreated for one hour by injecting 500 µl (i.p.) the following: Honey (800 mg/kg, 1:1 in $H_2O$); honey methanolic extract (HME) and honey ethyl acetate extract (HEAE) (180 mg/kg in 5% DMSO); indomethacin (5 mg/kg in 2% $NaHCO_3$ solution); and saline with 5% DMSO. All animals in both models were injected with (200 µl/paw) 1% g/ml carrageenan (λ-Carrageenan from Eucheuma Spinosa (Sigma)) in saline in the non-immune model; and with (200 µl/paw ) mg/ml LPS from Escherichia coli (sigma) in saline in the immune model. The paw volume was then measured every hour from 0 to 9 hours and also at 24 hours employing the volume displacement technique using a Plethysmometer (Ugo Basile, Italy). Edema was calculated as follows: Edema = paw volume at every hour - the paw volume at zero hours.

#### 2.2.5. Measurement of $PGE_2$ and NO in paw tissue

Twenty-four hours after injecting carrageenan and LPS, the rats were sacrificed and the paw tissues were removed. The tissues were centrifuged with 100 µl $dH_2O$ to extract the $PGE_2$ and NO products from the muscles and were stored at -20 °C until analysis. The analysis was done with an ELISA kit for $PGE_2$, following the manufacturers' (Cayman Chemical) guidelines. Nitrate *reductase* enzyme from *E.coli* was used to measure NO products also following the manufacturers' (Sigma) guidelines.

#### 2.2.6. Measurement of nociceptive activity

A plantar test was used to assess nociceptive responses to thermal stimuli according to the method introduced by Hargreaves [10]. Rats were placed in a transparent plastic chamber. The rats were allowed to habituate in this environment for 20 min prior to testing. After the acclimatization period, an infra-red (IR) source was positioned under the glass floor directly beneath the hind paw and activated. Paw withdrawal latency in response to radiant heat was measured using the plantar test apparatus (Ugo Basile, Comerio, Italy). A digital timer connected to the heat source automatically recorded the response latency for paw withdrawal to the nearest tenth of a second. A cut-off time of 22 seconds was used to prevent tissue damage. The reaction time was monitored at 15 and 30 minutes, and thereafter half- hourly, the total time of the study being 7 hrs. The paw withdrawal

latency of each rat was measured three times at each test interval and the median score was recorded.

### 2.2.7. Statistical analysis

Results are expressed as mean ± SD. Statistical analysis of the results was performed using one- way ANOVA followed by Tukey's multiple comparison test and the results were considered significant at $P<0.05$.

## 3. Results

### 3.1. Phenolic compounds in Gelam honey extracts fractionated by HPLC

The methanol in both conditions (acidic and neutral) and ethyl acetate extracts of Gelam honey were applied to SPE (C18) and HPLC, and similarly with commercial phenolic compounds. The identification of phenolic compounds in all conditions was compared with commercial standards depending on retention time and the wavelength of the phenolic compounds. Most of the phenolic acids appeared at 290 nm while most of the flavonoids and other polyphenolics appeared at 340 nm. All phenolic compounds were present in both extracts (methanol and ethyl acetate). However, the quantities of the compounds varied, being higher in the methanol extraction. In the acidic condition, some flavonoids and other polyphenolics appeared, while in the neutral condition the phenolic acids were absent. The highest levels of gallic acid were found in the acidic condition, whilst ellagic acid was found at the highest levels in neutral conditions as indicated in Figs. 1 and 2.

### 3.2. Measurement of paw edema volume

The subplantar injection of carrageenan and LPS to both models led to a time dependent increase in paw volume which peaked at 6 hrs for carrageenan, at 3 hrs for LPS and remained elevated thereafter for 24 hrs. Edema in the paw was measured by a plethysmometer in both models. HME, HEAE and honey significantly reduced the edema as shown in Figs. 3 and 4. The. $P$ value was <0.05.

### 3.3. Measurement of anti-nociceptive activity

The results depict the anti-nociceptive activity measured through infrared withdrawal latency in all groups. HME, HEAE

and honey significantly reduced the pain as shown in Fig. 5. The $P$ value was <0.05.

### 3.4. Measurement of NO and PGE₂ in paw edema

The concentrations of NO and $PGE_2$ in exudates of paw tissues in all groups of both models were measured. The LPS groups had higher concentrations compared with the carrageenan groups with the exception of the indomethacin groups (which showed approximately the same quantity). HME, HEAE and honey significantly inhibited the NO and $PGE_2$ as shown in Figs. 6 and 7. The $P$ values were significant when $P<0.05$.

## 4. Discussion

Our study investigates the anti-inflammatory and anti-nociceptive activities of Gelam honey in vivo and analytical conclusions about the potential therapeutic use of honey, a cheap and readily available natural product. To date, research findings have been inconclusive in terms of defining the role of honey in nociceptive activities. To the best of our knowledge, this is the first report on the inhibition of NO and $PGE_2$ specifically in inflamed paw tissue in immune and non immune animal models by using honey and its extracts. Honey and its extracts were found to downgrade inflammatory activity by reducing cardinal inflammatory signs and markers of inflammation. This was observed through the inhibition of swelling, the decrease in pain, as well as the reduction of the mediators of inflammation tested ($PGE_2$, NO). The anti-inflammatory activity of honey and its extracts is attributed to the phenolic compounds present in the honey. We are able to demonstrate enhanced anti-inflammatory activity in the methanol and ethyl acetate extracts of honey models as compared to the wholesome honey model.

It has been documented that carrageenan and LPS induced rat paw edema form a suitable in vivo model to predict the value of an agent's anti-inflammatory activity [11]. The results of this study (Figs. 3 and 4) indicate that the volume of edema differed between the two models. The LPS model showed a faster development of edema, with the largest edema volume being recorded at 3 hrs. On the other hand, the carrageenan model induced a larger edema volume, and the development of edema occurred over a longer period with the largest edema volume being recorded at 6 hrs. The effects of honey and its extracts were significant in both models but
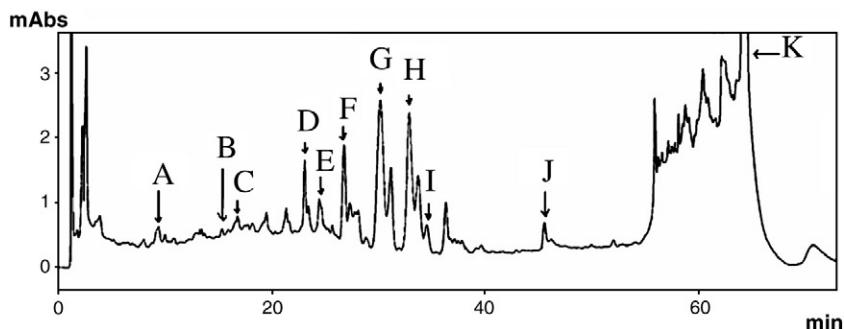


**Fig. 1.** Chromatograms of acidic condition of methanolic extracts of Gelam honey by using SPE (C18) and detected by HPLC-UV absorption at 290 nm. A = Caffeic acid, B = *p*-Coumaric acid, C = Ferulic acid, D = Ellagic acid, E = Myrectin, F = Qurecetin, G = Hesperetin, H = Luteulin, I = Kaempferol, J = Chrysin, K = Gallic acid.
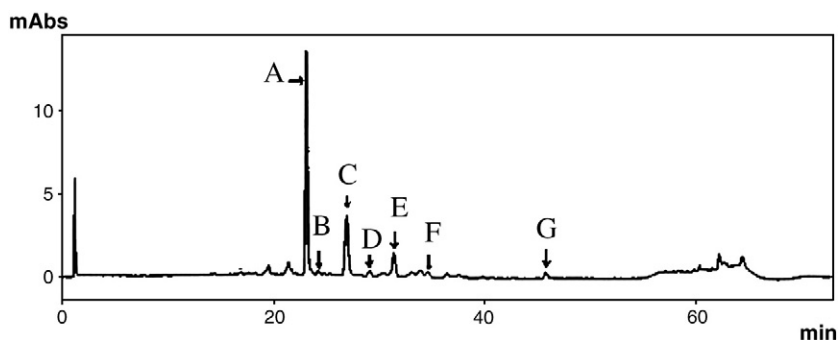
**Fig. 2.** Chromatograms of neutral condition of methanolic extortion of Gelam honey by using SPE (C18) and detected by HPLC-UV absorption at 340 nm. A = Ellagic acid, B = Myrectin, C = Qurecetin, D = Hesperetin, E = Luteulin, F = Kaempferol, G = Chrysin.

were more pronounced in the carrageenan model. This may be attributed to the fact that carrageenan is known to destroy macrophages [12].

The extracts (HME and HEAE) showed higher inhibition of edema in both of the models compared with honey. This was indicative of the role of phenolic compounds in the inhibition of edema, and it appeared that, particularly in HME, it contains the highest concentrations of phenolic compounds, specifically ellagic acid and gallic acid. The role of ellagic acid will be explained below, while gallic acid has been reported to inhibit iNOS, COX2, decrease histamine release, and suppress pro-inflammatory cytokine production in macrophage and P-selectin-mediated inflammation both in vitro and in vivo [13,14]. It is suggested that the mechanism of action of phenolic compounds may be the inhibition of molecular vasodilators, such as NO, as well as the inhibition of $PGE_2$.

Pain is a common symptom of injuries and inflammatory-related conditions. The perception of pain, commonly known as nociception, depends on integrated receptors and molecular pathways. Inflammatory mediators are involved in the genesis, persistence, and severity of pain [15]. The inflammatory milieu that usually precedes and accompanies pain is transcriptionally regulated [16]. The nuclear factor NF-κB is a transcription factor essentially involved in controlling the release of inflammatory mediators, which may exacer-

bate pain, hyperalgesia and nociception [17]. Carrageenan is used in hyperalgesia as a thermal stimulus as indicated by decreased withdrawal latency [18]. $PGE_2$ and NO were established as playing a significant role in nociceptive processing [19]. In this model, a decrease in paw withdrawal latency to radiant heat and withdrawal threshold was observed throughout the 30 min - 7 hrs time period after induction of the paws by carrageenan. The results in Fig. 5 depict that honey and its extracts showed potent anti-nociceptive activity which is caused by the inhibition of $PGE_2$ and NO. The extracts were more significant supporting the above suggestion for the involvement of phenolic compounds in this activity.

Nitric oxide (NO) is known to be an important mediator of acute and chronic inflammation. The inducible nitric oxide synthase (iNOS) is up-regulated in response to inflammatory and pro-inflammatory mediators, and their products can influence many aspects of the inflammatory cascade. Aspirin (widely used to treat inflammation) and indomethacin inhibit NF-kB activation [20]. Certain natural products inhibit NF-kB activation and decrease the level of iNOS and COX-2 expression caused by stimulation with LPS [21]. The results in Fig. 6 show that honey and it extracts inhibited NO in inflammatory tissues in both models. In the carrageenan model, the inhibition was more pronounced than that of the LPS model. The inhibition activity was more significant in



**Fig. 3.** The edema volume of the rats' paws after injection with carrageenan for all groups. Saline, indomethacin, honey, honey methanolic extract (HME), and honey ethyl acetate extract (HEAE), *P* value is significant when *P*<0.05.



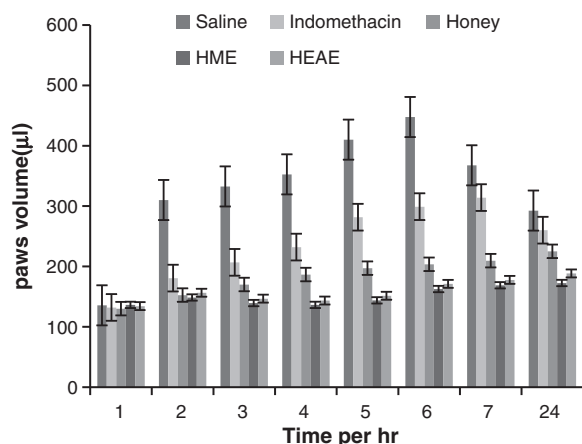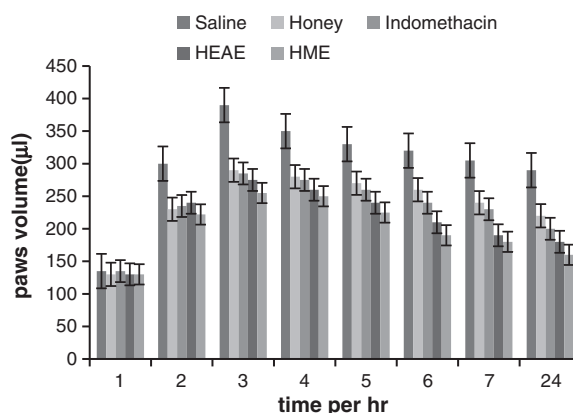**Fig. 4.** The edema volume of the rats' paws after injection with LPS for all groups. Saline, indomethacin, honey, honey methanolic extract (HME), and honey ethyl acetate extract (HEAE), P value is significant when *P*<0.05.
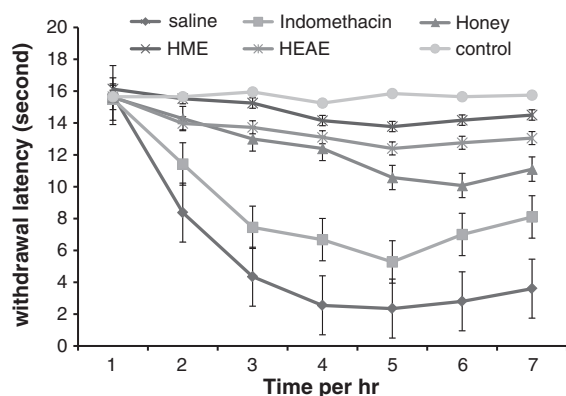
Fig. 5. Antinociceptive effect of honey and its extracts on rats' paws using infrared light after injection with carrageenan for 7 hrs. Tested honey, indomethacin, honey methanolic extract (HME) and honey ethyl acetate extract (HEAE) significantly inhibited the nociceptive response; P value is significant when $P < 0.05$.

honey extracts. Phenolic compounds are fully implicated for NO inhibition but the mechanism is still unclear. The major phenolic compounds in the methanol and ethyl acetate extracts were gallic acid, ellagic acid, caffeic acid, luteolin, chrysin and quercetin. The anti-inflammatory activity correlates positively with the radical-scavenging activity and total phenolic content [22]. It has been reported that Gelam honey has potent free radical scavenging activity [5]. Phenolic compounds showed a clear and strong correlation between ROS scavenging activity and decreased cytotoxicity. Phenolic compounds in Gelam honey such as quercetin, caffeic acid, chrysin and ellagic acid are known for their downregulation of NF-κB. This, in turn, reduces the biosynthesis of iNOS [23], and ultimately inhibits the production of nitric oxide. Phenolic compounds in honey and its extracts may be able to inhibit NO through the inhibition of NF-kB and scavenging activity of the NO radical.
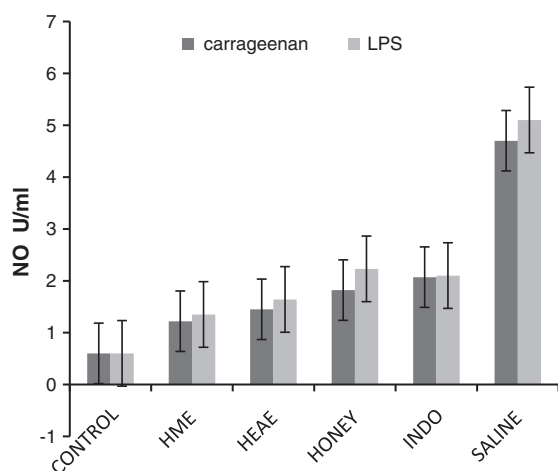


Fig. 6. Effect of honey and its extracts on NO production in paw tissue in the carrageenan and LPS models, indomethacin (INDO), honey methanolic extract (HME), and honey ethyl acetate extract (HEAE), P value is significant when $P < 0.05$.



Fig. 7. Effect of honey and its extracts on PGE$_2$ production in paw tissues in the carrageenan and LPS models. Indomethacin (INDO), honey methanolic extract (HME), and honey ethyl acetate extract (HEAE), P value is significant when $P < 0.05$.
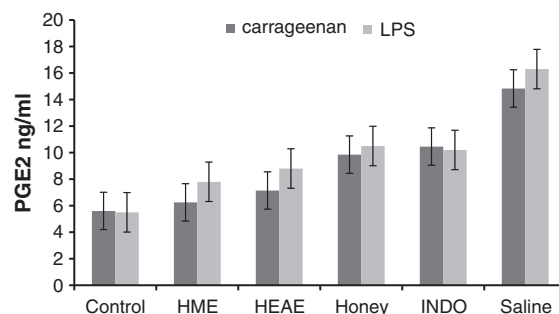
Prostaglandin is a very important mediator of all types of inflammation. It is synthesized by the enzyme cyclooxygenase (COX) which is stimulated in the inflammatory phase by pro-inflammatory mediators, such as cytokines, LPS and carrageenan. Previous studies have shown that COX-2 is responsible for increased prostaglandin production in inflamed tissue [24]. The results shown in Fig. 7 indicate that honey and its extracts inhibit the PGE$_2$ in inflammatory tissues of both inflammation models. Phenolic compounds have a major role in the inhibition of PGE$_2$ in inflammatory tissues since methanol and ethyl acetate extracts were more involved in the inhibition of PGE$_2$ production than the whole honey. Nevertheless, t the mechanism is still unclear. Honey has been proven to have a potent activity against gastritis and stomach ulcers [4]. Specific inhibition of COX-2 expression at the transcriptional level is a potent mechanism in the treatment of inflammatory disease [25]. It is possible that honey and its extracts are selective inhibitors of COX-2 because honey has no side effects on the gastrointestinal system. In relation with the above results, the inhibition of PGE$_2$ by honey extracts is more pronounced. Ellagic acid, the major phenolic compound in Gelam honey, has an inhibitory effect on PGE$_2$ release from monocytes and other phenolic compounds in Gelam honey such as quercetin, chrysin and luteolin which have been demonstrated to have inhibitory effects on interleukin, 1β, and cyclooxygenase-2 (COX-2) expression, prostaglandin E2 (PGE$_2$) synthesis and NF-κB [23,26]. These phenolics in Gelam honey and its extracts may have inhibited PGE$_2$ through the inhibition of COX-2 and NF-kB.

In conclusion, phenolic compounds in Gelam honey and its extracts do appear to have anti-inflammatory effects against the inflammatory mediators NO and PGE$_2$ in tissues. Effects on NO and PGE$_2$ correspond with the reduction in paw edema volume and the inhibition of pain. Honey and its extracts are, therefore, potentially useful for treating inflammatory conditions.

## Acknowledgements

# References

[1] Havsteen BH. The biochemistry and medical significance of the flavonoids. Pharmacol Ther 2002;96:67–202.

[2] Lusby PE, Coombes AL, Wilkinson JM. Bactericidal activity of different honeys against pathogenic bacteria. Arch Med Res 2005;36:464–7.

[3] Agbaje EO, Ogunsanya T, Aiwerioba OR. Conventional use of honey as antibacterial agent. Ann Afr Med 2006;5:78–81.

[4] Kandil M, El-Banby M, Abdel-Wahed K, Abdel-Gawwad M, Fayez M. Curative properties of true floral and false non-floral honeys on induced gastric ulcers. J Drug Res 1987;17:103–6.

[5] Aljadi AM, Kamaruddin MY. Evaluation of the phenolic contents and antioxidant capacities of two Malaysian floral honeys. Food Chem 2004;85:513–8.

[6] Marletta MA. Nitric oxide synthase: aspects concerning structure and catalysis. Cell 1994;78:927–30.

[7] Crane BR, Arvai AS, Gachhui R, Wu C, Ghosh DK, Getzoff ED, et al. The structure of nitric oxide synthase oxygenase domain and inhibitor complexes. Science 1997;278:425–31.

[8] Chen H, Zuo Y, Deng Y. Separation and determination of flavonoids and other phenolic compounds in cranberry juice by high-performance liquid chromatography. J Chromatogr A 2001;913:387–95.

[9] Martos I, Cossentini M, Ferreres F, Tomas-Barberan FA. Flavonoid composition of Tunisian honeys and propolis. J Agric Food Chem 1997;45: 2824–9.

[10] Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 1988;32:77–88.

[11] Morebise O, Fafunso MA, Makinde JM, Olajide OA, Awe EO. Antiinflammatory property of the leaves of *Gongronema latifolium*. Phytother Res 2002;16(Suppl 1):S75–7.

[12] Ogata M, Matsui T, Kita T, Shigematsu A. Carrageenan primes leukocytes to enhance lipopolysaccharide-induced tumor necrosis factor alpha production. Infect Immun 1999;67:3284–9.

[13] Kwon KH, Murakami A, Ohigashi H. Suppressive effects of natural and synthetic agents on dextran sulfate sodium-induced interleukin-1beta release from murine peritoneal macrophages. Biosci Biotechnol Biochem 2004;68:436–9.

[14] Matsuo N, Yamada K, Shoji K, Mori M, Sugano M. Effect of tea polyphenols on histamine release from rat basophilic leukemia (RBL-2H3) cells: the structure-inhibitory activity relationship. Allergy 1997;52:58–64.

[15] Haddad JJ. On the enigma of pain and hyperalgesia: a molecular perspective. Biochem Biophys Res Commun 2007;353:217–24.

[16] Sung YJ, Ambron RT. Pathways that elicit long-term changes in gene expression in nociceptive neurons following nerve injury: contributions to neuropathic pain. Neurol Res 2004;26:195–203.

[17] Zhang JH, Huang YG. The immune system: a new look at pain. Chin Med J (Engl) 2006;119:930–8.

[18] Jackson DL, Graff CB, Richardson JD, Hargreaves KM. Glutamate participates in the peripheral modulation of thermal hyperalgesia in rats. Eur J Pharmacol 1995;284:321–5.

[19] Meller ST, Gebhart GF. Nitric oxide (NO) and nociceptive processing in the spinal cord. Pain 1993;52:127–36.

[20] Yoneda H, Miura K, Matsushima H, Sugi K, Murakami T, Ouchi K, et al. Aspirin inhibits Chlamydia pneumoniae-induced NF-kappa B activation, cyclo-oxygenase-2 expression and prostaglandin E2 synthesis and attenuates chlamydial growth. J Med Microbiol 2003;52:409–15.

[21] Han WJ, Lee GB, Kim KY, Yoon WJ, Jin KH, Hong SW, et al. Ergolide, sesquiterpene lactone from Inula britannica, inhibits inducible nitric oxide synthase and cyclo-oxygenase-2 expression in RAW 264.7 macrophages through the inactivation of NF-. kappaB. Br J Pharmacol 2001;133:503–12.

[22] Terra X, Valls J, Vitrac X, Merrillon JM, Arola L, Ardevol A, et al. Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. J Agric Food Chem 2007;55:4357–65.

[23] Romier B, Van De Walle J, During A, Larondelle Y, Schneider YJ. Modulation of signalling nuclear factor-kappaB activation pathway by polyphenols in human intestinal Caco-2 cells. Br J Nutr 2008;100:542–51.

[24] DeWitt DL. Prostaglandin endoperoxide synthase: regulation of enzyme expression. Biochim Biophys Acta 1991;1083:121–34.

[25] O'Banion MK, Winn VD, Young DA. cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. Proc Natl Acad Sci U S A 1992;89:4888–92.

[26] Gutierrez-Venegas G, Jimenez-Estrada M, Maldonado S. The effect of flavonoids on transduction mechanisms in lipopolysaccharide-treated human gingival fibroblasts. Int Immunopharmacol 2007;7:1199–210.

**3.3 Gelam honey inhibits lipopolysaccharide-induced endotoxmeia in rats through the induction of heme oxygenase-1 and the inhibition of cytokines, nitric oxide, and high-mobility group protein B1**

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# Gelam honey inhibits lipopolysaccharide-induced endotoxemia in rats through the induction of heme oxygenase-1 and the inhibition of cytokines, nitric oxide, and high-mobility group protein B1

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## ARTICLE INFO

## ABSTRACT

Malaysian Gelam honey has anti-inflammatory and antibacterial properties, a high antioxidant capacity, and free radical-scavenging activity. Lipopolysaccharide (LPS) stimulates immune cells to sequentially release early pro- and anti-inflammatory cytokines and induces the synthesis of several related enzymes. The aim of this study was to investigate the effect of the intravenous injection of honey in rats with LPS-induced endotoxemia. The results showed that after 4 h of treatment, honey reduced cytokine (tumor necrosis factor-$\alpha$, interleukins 1$\beta$, and 10) and NO levels and increased heme oxygenase-1 levels. After 24 h, a decrease in cytokines and NO and an increase in HO-1 were seen in all groups, whereas a reduction in HMGB1 occurred only in the honey-treated groups. These results support the further examination of honey as a natural compound for the treatment of a wide range of inflammatory diseases.

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## 1. Introduction

Honey is a naturally sweet viscous fluid produced by bees from floral nectar. To date, more than 400 different chemical compounds have been identified in many varieties of honey [1], including proteins, enzymes, organic acids, mineral salts, vitamins, phenolic acids, flavonoids, free amino acids, fatty acids and small quantities of volatile compounds [2,3]. As early as 5000 BC, honey was used by Egyptians in wound management, while the Greeks, Chinese, and Romans exploited its antiseptic properties as a topical agent for the treatment of sores and skin ulcers [4]. The ability of honey to induce the activation and proliferation of peripheral blood cells, including lymphocytic and phagocytic activity, is well-established, as its role in combating infection by stimulating the anti-inflammatory,

antioxidant, and proliferative activities of the immune system [5,6]. It was reported in a clinical experiment that when wound infected with bacteria was treated with honey, infection was more quickly eradicated [7–9]. Immunomodulatory effects were demonstrated *in vitro* by cytokine release from human peripheral monocytes and the monocytic cell line Mono Mac 6 after incubation with honey [10]. All of these properties have been determined in Gelam honey. Specifically, Gelam honey inhibits the release of both nitric oxide (NO) and tumor necrosis factor (TNF)-$\alpha$ *in vitro* and *in vivo* [11]. The floral source of Gelam honey is *Melaleuca cajuputi* Powell, it has medicinal antiseptic, antibacterial, anti-inflammatory and anodyne properties, and it is used traditionally against pain, burns, colds, influenza and dyspepsia. Cajeput oil is produced from the *M. cajuputi* leaves by steam distillation. It is used for the treatment of coughs and colds, against stomach cramps, colic, asthma, relief of neuralgia and rheumatism. It has been approved for food use by the Food and Drug Administration (FDA) of the

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United States [12,13]. The active compounds in Gelam honey include ellagic acid, gallic acid ,chrysin, quercetin, caffeic acid phenethyl ester, luteolin, kaempferol, and hesperetin [11,14], many of which have anti-inflammatory and immunomodulatory properties [15–17]. Gelam honey also antagonizes the lipopolysaccharide (LPS)-induced immune response *in vitro* and *in vivo* [11,14]. LPS is a cell-wall component of Gram negative bacteria and a potent inducer of the host immune system, including the overproduction of numerous pro- and anti-inflammatory cytokines, an increase in oxidative stress, and the induction of nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1). Together, these events result in severe tissue injury. Moreover, LPS causes endotoxemia, which is associated with multiple organ failure and is often lethal [18,19]. Given the complexity of the immune response to LPS-induced endotoxemia and the many anti-inflammatory properties of honey, we examined the ability of Gelam honey to induce several key immunomodulators (TNF-α, IL-1, IL-6, IL-10, NO, and HO-1) and HMGB1 in a rat model of LPS-induced endotoxemia.

## 2. Materials and methods

### 2.1. Materials

Fresh Malaysian honey (Gelam, from *Apis mellifera*) was obtained from the National Apiary (Department of Agriculture, Parit Botak, Johor, Malaysia) and then sent to the Malaysian Nuclear Agency for sterilization using a cobalt-60 source (model JS10000). Prior to use, the Gelam honey was diluted in saline and then filter-sterilized through a 0.20-μm syringe filter. All chemicals and reagents used were of analytical grade.

### 2.2. Extraction of phenolic compounds from honey by strong acid hydrolysis

Extraction and hydrolysis conditions for the honey sample were performed to obtain their corresponding aglycones in 50% (v/v) aqueous methanol, containing hydrochloric acid (6 M) as described in Ref. [20] with a modified. For the extraction of phenolic compounds of Gelam honey, 5 g was dissolved in 30 ml 50% (V/V) aqueous methanol with added HCl. The mixture was stored at 35 °C for 24 h. Then the extract was evaporated under pressure at 40 °C after that, the residues were diluted with 5 ml water and 5 ml ethyl acetate repeated three times. All ethyl acetate extracts were collected and then flushed with $N_2$; the dry residues were redissolved in methanol, and then filtrated through a membrane (45 μml). 20 μml of resultants extract was injected to Liquid chromatography–mass spectrometry (LC–MS) to identify the compounds present. The LC–MS conditions were similar to the previously describe [11]

### 2.3. Animals

Male Sprague Dawley (SD) rats weighing 300–350 g were kept in individual cages under standard conditions (12-h light and 12-h dark conditions). They were fed a diet of Purina lab chow and given water *ad libitum*. The study was carried out in accordance with the University of Malaya Animal Ethics Committee guidelines for animal experimentation. Approved protocols were followed and a project license, ANES/14/07/2010/MKAK (R), was obtained.

### 2.4. Toxicity test

The toxicity of Gelam honey in rats (n = 8) was evaluated for 1 month prior to the study. Four different doses of honey (10, 60, 300, and 600 mg/kg diluted in 1 ml of saline) were injected daily through the tail vein. The control group received a similar volume of saline. Both the honey- and the saline-treated rats were observed for 3 h after injection.

Symptoms and mortality were recorded for all groups. At the end of the study, all of the rats were sacrificed and their blood and organs collected. Compared with the control group, the treated groups showed no abnormalities as determined by biochemical and histopathological analyses of the liver, lungs, and kidneys (data not shown).

### 2.5. Induction of endotoxemia in rats by LPS stimulation and treatment with honey

The rats were divided into six groups (n = 6/group) and were treated as described below. Endotoxemia was induced in four groups by intravenous injection of 5 mg/kg LPS (B: 0111; Sigma, St. Louis, MO, USA) prepared in saline. One of the four groups served as the positive control (LPS only), while the other three received one of three different concentrations of honey: 60 mg/kg (H60), 300 mg/kg (H300), and 600 mg/kg (H600), diluted in saline. The fifth group served as the negative control and was given saline only, while the sixth group was given honey (600 mg/kg in saline) but no LPS. All doses were administered in a volume of 1 ml and were prepared immediately prior to injection.

Five groups of 10 rats were used for survival rate analysis. Endotoxemia was induced in four groups by intravenous injection of 5 mg/kg LPS as described above; the fifth group was left untreated (control). The viability of all 50 rats was monitored every 12 h for 15 days.

### 2.6. Quantification of cytokines, NO, HO-1, and HMGB1 levels

Blood samples were collected 4 and 24 h after treatment, after which all of the rats were killed. Samples were collected after 4 h of treatment and serum levels of TNF-α, IL-1, IL-6, IL-10, NO, and HO-1 were measured using an enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA). The ELISA was repeated after 24 h. Serum HMGB1 levels were also examined after 24 h using an ELISA (Shino-Test: 326054329, Japan) according to the manufacturer's instructions.

### 2.7. Statistical analysis

Data are expressed as the mean ± standard deviation and analyzed using a non-parametric one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. All analyses were carried out using GraphPad Prism 5 statistical software (San Diego, CA, USA). Survival data were subjected to Kaplan–Meier analysis. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Identification of phenolic compounds in Gelam honey by LC–MS

LC–MS was used for the identification of some phenolic compounds. Fig. 1S shows the peaks of gellic acid, ferulic acid, quercetin, ellagic acid, Hesperetin, and chrysin detected in Gelam honey using positive and negative ESI-MS. Figs. 2S–6S show the fragments of the identified compounds using positive and negative ionization (ESI-MS). Some compounds did not ionize under the conditions used for analysis. The negative ionization was more useful for identifying compounds in the extracts than positive ESI-MS.LC–MS analysis.

### 3.2. Effect of honey on cytokines, HMGB1, NO, and HO-1

Cytokine production was lower in rats injected with LPS and subsequently treated with honey than in rats injected with LPS alone. A significant reduction in TNF-α level occurred at 4 h, but was no longer apparent at 24 h (Fig. 1). Honey also showed potent inhibitory activity against IL-1β and IL-10; however, in contrast to its short-lived effect on TNF-α level, highly significant differences in the levels of
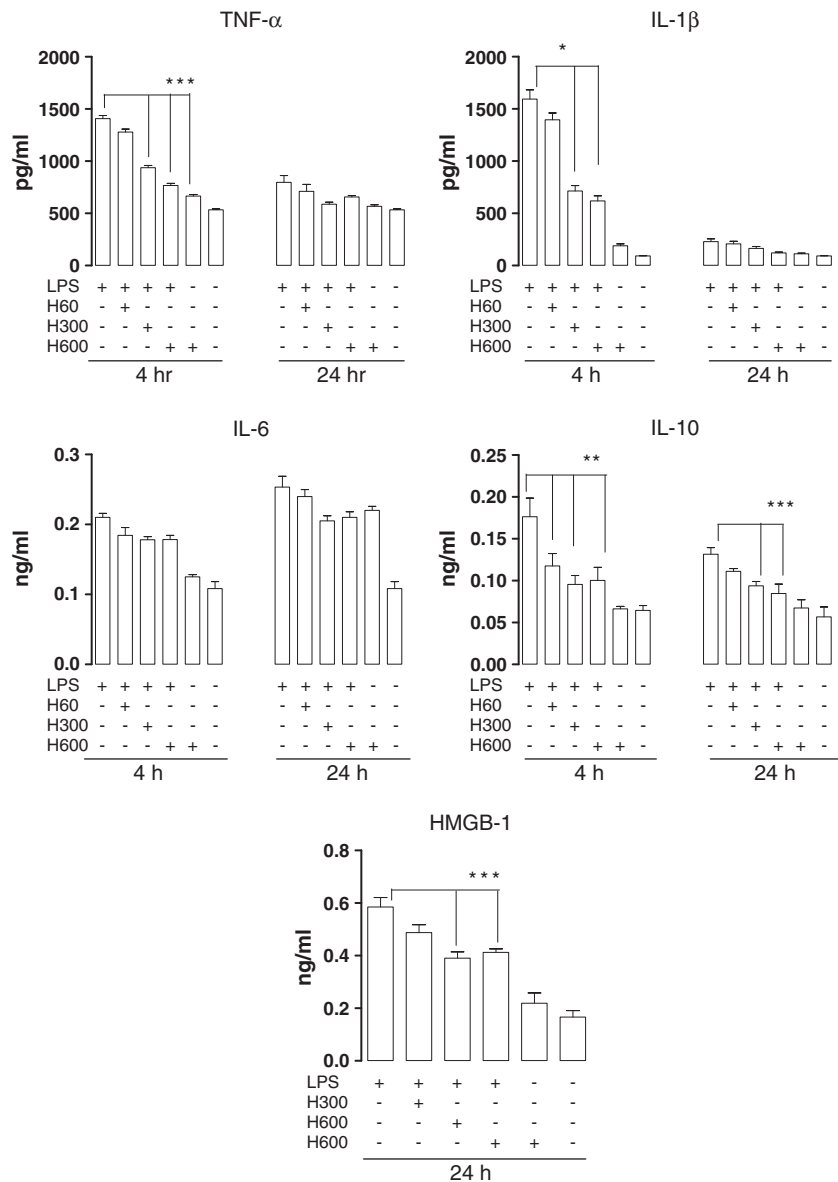


Fig. 1. Effect of honey on cytokine and high mobility group protein B1 (HMGB1) levels in rats. Rats were injected with lipopolysaccharide (LPS) and then treated with varying doses of honey. Cytokines and HMGB1 were measured using an ELISA at 4 h and 24 h. Six groups were examined (n = 6/group), and all groups received injections into the tail vein. The LPS group was treated with 5 mg/kg LPS in 1 ml saline, the negative control group with 1 ml saline, the honey-treated groups with injection of 60, 300, and 600 mg/kg honey plus 5 mg/kg LPS in 1 ml saline, and the final control group with only 600 mg/kg honey in 1 ml saline. Data are presented as the mean ± standard deviation. (***P<0.005; **P<0.003; and *P<0.001).

these two cytokines between the honey-treated groups and the LPS-only control group were evident both at 4 h and 24 h (Fig. 1). The specific immunomodulatory effects of honey were demonstrated by the observation that IL-6 levels remained unchanged after honey treatment, and did not differ from those of the control groups, while serum HMGB1 levels decreased only at 24 h (Fig. 1). Furthermore, honey induced a significant reduction in NO production at 4 h and to a lesser extent at 24 h (Fig. 2). Honey was also a potent inducer of HO-1, with significant differences between the honey-treated groups and the LPS-only control group evident at 4 h and at 24 h (Fig. 3).

### 3.3. Survival

At 12 h after LPS injection, only 70% of the rats in the H60 group survived; however, all the LPS-injected rats in the H300 or H600 groups were still alive. At 24 h, survival in the LPS, H60, H300, and H600 groups decreased to 30%. By 36 h, all rats in the LPS and H60 groups had died, while survival in the H300 and H600 groups decreased to 38%. By contrast, the negative control group, which received saline only, survived for an average of 15 days. Kaplan–Meier analysis revealed a significantly shorter time to death in the LPS-only group than in the H300 and H600 groups (Fig. 4).

## 4. Discussion

In our study, Gelam honey was injected intravenously, as this is the fastest route of delivery for the majority of drugs. The rapid transit of the injected agent through the bloodstream allows immediate exposure to the blood and immune cells. In addition, intravenous injection preserves the activity of the many vitamins, minerals, enzymes, and active compounds present in the honey, whereas the acid environment of the stomach encountered following oral administration
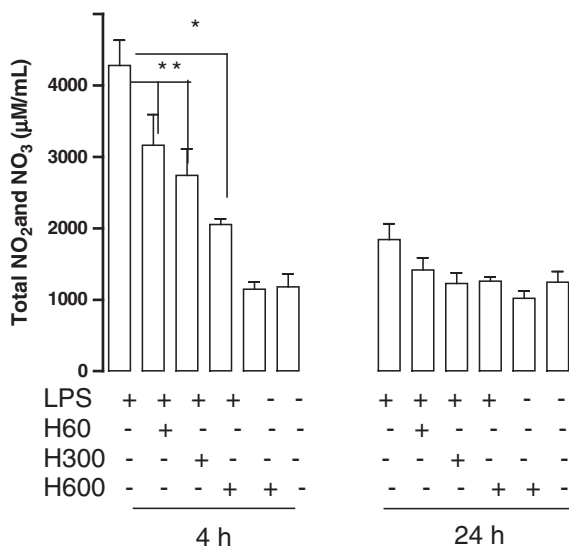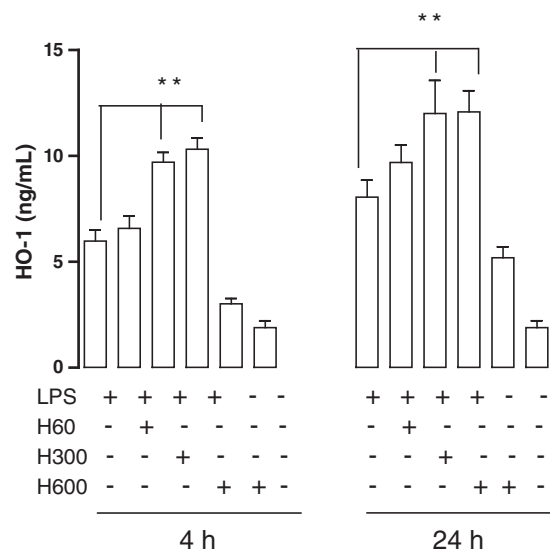


**Fig. 3.** Effect of honey on heme oxygenase-1 (HO-1) levels. Rats were injected with 5 mg/kg LPS and HO-1 levels were measured 4 h and 24 h later. The six groups of rats (n = 6/group) were the same as those described in Fig. 1. Data are presented as the mean ± standard deviation. (**$P<0.003$).

would result in their destruction [21–23]. Previous study reported that no side effects with the use of intravenous honey in sheep [24]. This study demonstrated that intravenous injection of honey into LPS-treated rats inhibited cytokine production, including that of TNF-α, IL-1, and IL-10, as well as HMGB1 and NO release, while at the same time inducing HO-1. Thus, consistent with *in vitro* studies demonstrating the immunomodulatory effects of Gelam honey on cytokines and NO released in L929 and RAW 264.7 [11], our results show that Gelam honey inhibits cytokines, NO and protects rats from endotoxemia. Upregulation of HO-1 inhibits the release of cytokines, HMGB1 and NO. Furthermore, upregulation of HO-1 may protect rats from the effects of endotoxemia, which may reflect the decrease in systemic levels of cytokines, HMGB1, and NO. The cytokine levels observed in



**Fig. 2.** Effect of honey on nitric oxide (NO). NO production was measured (as nitrate and nitrite) in rats stimulated with 5 mg/kg LPS 4 h and 24 h after treatment. The groups of rats were treated as described in Fig. 1. Data are presented as the mean ± standard deviation. (***$P<0.003$ and *$P<0.001$).
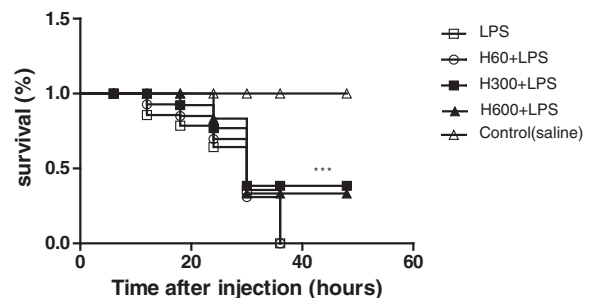


**Fig. 4.** Effect of honey on survival. Five groups of rats (n = 10/group) received a 1-ml injection of LPS (5 mg/kg) into the tail vein. The survival rate of the group injected with LPS alone is depicted by white squares. The survival rates of the groups treated with LPS plus 60, 300, 600 mg/kg honey are depicted by white circles, black squares, and black triangles, respectively. Control rats received saline only (white triangles). Honey was injected daily for 3 days after LPS treatment. Kaplan–Meier analysis showed a significantly shorter time to death for the untreated LPS group (LPS) than for the groups treated with LPS + 300 mg/kg honey (LPS + H300) or LPS + 600 mg/kg honey (LPS + H600) (***$P<0.005$).

the blood and tissues are attributable to activation of neutrophils, macrophages, and lymphocytes and their subsequent infiltration into the tissues, and to the activation of other cells such as endothelial in different tissues such as blood, vessels, lung and liver. In endotoxemia, the levels of cytokines, HMGB1 and NO, are increased in the blood and tissues due to activation of nuclear factor (NF)-κB. Inhibition of cytokines, NO, and HMGB1, and the induction of HO-1 induced in response to LPS are important for protection against endotoxemia [25]. The release of cytokines and NO contributes to inflammation-related pathologies and mortality; therefore, inhibition of cytokines and NO provides protection from endotoxemia-induced mortality in both animals and humans [26]. The mechanism by which honey inhibits both pro-inflammatory cytokines (such as TNF-α and IL-1β), and NO is unclear, but it may involve the inhibition of NF-κB. A previous study shows that NF-κB prevents the release pro-inflammatory cytokines and inhibits the release of iNOS [27,28]. However, the inhibitory effect of honey on the anti-inflammatory cytokine, IL-10, remains a matter for speculation. IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of TNF-α, IL-1α, IL-1β, and IL-6 *in vitro* [29–31]. It is also an important mediator of endotoxemia-induced immunosuppression and of macrophage deactivation during LPS desensitization and endotoxemia [30,32]. High circulating levels of IL-10 lead to immunoparalysis [31,33], an effect that is compounded by the presence of secondary factors, including LPS; in such cases, temporary immunoparalysis can become chronic, with a concomitantly higher risk of infection [34,35]. The enzyme HO-1 protects animals from severe inflammation, and a clear relationship has been determined between HO-1 activation and decreased HMGB1 levels which, in turn, protects animals from endotoxemia. In addition, the induction of HO-1 improves animal survival during lethal endotoxemia, and inhibits the production of both NO and cytokines such as TNF-α and IL-1β [36]. Consistent with our suggestion that honey exerts its effects, at least in part, *via* NF-κB, HO-1 also inhibits NF-κB, thereby modulating cytokine release and inhibiting iNOS, with a subsequent decrease in NO [37,38]. Our results showed that honey inhibited HMGB1 while inducing HO-1 and increasing the survival of LPS-treated rats. Similarly, potent HO-1-inducing abilities were identified in other natural products (such as (−)-epigallocatechin-3-gallate (EGCG)); moreover, these natural products include immunomodulators of LPS-induced HMGB1 release, and their administration increases the survival of HO-1-deficient mice [19,39].

The active components in honey include phenolic acid, flavonoids, and polyphenols such as caffeic acid phenethyl ester and quercetin [40–42], which inhibit HMGB1.

## 5. Conclusion

In addition to its well-known properties as a natural sweetener, honey has many anti-inflammatory properties. These include the ability to stimulate HO-1 production and to inhibit the release of both pro- and anti-inflammatory cytokines (TNF-α, IL-1, IL-10), HMGB1, and NO. Together, these effects suggest a mechanism by which honey is able to protect animals from the lethal effects of LPS-induced endotoxemia. Therefore, honey should be further explored with respect to its anti-inflammatory and immunomodulatory properties for the use in the treatment of inflammatory diseases.

## Declaration of competing interests

There are no competing interests to declare.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2012.05.008.

## References

[1] Lazaridou A, Biliaderis CG, Bacandritsos N, Sabatini AG. Composition, thermal and rheological behaviour of selected Greek honeys. J Food Eng 2004;64:9–21.
[2] Gheldof N, Wang XH, Engeseth NJ. Buckwheat honey increases serum antioxidant capacity in humans. J Agric Food Chem 2003;51:1500–5.
[3] Weston RJ, Brocklebank LK. The oligosaccharide composition of some New Zealand honeys. Food Chem 1999;64:33–7.
[4] White R. The benefits of honey in wound management. Nurs Stand 2005;20:57–64 [quiz 66].
[5] Molan PC. Potential of honey in the treatment of wounds and burns. Am J Clin Dermatol 2001;2:13–9.
[6] Al-Jabri AA. Honey, milk and antibiotics. Afr J Biotechnol 2005;4:1580–7.
[7] Al-Waili NS, Saloom KY. Effects of topical honey on post-operative wound infections due to gram positive and gram negative bacteria following caesarean sections and hysterectomies. Eur J Med Res 1999;4:126–30.
[8] Albietz JM, Lenton LM. Effect of antibacterial honey on the ocular flora in tear deficiency and meibomian gland disease. Cornea 2006;25:1012–9.
[9] Simon A, Traynor K, Santos K, Blaser G, Bode U, Molan P. Medical honey for wound care—still the 'latest resort'? Evid Based Complement Alternat Med 2009;6:165–73.
[10] Tonks AJ, Cooper RA, Jones KP, Blair S, Parton J, Tonks A. Honey stimulates inflammatory cytokine production from monocytes. Cytokine 2003;21:242–7.
[11] Kassim M, Achoui M, Mustafa MR, Mohd MA, Yusoff KM. Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate *in vitro* anti-inflammatory activity. Nutr Res 2010;30:650–9.
[12] Hassan UV, Jambari A, Aspllaha SM. Biocidal and medicinal properties of a triterpene from the bark of *Melaleuca cajuputi*. Weed Sci Soc Pak 2003;9:241–2.
[13] Rattiwanich T, Karuhapattana B, Kittirattrakarn T, Anantachoke C. *Melaleuca cajuputi* Powell leaves oil from Toedaeng swamp forest. Annual Conference of Forestry; 1992. p. 361–7.
[14] Kassim M, Achoui M, Mansor M, Yusoff KM. The inhibitory effects of Gelam honey and its extracts on nitric oxide and prostaglandin E(2) in inflammatory tissues. Fitoterapia 2010;81:1196–201.
[15] Kotanidou A, Xagorari A, Bagli E, Kitsanta P, Fotsis T, Papapetropoulos A, et al. Luteolin reduces lipopolysaccharide-induced lethal toxicity and expression of proinflammatory molecules in mice. Am J Respir Crit Care Med 2002;165:818–23.
[16] Fidan H, Sahin O, Yavuz Y, Kilbas A, Cetinkaya Z, Ela Y, et al. Caffeic acid phenethyl ester reduces mortality and sepsis-induced lung injury in rats. Crit Care Med 2007;35:2822–9.
[17] Tang D, Kang R, Xiao W, Zhang H, Lotze MT, Wang H, et al. Quercetin prevents LPS-induced high-mobility group box 1 release and proinflammatory function. Am J Respir Cell Mol Biol 2009;41:651–60.
[18] Oberholzer A, Oberholzer C, Moldawer LL. Sepsis syndromes: understanding the role of innate and acquired immunity. Shock 2001;16:83–96.
[19] Takamiya R, Hung CC, Hall SR, Fukunaga K, Nagaishi T, Maeno T, et al. High-mobility group box 1 contributes to lethality of endotoxemia in

heme oxygenase-1-deficient mice. Am J Respir Cell Mol Biol 2009;41: 129–35.

[20] Hertog MGL, Hollman PCH, Katan MB. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. J Agric Food Chem 1992;40:2379–83.

[21] Shire SJ, Shahrokh Z, Liu J. Challenges in the development of high protein concentration formulations. J Pharm Sci 2004;93:1390–402.

[22] Shaji J, Patole V. Protein and peptide drug delivery: oral approaches. Indian J Pharm Sci 2008;70:269–77.

[23] Chang Q, Zuo Z, Ho WK, Chow MS. Comparison of the pharmacokinetics of hawthorn phenolics in extract versus individual pure compound. J Clin Pharmacol 2005;45:106–12.

[24] Al-Waili NS. Identification of nitric oxide metabolites in various honeys: effects of intravenous honey on plasma and urinary nitric oxide metabolites concentrations. J Med Food 2003;6:359–64.

[25] Tsoyi K, Lee TY, Lee YS, Kim HJ, Seo HG, Lee JH, et al. Heme-oxygenase-1 induction and carbon monoxide-releasing molecule inhibit lipopolysaccharide (LPS)-induced high-mobility group box 1 release *in vitro* and improve survival of mice in LPS- and cecal ligation and puncture-induced sepsis model *in vivo*. Mol Pharmacol 2009;76:173–82.

[26] Sunden-Cullberg J, Norrby-Teglund A, Rouhiainen A, Rauvala H, Herman G, Tracey KJ, et al. Persistent elevation of high mobility group box-1 protein (HMGB1) in patients with severe sepsis and septic shock. Crit Care Med 2005;33:564–73.

[27] Abraham E. Nuclear factor-kappaB and its role in sepsis-associated organ failure. J Infect Dis 2003;187(Suppl. 2):S364–9.

[28] Matsumori A, Nunokawa Y, Yamaki A, Yamamoto K, Hwang MW, Miyamoto T, et al. Suppression of cytokines and nitric oxide production, and protection against lethal endotoxemia and viral myocarditis by a new NF-kappaB inhibitor. Eur J Heart Fail 2004;6:137–44.

[29] Chen J, Raj N, Kim P, Andrejko KM, Deutschman CS. Intrahepatic nuclear factor-kappa B activity and alpha 1-acid glycoprotein transcription do not predict outcome after cecal ligation and puncture in the rat. Crit Care Med 2001;29:589–96.

[30] Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. J Immunol 1991;147:3815–22.

[31] de Vries JE. Immunosuppressive and anti-inflammatory properties of interleukin 10. Ann Med 1995;27:537–41.

[32] Steinhauser ML, Hogaboam CM, Kunkel SL, Lukacs NW, Strieter RM, Standiford TJ. IL-10 is a major mediator of sepsis-induced impairment in lung antibacterial host defense. J Immunol 1999;162:392–9.

[33] Allen ML, Hoschtitzky JA, Peters MJ, Elliott M, Goldman A, James I, et al. Interleukin-10 and its role in clinical immunoparalysis following pediatric cardiac surgery. Crit Care Med 2006;34:2658–65.

[34] Randow F, Syrbe U, Meisel C, Krausch D, Zuckermann H, Platzer C, et al. Mechanism of endotoxin desensitization: involvement of interleukin 10 and transforming growth factor beta. J Exp Med 1995;181:1887–92.

[35] Wolk K, Docke W, von Baehr V, Volk H, Sabat R. Comparison of monocyte functions after LPS- or IL-10-induced reorientation: importance in clinical immunoparalysis. Pathobiology 1999;67:253–6.

[36] Yu JB, Yao SL. Protective effects of hemin pretreatment combined with ulinastatin on septic shock in rats. Chin Med J (Engl) 2008;121:49–55.

[37] Bellezza I, Tucci A, Galli F, Grottelli S, Mierla AL, Pilolli F, et al. Inhibition of NF-kappaB nuclear translocation via HO-1 activation underlies alpha-tocopheryl succinate toxicity. J Nutr Biochem. in press, http://dx.doi.org/10.1016/j.jnutbio.2011.10.012

[38] Brouard S, Berberat PO, Tobiasch E, Seldon MP, Bach FH, Soares MP. Heme oxygenase-1-derived carbon monoxide requires the activation of transcription factor NF-kappa B to protect endothelial cells from tumor necrosis factor-alpha-mediated apoptosis. J Biol Chem 2002; 277:17950–61.

[39] Li W, Ashok M, Li J, Yang H, Sama AE, Wang H. A major ingredient of green tea rescues mice from lethal sepsis partly by inhibiting HMGB1. PLoS One 2007;2:e1153.

[40] Lin HC, Cheng TH, Chen YC, Juan SH. Mechanism of heme oxygenase-1 gene induction by quercetin in rat aortic smooth muscle cells. Pharmacology 2004;71:107–12.

[41] Scapagnini G, Foresti R, Calabrese V, Giuffrida Stella AM, Green CJ, Motterlini R. Caffeic acid phenethyl ester and curcumin: a novel class of heme oxygenase-1 inducers. Mol Pharmacol 2002;61:554–61.

[42] Min KS, Lee HJ, Kim SH, Lee SK, Kim HR, Pae HO, et al. Hydrogen peroxide induces heme oxygenase-1 and dentin sialophosphoprotein mRNA in human pulp cells. J Endod 2008;34:983–9.

## 3.4  Gelam honey has a protective effect against lipopolysaccharide (LPS)-induced organ failure

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*Article*

# Gelam Honey Has a Protective Effect against Lipopolysaccharide (LPS)-Induced Organ Failure

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**Abstract:** Gelam honey exerts anti-inflammatory and antioxidant activities and is thought to have potent effects in reducing infections and healing wounds. The aim of this study was to investigate the effects of intravenously-injected Gelam honey in protecting organs from lethal doses of lipopolysaccharide (LPS). Six groups of rabbits ($N = 6$) were used in this study. Two groups acted as controls and received only saline and no LPS injections. For the test groups, 1 mL honey (500 mg/kg in saline) was intravenously injected into two groups (treated), while saline (1 mL) was injected into the other two groups (untreated); after 1 h, all four test groups were intravenously-injected with LPS (0.5 mg/kg). Eight hours after the LPS injection, blood and organs were collected from three groups (one from each treatment stream) and blood parameters were measured and biochemical tests, histopathology, and myeloperoxidase assessment were performed. For survival rate tests, rabbits from the remaining three groups were monitored over a 2-week period. Treatment with honey showed protective effects on organs through the improvement of organ blood parameters, reduced infiltration of neutrophils, and decreased myeloperoxidase activity. Honey-treated rabbits also showed reduced mortality after LPS injection compared with untreated rabbits. Honey may have a therapeutic effect in protecting organs during inflammatory diseases.

## 1. Introduction

Honey is a natural, sweet and viscous fluid produced by bees from floral nectar, which comprises more than 400 different chemical compounds [1], including proteins, enzymes, organic acids, mineral salts, vitamins, phenolic acids, flavonoids, free amino acids, and small quantities of volatile compounds [2]. Historically, honey has been used as a treatment for a broad spectrum of injuries, including wounds, burns and ulcers [3,4]. Honey has also been reported to stimulate the immune system (monocytes, neutrophils) [5–7]. It also clears infection by boosting the immune system, exerting anti-inflammatory and antioxidant activities, and stimulating cell growth [8]. Gelam honey inhibits nitric oxide (NO) and cytokine release both *in vitro* and *in vivo* [9,10].

Lipopolysaccharide (LPS) stimulates innate immune responses that mediate the cellular release of NO and various proinflammatory cytokines and chemokines, as well as inducing macrophage migration and contributing to the pathogenesis of sepsis [11]. Injection of animals with high doses of LPS causes multiple organ failure, characterized by circulatory failure, systemic hypotension, hypo-reactivity to vasoconstrictors, subsequent problems with organ perfusion and the development of functional abnormalities [12], which reflect systemic inflammatory response syndrome and septic shock, rather than endotoxin-induced failure of lung, liver, and renal tissues [13].

Sepsis is the leading cause of death worldwide, with more than 750,000 cases of sepsis diagnosed annually and mortality rates ranging from 30 to 60%; this systemic inflammation accounts for approximately 200,000 deaths per year in the US alone [14]. Sepsis causes endothelial injury and neutrophil infiltration into tissues, leading to local injury, disturbed capillary blood flow and enhanced microvascular permeability, disseminated intravascular coagulation, circulatory collapse, hypoxia and, ultimately, multiple organ failure [15]. The aim of the current study was to investigate whether intravenous injection of honey can protect organs from lethal doses of LPS that induce sepsis in rabbits.

## 2. Results

### 2.1. Effect of Gelam Honey on Biochemical and Hematological Tests, Histopathology, and MPO Activity

Intravenous injection of honey resulted in potent protection against a lethal dose of LPS as evidenced by improved liver, kidney, cardiac and lipid profiles. Compared to the untreated group, the honey-treated group showed significant reductions in the levels of alanine transaminase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (γ-GT), alkaline phosphatase (ALP), cholesterol, triglycerides, creatine kinase, creatinine, urea and amylase. Moreover, the honey-treated group showed higher RBC, WBC and thrombocyte counts than the untreated group (Table 1). Arterial blood gases and pH values were determined for all groups (Table 1). The honey-treated group showed mild respiratory alkalosis, while in the untreated group, the arterial blood pH was closer to acidosis. Blood $pCO_2$ was lowered by LPS injection, but to the same level in the honey-treated group and

untreated group, indicating that honey injection did not prevent the reduction in $pCO_2$. Blood $HCO_3$ and $PO_2$ were higher in the honey-treated group than in the untreated group. There was clear evidence of hypoxia in the untreated group, as shown by the reduction in the $pO_2$ value (Table 1). Neutrophil infiltration was reduced in the treated group; however, MPO activity in the honey-treated group was significantly lower than that in the untreated group (Figure 1). In addition, more histopathological changes were observed in the untreated group, as evidenced by cellular infiltration of the lungs (Figure 2). Finally, 66.7% of rabbits in the untreated group died compared with 33.3% in the treated group (Figure 3). Survival rates were monitored over a 2-week period.

**Figure 1.** Effect of honey on neutrophil infiltration into lung tissues induced by a lethal dose of lipopolysaccharide (LPS). Myeloperoxidase (MPO) activity was measured in all groups ($n = 6$ per group) 8 h after LPS injection. MPO activity was significantly higher in the untreated (saline + LPS 0.5 mg/kg) group than in the treated (honey, 500 mg/kg + LPS 0.5 mg/kg) group. *** $P < 0.002$.



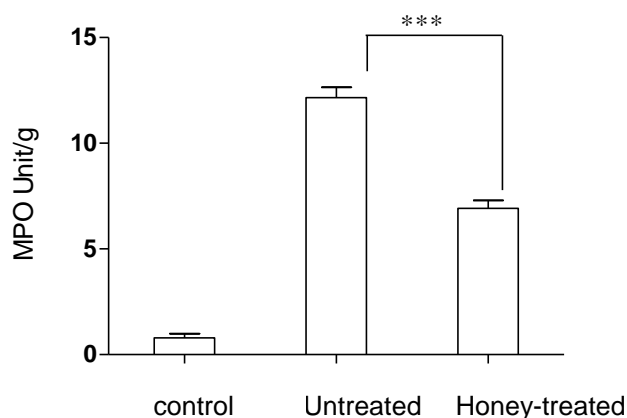**Figure 2.** (**A**) Immune-cell infiltration and tissue damage in the lungs of rabbits from the untreated group 8 h after LPS injection; (**B**) Immune-cell infiltration and tissue damage in the lungs of rabbits from the honey-treated group 8 h after LPS injection; (**C**) Normal lung tissues in rabbits treated with saline. Hematoxylin and eosin staining; magnification 10×; scale bar, 30 μm.
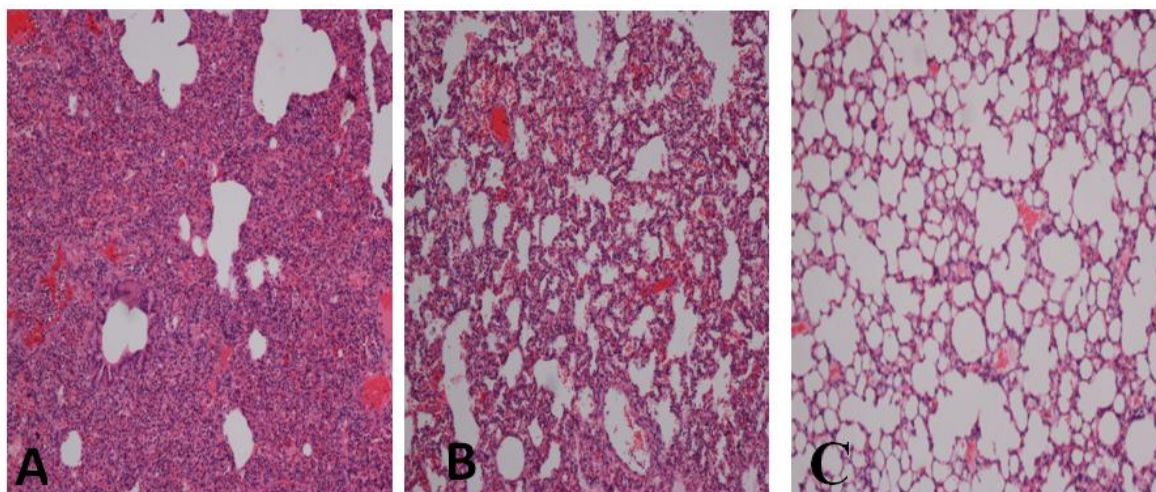
**Figure 3.** The effect of honey on the survival of rabbits injected with LPS (0.5 mg/kg). Rabbits in all three groups (*n* = 6 per group) received 1 mL injections of LPS into the ear vein. The survival rates in the untreated and honey-treated (60, 300, and 600 mg/kg) groups injected with 0.5 mg/kg LPS are shown as black triangles and black squares, respectively. Control rabbits received saline only (black circles). Honey was administered daily for 3 days after LPS treatment. Kaplan–Meier analysis showed significantly better survival rates in the honey-treated group (500 mg/kg + LPS) than in the untreated LPS group (LPS). *** $P < 0.005$.
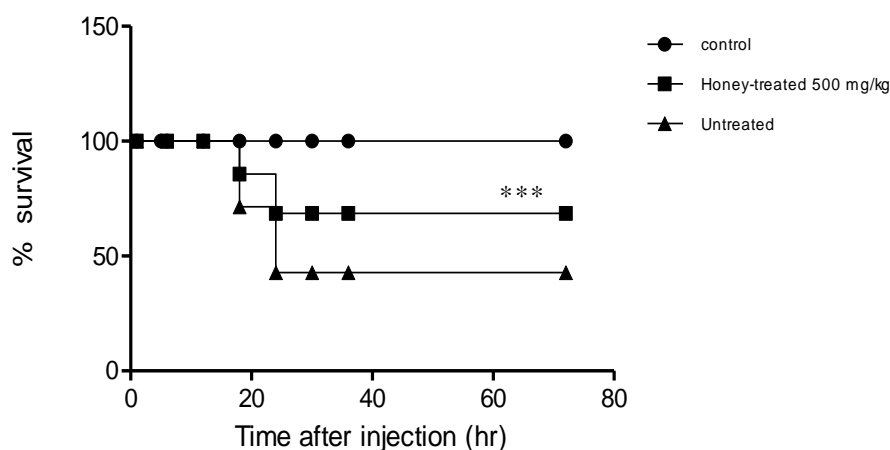


**Table 1.** Assessment of organ damage in the control group and in honey-treated and untreated groups given a single intravenous injection of lipopolysaccharide.

| Parameter | Normal Rabbits (*N* = 6) | Untreated (*N* = 6) | Honey-treated (*N* = 6) |
|---|---|---|---|
| Urea (mmol/L) | 5.85 ±0.20 | 55.85 ±2.5 | 10.5 ±0.23 [a] |
| Creatinine (mmol/L) | 83.71 ±2.5 | 154 ±6.16 | 72 ±2.87 [a] |
| ALT (IU/L) | 54.125 ±1.8 | 108.75 ±3.6 | 78.75 ±1.98 [a] |
| AST (IU/L) | 27.75 ±0.9 | 577.33 ±19.2 | 231.5 ±7.6 [a] |
| ALP (IU/L) | 131.5 ±4.1 | 542.75 ±15.5 | 308.75 ±11.4 [a] |
| GGT (IU/L) | 10 ±0.32 | 38.4 ±0.6 | 25 ±1.7 [b] |
| Triglyceride (mmol/L) | 0.885 ±0.03 | 10.434 ±0.4 | 2.47 ±0.13 [a] |
| Total cholesterol (mmol/L) | 1.1125 ±0.04 | 3.16 ±0.1 | 1.8 ±0.08 [c] |
| HDL (mmol/L) | 0.65 ±0.03 | 0.25 ±0.006 | 0.545 ±0.02 [b] |
| LDL (mmol/L) | 0.202 ±0.005 | 0.45 ±0.01 | 0.32 ±0.012 |
| Creatine kinase (IU/L) | 1327.4 ±5.3 | 2168.3 ±34 | 998.6 ±26.8 [a] |
| pH (KPa) | 7.38 ±0.3 | 7.36 ±0.3 | 7.5 ±0.27 [b] |
| pCO$_2$ (KPa) | 4.3 ±0.17 | 3 ±0.12 | 3 ±0.12 |
| pO$_2$ (KPa) | 16.21 ±0.53 | 7.65 ±0.23 | 19.3 ±0 .77 [b] |
| HCO$_3$ (mmol/L) | 19 ±0.71 | 15 ±0.51 | 19 ±0.54 [a] |
| Platelet (10e9/L) | 194.3 ±6.4 | 144.5 ±4.3 | 183.4 ±7.6 [b] |
| Amylase (IU/L) | 181.3 ±7.2 | 215.5 ±6.2 | 180 ±5.2 [b] |
| RBC (10e12/L) | 9.27 ±0.31 | 4.83 ±0.15 | 8.3475 ±0.32 [a] |
| WBC (10e9/L) | 15 ±0.6 | 6.05 ±0.25 | 11.65 ±0.36 [a] |

[a] $P < 0.001$; significant effect of untreated group *vs.* honey-treated group; [b] $P < 0.003$; significant effect of untreated group *vs.* honey-treated group; [c] $P < 0.005$; significant effect of untreated group *vs.* honey-treated group.

## 3. Discussion

Previous studies have shown that honey has antioxidant, antimicrobial, and anti-inflammatory properties [16]. This study identified a protective role for honey against systemic damage induced by lethal doses of LPS in a rabbit model. These effects were evidenced by decreased blood chemistry parameters of organ dysfunction, decreased cellular infiltration into the tissues, and decreased mortality. To the best of our knowledge, this is the first study showing that honey can protect organs from lethal doses of LPS. The results indicate that honey can counteract the effects of LPS, which is a compound that can lead to organ and multi-organ failure.

When immune responses are insufficient, infections can lead to sepsis [17]. Many studies report that sepsis is a complicated pathophysiological and immunological process that causes alterations in the structure and characteristics of blood cells and tissues, leading to multi-organ failure. Lethal doses of LPS in animals induce a variety of organ and systemic changes that lead to organ failure and, ultimately, to death [18,19]. Previous studies have shown that the acute exposure of rabbits to LPS is associated with necrosis in organs such as the lungs and liver. The presence of polymorphonuclear leukocytes (PMNLs) was noted in association with necrosis in the lung and liver as well as an apoptotic cellular appearance in the LPS group. In addition, LPS stimulates the production of many cellular substances, such as cytokines, NO, vasoactive peptides, pro-coagulant factors, and prostaglandins, both *in vitro* and *in vivo* [15]. Earlier reports indicate that LPS and cytokines, such as TNF-$\alpha$ and IL-1$\beta$, induce apoptotic necrosis in cells and tissues [20,21]. Furthermore, LPS activates NF-$\kappa$B, which activates many mediators including pro- and anti-inflammatory cytokines such as TNF-$\alpha$ ,IL-1$\beta$, IL6 and IL-10 [22]. These cytokines enhance vascular permeability, stimulate the expression of adhesion molecules on endothelial cells, and induce infiltration of cells from the blood to tissues [23]. Sepsis-induced acute lung injury is a major clinical problem with significant morbidity and mortality [24–26]. PMNLs are thought to contribute significantly to the pathophysiologic features of acute lung injury [27–31]. A pathological hallmark of acute lung injury is subsequent tissue infiltration of neutrophils and pulmonary microvascular sequestration [32,33]. Enhanced pulmonary neutrophil sequestration and infiltration during sepsis changes the neutrophil profile by increasing neutrophil surface expression and activating cell-cell adhesion molecules, and enhancing the release of soluble mediators, production of cytokines, and generation of reactive oxygen species, NO, and ONOO$^-$ [34–38]. Acute lung injury is characterized by increased MPO activity, a marker of neutrophil infiltration, increased expression and activity of cytokines and iNOS, high-protein pulmonary edema, and oxidant stress [31,39]. Pulmonary microvascular neutrophil sequestration and tissue infiltration are hallmarks of the pathogenesis of acute lung injury [33,40,41]. The present study is in agreement with previous studies showing that sepsis induces changes in pulmonary microvascular neutrophil sequestration and alveolar neutrophil infiltration, [34–36,42] as clearly shown in the untreated group but not in the honey-treated group (Figures 1 and 2). In addition, honey treatment decreased lung injury by inhibiting MPO activity. Therefore, as reported in our previous studies, honey may decrease lung injury through systemic inhibition of cytokines such as PGE$_2$ and NO [9,10].

In this study, the reductions in RBC, WBC, and platelet counts observed in the untreated group confirm those seen in earlier reports [43,44]. Treatment with honey significantly attenuated the severe reductions in blood counts (WBC and RBC) and thrombocytopenia, suggesting that honey has a

protective role against sepsis-induced disseminated intravascular coagulation. LPS causes disseminated intravascular coagulation, which is associated with coagulation disorders and loss of platelets. In the liver, LPS causes increases in AST, ALT, γ-GT, and lipid profiles [43,45–49], which are all markers of hepatic damage [44,49,50]. Our results confirm that sepsis caused liver failure, as shown by significantly elevated serum levels of AST, ALP, and γ-GT in the untreated group; honey inhibited these increases. Improved liver function tests after honey treatment indicate that honey may potentially protect liver cells from sepsis. Lipid profiles showed that cholesterol, triglycerides, and LDL levels were significantly increased in the LPS-induced untreated sepsis group but not in the honey-treated group. However, the HDL levels were significantly lower in the untreated group. Injection of LPS into animals induces renal dysfunction characterized by increased blood urea nitrogen and plasma creatinine levels [45,51]. Urea nitrogen and plasma creatinine levels were increased by LPS injection, but were lower in the honey-treated group than in the untreated group. Both our previous studies and the above results show that LPS increased the levels of hepatic damage markers, modified lipid metabolism, and increased lipid profiles, hematological values, and renal dysfunction [52]. Our results also show that Gelam honey protects organs from immune responses induced by lethal doses of LPS. Our previous study showed that Gelam honey contains many phenolic compounds with antioxidant and anti-inflammatory activity. In addition, its inhibitory effect on cytokines (TNF-α, IL-1β, and IL-10), high-mobility group protein 1 (HMG-1), and NO both *in vitro* and *in vivo* were studied [9,10,53]. Gelam honey also showed potent induction of HO-1, a molecule related to oxidative stress [53]. These activities, including the inhibition of cytokines and NO during severe sepsis, suggest that honey may be useful for the treatment of sepsis. The phenolic compounds in Gelam honey play a role in protecting tissues from LPS and free radicals due to their antioxidant activity, such as scavenging oxygen radicals, NO, and lipid radicals [54], and preventing cancer and various inflammatory disorders, such as arthritis and septic shock induced by endotoxemia [55–58]. The beneficial effects of honey, which include preventing histological changes and hypoxia in the organs of rabbits treated with LPS, may be directly related to its antioxidant activity, or indirectly related to the inhibition of PMNL chemotaxis, thereby preventing the production of the chemotactic agents implicated in tissue damage. We showed previously that Gelam honey has potent antioxidant activity and inhibits mediators of inflammation, such as cytokines, NO and PGE2 [9,10,53]. Allergic reactions constitute a potentially serious contraindication for injecting people with honey because honey contains bee-secreted and plant pollen-derived proteins that are known to induce allergic reactions [59].

## 4. Experimental Section

### 4.1. Preparation of Honey

Malaysian Gelam honey (*Melaleuca* spp.) was purchased from the department of Agriculture, Batu Pahat, Johor, Malaysia, and sent to Malaysian Nuclear Agency for sterilization using a Cobalt-60 source (Model JS10000). Honey was mixed with saline and filtered through a 0.20 μm syringe filter before injection.

### 4.2. Animals

Mice Balb/c mice (6–7 weeks of age) and New Zealand white male rabbits weighing 25 g and 2 kg, respectively, were kept in individual cages under standard conditions (12 h light and 12 h dark conditions); water and chow diet were available *ad libitum*. The study was carried out in accordance with the University of Malaya Animal Ethics Committee guidelines for animal experimentation and followed the approved protocols outlined in the project license (ANES/14/07/2010/MKAK (R)).

### 4.3. Toxicity Tests

The toxicity of Gelam honey was evaluated in mice ($n = 8$) for 1 month prior to the study. Four different doses of honey (10, 60, 300, and 600 mg/kg diluted in saline) were administered daily by injection into the tail vein (final volume, 100 μL). The control group received a similar volume of saline. Mice were observed for 3 h after injection. Symptoms and mortality were recorded for all groups. At the end of the study, all mice were sacrificed, and blood and organs were collected. Compared with the control group, the treated groups showed no abnormalities on biochemical and histopathological analysis of the liver, lungs, and kidneys (data not shown).

### 4.4. Induction of an Immune Response in Rabbits by LPS Stimulation and Treatment with Honey

New Zealand white male rabbits were divided into six groups ($N = 6$) of six animals ($n = 6$) and each group was treated as described below. An immune response was induced in four groups by intravenous injection of 0.5 mg/kg LPS (B: 0111; Sigma, St. Louis, MO, USA) diluted in saline. One hour before LPS injection, honey (500 mg/kg diluted in saline) was injected into the rabbits from two groups (treated group), while saline was injected into the rabbits from another two groups (untreated groups). The two remaining groups acted as negative controls and were given saline only and no LPS. All doses were administered in a final volume of 1 mL and were mixed immediately prior to injection. Three groups, one from each treatment stream, were used for biochemical and histopathological studies and assessment of myeloperoxidase (MPO) activity as described below, while the remaining three groups were used to assess survival rates. Survival was monitored every 12 h for 15 days.

### 4.5. Biochemical Analysis

Blood samples were collected from the ears of rabbits after 8 h of LPS injection. Serum was separated by centrifugation at $3000 \times g$ at 23 ℃, and hematological and biochemistry analysis were performed using an automated hematology cell counter analyzer (Sysmex XE-2100, Sysmex America, Inc.) and Advia 2400 Chemistry System (Siemen, Eschborn, Germany), respectively, in the clinical diagnostic laboratory at University of Malaya Medical Center. Biochemical analyses included measurement of glucose, liver, and kidney functions. The parameters used for hematological analysis were red blood cell count (RBC), white blood cell count (WBC), and platelet counts. Arterial blood samples were collected to measure pH, $pO_2$, $pCO_2$, and $HCO_3$ using a blood gas analyser at the same time as the other biomedical tests were performed.

*4.6. Myeloperoxidase Assay*

Neutrophil infiltration into the lungs was monitored by measuring MPO activity as previously reported [60]. Briefly, tissue specimens were homogenized at 50 mg/mL in PBS (50 mM, pH 6.0) containing 0.5% exadecyltrimethylammonium bromide (Sigma-Aldrich). Samples were freeze-thawed three times and centrifuged at 13,000 rpm for 20 min. The supernatants were diluted 1:30 in assay buffer (50 mM PBS pH 6.0 containing 0.167 mg/mL *o*-dianisidine; (Sigma-Aldrich) and 0.0005% $H_2O_2$), and the colorimetric reaction was measured at 450 nm for between 1 and 3 min in a spectrophotometer (Microplate reader, Model 680, Life Science Research, Bio-Rad). MPO activity/g of wet tissue was calculated as follows: MPO activity (U/g wet tissue) = (A450) (13.5)/tissue weight (g), where A450 is the change in the absorbance of 450 nm light between 1 and 3 min after the initiation of the reaction. The coefficient 13.5 was empirically determined such that 1 U MPO activity corresponded to the amount of enzyme that reduced 1 μmol peroxide/min.

*4.7. Histopathology*

Liver, lung, heart, and kidney tissues were fixed in 10% formalin after the organs were dehydrated using graded ethanol solutions, cleared with xylene, paraffin embedded, sectioned, and stained with hematoxylin and eosin. Pathological changes were evaluated under a light microscope by a pathologist.

*4.8. Statistical Analysis*

All data are expressed as the mean ± confidence interval. Data were analysed using GraphPad prism statistical software (San Diego, CA, USA) for non-parametric analysis of variance. Kaplan–Meier analysis was used to compare survival rates. Differences were considered statistically significant at $P < 0.05$.

## 5. Conclusions

In summary, Gelam honey protects organs from lethal doses of LPS by improving organ functions, reducing infiltration by PMNs that cause tissue damage, reducing MPO activity and increasing the survival rate.

**Conflict of Interest**

The authors declare no conflict of interest.

## References

1. Lazaridou, A.; Biliaderis, C.G.; Bacandritsos, N.; Sabatini, A.G. Composition, thermal and rheological behaviour of selected greek honeys. *J. Food Eng.* **2004**, *64*, 9–21.
2. Gheldof, N.; Wang, X.H.; Engeseth, N.J. Buckwheat honey increases serum antioxidant capacity in humans. *J. Agric. Food Chem.* **2003**, *51*, 1500–1505.
3. White, R. The benefits of honey in wound management. *Nurs. Stand.* **2005**, *20*, 57–64; quiz 66.
4. Molan, P.C. The evidence supporting the use of honey as a wound dressing. *Int. J. Low. Extrem. Wounds* **2006**, *5*, 40–54.
5. Tonks, A.J.; Dudley, E.; Porter, N.G.; Parton, J.; Brazier, J.; Smith, E.L.; Tonks, A. A 5.8-kda component of manuka honey stimulates immune cells via TLR4. *J. Leukoc. Biol.* **2007**, *82*, 1147–1155.
6. Molan, P.C. Potential of honey in the treatment of wounds and burns. *Am. J. Clin. Dermatol.* **2001**, *2*, 13–19.
7. Majtan, J.; Kovacova, E.; Bilikova, K.; Simuth, J. The immunostimulatory effect of the recombinant apalbumin 1-major honeybee royal jelly protein-on tnfalpha release. *Int. Immunopharmacol.* **2006**, *6*, 269–278.
8. Leong, A.G.; Herst, P.M.; Harper, J.L. Indigenous new zealand honeys exhibit multiple anti-inflammatory activities. *Innate Immun.* **2011**, doi:10.1177/1753425911422263.
9. Kassim, M.; Achoui, M.; Mansor, M.; Yusoff, K.M. The inhibitory effects of gelam honey and its extracts on nitric oxide and prostaglandin E(2) in inflammatory tissues. *Fitoterapia* **2010**, *81*, 1196–1201.
10. Kassim, M.; Achoui, M.; Mustafa, M.R.; Mohd, M.A.; Yusoff, K.M. Ellagic acid, phenolic acids, and flavonoids in malaysian honey extracts demonstrate *in vitro* anti-inflammatory activity. *Nutr. Res.* **2010**, *30*, 650–659.
11. Wang, H.; Li, W.; Li, J.; Rendon-Mitchell, B.; Ochani, M.; Ashok, M.; Yang, L.; Yang, H.; Tracey, K.J.; Wang, P.; *et al.* The aqueous extract of a popular herbal nutrient supplement, angelica sinensis, protects mice against lethal endotoxemia and sepsis. *J. Nutr.* **2006**, *136*, 360–365.
12. Bone, R.C.; Grodzin, C.J.; Balk, R.A. Sepsis: A new hypothesis for pathogenesis of the disease process. *Chest* **1997**, *112*, 235–243.
13. Bohlinger, I.; Leist, M.; Gantner, F.; Angermuller, S.; Tiegs, G.; Wendel, A. DNA fragmentation in mouse organs during endotoxic shock. *Am. J. Pathol.* **1996**, *149*, 1381–1393.
14. Angus, D.C.; Linde-Zwirble, W.T.; Lidicker, J.; Clermont, G.; Carcillo, J.; Pinsky, M.R. Epidemiology of severe sepsis in the united states: Analysis of incidence, outcome, and associated costs of care. *Crit. Care Med.* **2001**, *29*, 1303–1310.
15. Cohen, J. The immunopathogenesis of sepsis. *Nature* **2002**, *420*, 885–891.
16. Al-Jabri, A.A. Honey, milk and antibiotics. *Afr. J. Biotechnol.* **2005**, *4*, 1580–1587.
17. Molan, P.C. The potential of honey to promote oral wellness. *Gen. Dent.* **2001**, *49*, 584–589.
18. Van Deventer, S.J.; ten Cate, J.W.; Tytgat, G.N. Intestinal endotoxemia. Clinical significance. *Gastroenterology* **1988**, *94*, 825–831.

19. Wenzel, R.P.; Pinsky, M.R.; Ulevitch, R.J.; Young, L. Current understanding of sepsis. *Clin. Infect. Dis.* **1996**, *22*, 407–412.

20. Tracey, K.J.; Lowry, S.F.; Beutler, B.; Cerami, A.; Albert, J.D.; Shires, G.T. Cachectin/tumor necrosis factor mediates changes of skeletal muscle plasma membrane potential. *J. Exp. Med.* **1986**, *164*, 1368–1373.

21. Schafer, T.; Scheuer, C.; Roemer, K.; Menger, M.D.; Vollmar, B. Inhibition of p53 protects liver tissue against endotoxin-induced apoptotic and necrotic cell death. *FASEB J.* **2003**, *17*, 660–667.

22. Essani, N.A.; McGuire, G.M.; Manning, A.M.; Jaeschke, H. Endotoxin-induced activation of the nuclear transcription factor kappa B and expression of E-selectin messenger RNA in hepatocytes, Kupffer cells, and endothelial cells *in vivo*. *J. Immunol.* **1996**, *156*, 2956–2963.

23. Dustin, M.L.; Springer, T.A. Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. *Annu. Rev. Immunol.* **1991**, *9*, 27–66.

24. Luce, J.M. Acute lung injury and the acute respiratory distress syndrome. *Crit. Care Med.* **1998**, *26*, 369–376.

25. Vincent, J.L.; Sakr, Y.; Ranieri, V.M. Epidemiology and outcome of acute respiratory failure in intensive care unit patients. *Crit. Care Med.* **2003**, *31*, S296–S299.

26. Bernard, G.R.; Artigas, A.; Brigham, K.L.; Carlet, J.; Falke, K.; Hudson, L.; Lamy, M.; Legall, J.R.; Morris, A.; Spragg, R. The american-european consensus conference on ards. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. *Am. J. Respir. Crit. Care Med.* **1994**, *149*, 818–824.

27. Braude, S.; Nolop, K.B.; Hughes, J.M.; Barnes, P.J.; Royston, D. Comparison of lung vascular and epithelial permeability indices in the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* **1986**, *133*, 1002–1005.

28. Patterson, C.E.; Barnard, J.W.; Lafuze, J.E.; Hull, M.T.; Baldwin, S.J.; Rhoades, R.A. The role of activation of neutrophils and microvascular pressure in acute pulmonary edema. *Am. Rev. Respir. Dis.* **1989**, *140*, 1052–1062.

29. Tomashefski, J.F., Jr. Pulmonary pathology of the adult respiratory distress syndrome. *Clin. Chest Med.* **1990**, *11*, 593–619.

30. Singh, R.; Barden, A.; Mori, T.; Beilin, L. Advanced glycation end-products: A review. *Diabetologia* **2001**, *44*, 129–146.

31. Wang, L.F.; Patel, M.; Razavi, H.M.; Weicker, S.; Joseph, M.G.; McCormack, D.G.; Mehta, S. Role of inducible nitric oxide synthase in pulmonary microvascular protein leak in murine sepsis. *Am. J. Respir. Crit. Care Med.* **2002**, *165*, 1634–1639.

32. Tate, R.M.; Repine, J.E. Neutrophils and the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* **1983**, *128*, 552–559.

33. Kindt, G.C.; Gadek, J.E.; Weiland, J.E. Initial recruitment of neutrophils to alveolar structures in acute lung injury. *J. Appl. Physiol.* **1991**, *70*, 1575–1585.

34. Doerschuk, C.M. Mechanisms of leukocyte sequestration in inflamed lungs. *Microcirculation* **2001**, *8*, 71–88.

35. Brown, D.M.; Drost, E.; Donaldson, K.; MacNee, W. Deformability and *cd11/cd18* expression of sequestered neutrophils in normal and inflamed lungs. *Am. J. Respir. Cell Mol. Biol.* **1995**, *13*, 531–539.

36. Skoutelis, A.T.; Kaleridis, V.; Athanassiou, G.M.; Kokkinis, K.I.; Missirlis, Y.F.; Bassaris, H.P. Neutrophil deformability in patients with sepsis, septic shock, and adult respiratory distress syndrome. *Crit. Care Med.* **2000**, *28*, 2355–2359.

37. Goode, H.F.; Webster, N.R. Free radicals and antioxidants in sepsis. *Crit. Care Med.* **1993**, *21*, 1770–1776.

38. Novelli, G.P. Role of free radicals in septic shock. *J. Physiol. Pharmacol.* **1997**, *48*, 517–527.

39. Razavi, H.M.; Werhun, R.; Scott, J.A.; Weicker, S.; Wang, L.F.; McCormack, D.G.; Mehta, S. Effects of inhaled nitric oxide in a mouse model of sepsis-induced acute lung injury. *Crit. Care Med.* **2002**, *30*, 868–873.

40. Hogg, J.C.; Doerschuk, C.M. Leukocyte traffic in the lung. *Annu. Rev. Physiol.* **1995**, *57*, 97–114.

41. Downey, G.P.; Fialkow, L.; Fukushima, T. Initial interaction of leukocytes within the microvasculature: Deformability, adhesion, and transmigration. *New Horiz.* **1995**, *3*, 219–228.

42. Razavi, H.M.; Wang, L.F.; Weicker, S.; Rohan, M.; Law, C.; McCormack, D.G.; Mehta, S. Pulmonary neutrophil infiltration in murine sepsis: Role of inducible nitric oxide synthase. *Am. J. Respir. Crit. Care Med.* **2004**, *170*, 227–233.

43. Aoki, Y.; Ota, M.; Katsuura, Y.; Komoriya, K.; Nakagaki, T. Effect of activated human protein c on disseminated intravascular coagulation induced by lipopolysaccharide in rats. *Arzneimittelforschung* **2000**, *50*, 809–815.

44. Chiou, W.F.; Ko, H.C.; Chen, C.F.; Chou, C.J. Evodia rutaecarpa protects against circulation failure and organ dysfunction in endotoxaemic rats through modulating nitric oxide release. *J. Pharm. Pharmacol.* **2002**, *54*, 1399–1405.

45. Chen, C.P.; Yokozawa, T.; Kitani, K. Beneficial effects of sanguisorbae radix in renal dysfunction caused by endotoxin *in vivo*. *Biol. Pharm. Bull.* **1999**, *22*, 1327–1330.

46. Cunha, F.Q.; Assreuy, J.; Moncada, S.; Liew, F.Y. Phagocytosis and induction of nitric oxide synthase in murine macrophages. *Immunology* **1993**, *79*, 408–411.

47. Deaciuc, I.V.; D'Souza, N.B.; de Villiers, W.J.; Burikhanov, R.; Sarphie, T.G.; Hill, D.B.; McClain, C.J. Inhibition of caspases *in vivo* protects the rat liver against alcohol-induced sensitization to bacterial lipopolysaccharide. *Alcohol. Clin. Exp. Res.* **2001**, *25*, 935–943.

48. Hong, K.W.; Kim, K.E.; Rhim, B.Y.; Lee, W.S.; Kim, C.D. Effect of rebamipide on liver damage and increased tumor necrosis factor in a rat model of endotoxin shock. *Dig. Dis. Sci.* **1998**, *43*, 154S–159S.

49. Jiang, J.; Chen, H.; Diao, Y.; Tian, K.; Zhu, P.; Wang, Z. Distribution of endotoxins in tissues and circulation and its effects following hemorrhagic shock. *Chin. Med. J. (Engl.)* **1998**, *111*, 118–122.

50. Barton, C.C.; Ganey, P.E.; Roth, R.A. Lipopolysaccharide augments aflatoxin B(1)-induced liver injury through neutrophil-dependent and -independent mechanisms. *Toxicol. Sci.* **2000**, *58*, 208–215.

51. Wellings, R.P.; Corder, R.; Vane, J.R. Lack of effect of ET antibody or SB 209670 on endotoxin-induced renal failure. *J. Cardiovasc. Pharmacol.* **1995**, *26*, S476–S478.

52. Memon, R.A.; Grunfeld, C.; Moser, A.H.; Feingold, K.R. Tumor necrosis factor mediates the effects of endotoxin on cholesterol and triglyceride metabolism in mice. *Endocrinology* **1993**, *132*, 2246–2253.

53. Kassim, M.; Mansor, M.; Achoui, M.; Ong, G.S.Y.; Sekaran, S.D.; Yusoff, K.M. Honey as an immunomodulator during sepsis in animal models. *Crit. Care* **2009**, *13*, doi:10.1186/cc8096.

54. Salah, N.; Miller, N.J.; Paganga, G.; Tijburg, L.; Bolwell, G.P.; Rice-Evans, C. Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Arch. Biochem. Biophys.* **1995**, *322*, 339–346.

55. Unno, T.; Sakane, I.; Masumizu, T.; Kohno, M.; Kakuda, T. Antioxidant activity of water extracts of lagerstroemia speciosa leaves. *Biosci. Biotechnol. Biochem.* **1997**, *61*, 1772–1774.

56. Virgili, F.; Kim, D.; Packer, L. Procyanidins extracted from pine bark protect alpha-tocopherol in ECV 304 endothelial cells challenged by activated raw 264.7 macrophages: Role of nitric oxide and peroxynitrite. *Fed. Eur. Biochem. Soc. Lett.* **1998**, *431*, 315–318.

57. Yang, F.; de Villiers, W.J.; McClain, C.J.; Varilek, G.W. Green tea polyphenols block endotoxin-induced tumor necrosis factor-production and lethality in a murine model. *J. Nutr.* **1998**, *128*, 2334–2340.

58. Wakabayashi, I. Inhibitory effects of baicalein and wogonin on lipopolysaccharide-induced nitric oxide production in macrophages. *Basic Clin. Pharmacol. Toxicol.* **1999**, *84*, 288–291.

59. Bauer, L.; Kohlich, A.; Hirschwehr, R.; Siemann, U.; Ebner, H.; Scheiner, O.; Kraft, D.; Ebner, C. Food allergy to honey: Pollen or bee products? Characterization of allergenic proteins in honey by means of immunoblotting. *J. Allergy Clin. Immunol.* **1996**, *97*, 65–73.

60. Bradley, P.P.; Christensen, R.D.; Rothstein, G. Cellular and extracellular myeloperoxidase in pyogenic inflammation. *Blood* **1982**, *60*, 618–622.

## 3.5 Gelam honey scavenges peroxynitrite during the immune response

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*Article*

# Gelam Honey Scavenges Peroxynitrite During the Immune Response

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**Abstract:** Monocytes and macrophages are part of the first-line defense against bacterial, fungal, and viral infections during host immune responses; they express high levels of proinflammatory cytokines and cytotoxic molecules, including nitric oxide, reactive oxygen species, and their reaction product peroxynitrite. Peroxynitrite is a short-lived oxidant and a potent inducer of cell death. Honey, in addition to its well-known sweetening properties, is a natural antioxidant that has been used since ancient times in traditional medicine. We examined the ability of Gelam honey, derived from the Gelam tree (*Melaleuca* spp.), to scavenge peroxynitrite during immune responses mounted in the murine macrophage cell line RAW 264.7 when stimulated with lipopolysaccharide/ interferon-γ (LPS/IFN-γ) and in LPS-treated rats. Gelam honey significantly improved the viability of LPS/IFN-γ-treated RAW 264.7 cells and inhibited nitric oxide production—similar to the effects observed with an inhibitor of inducible nitric oxide synthase (1400W). Furthermore, honey, but not 1400W, inhibited peroxynitrite production from the synthetic substrate 3-morpholinosydnonimine (SIN-1) and prevented the peroxynitrite-mediated conversion of dihydrorhodamine 123 to its fluorescent oxidation product rhodamine 123. Honey inhibited peroxynitrite synthesis in LPS-treated rats. Thus,

honey may attenuate inflammatory responses that lead to cell damage and death, suggesting its therapeutic uses for several inflammatory disorders.

## 1. Introduction

Monocytes and macrophages play critical roles in innate immunity. Specifically, these cells act as a part of the first-line defense against bacterial, fungal, and viral infections during host immune responses [1] by expressing high levels of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-1β, and cytotoxic molecules such as nitric oxide (NO) and reactive oxygen species (ROS) [2,3]. The inflammatory response is accompanied by the upregulation of a lipopolysaccharide (LPS)-inducible isoform of NO synthase (iNOS) [4], the expression of which is correlated with the degree of inflammation [5] as well as the presence of NO. However, in addition to their defensive roles, proinflammatory responses can damage DNA and other cellular structures, activating necrosis, apoptosis, and potentially, tumorigenesis [6,7].

Of the inflammatory modulators produced in response to LPS, NO is implicated in both acute and chronic models of inflammation, including septic shock [8], where there is extensive damage to host tissues. The damage is, in part, due to the reaction of NO with superoxide radicals, resulting in the formation of peroxynitrite (ONOO⁻). This short-lived oxidant species profoundly influences the inflammatory response at multiple cellular levels and is a potent inducer of cell death [9]. The biological targets of peroxynitrite include membrane, cytosolic, and nuclear receptors [9]. During inflammation, peroxynitrite also reacts with inflammatory mediators such as interleukins, and with iNOS, either triggering or enhancing proinflammatory pathways mediated by nuclear factor (NF)-κB [10]. Furthermore, the reaction products of peroxynitrite are detected in several pathological conditions, including vascular diseases, ischemia-reperfusion injury, circulatory shock, inflammation, pain, and neurodegeneration. Conversely, studies in animal models of inflammation and reperfusion injury have shown a protective effect for compounds that either inhibit peroxynitrite formation or accelerate its decomposition [9,11–16].

Honey is a well-known natural sweetener; this property is conferred by its complex mixture of carbohydrates, which are the main constituents of honey and are produced by honeybees from nectar sucrose. Monosaccharides, including fructose and glucose, are the major carbohydrates in honey; disaccharides (such as maltose and sucrose), trisaccharides (such as maltotriose and panose), and oligosaccharides are also present in honey [17–22]. In addition, honey also contains minerals, vitamins, enzymes, flavonoids, and phenolic compounds, making it a natural antioxidant [23]. Indeed, honey has been used in traditional medicine since ancient times [24]. It has potential effects in high oxidative stress conditions, and its ability to modulate antioxidant enzymes as well as its antioxidative properties provide protective effects against oxidative stress [25,26]. Recent studies demonstrated a strong correlation between the content of phenolic compounds in honeys from various floral sources and their antioxidant capacity and beneficial effects in human health [27–31]. One of the potential benefits of phenolic compounds is that they stabilize cell membranes by reducing lipid peroxidation and

scavenging free radicals, [32] and they simultaneously enhance membrane integrity against several chemical and physical stress conditions [33]. Various studies investigated the potential protective effects of phenolic compounds against oxidative damage to red blood cells [34,35]. Honey demonstrated strong reducing activity against free radicals, with significant suppression/prevention of cell damage, complete inhibition of cell membrane oxidation and intracellular ROS production, and recovery of intracellular glutathione in cultured endothelial cells. This protective effect is mainly attributed to phenolic acids and flavonoids [36]. The phenolic compounds in honey were found to scavenge free radicals and prevent the production of malondialdehyde, a biomarker of oxidative damage, in a concentration-dependent manner [36,37].

Gelam honey is derived from the nectar of the Gelam tree (*Melaleuca* spp.), which grows in the forests of Malaysia. [38,39]. Gelam honey is reported to have a high phenolic content, with many phenolic compounds isolated from it, such as gallic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, ellagic acid, quercetin, hesperetin, luteolin, kaempferol, and chrysin [40]. A previous study showed that Gelam honey exhibited antibacterial activity and free radical scavenging activity because of its phenolic compounds; furthermore, Gelam honey inhibited inflammation *in vitro* and *in vivo* [38–42]. It is also a potent inducer of heme oxygenase-1 (HO-1) and significantly reduces DNA damage and plasma malondialdehyde levels [41,43]. In this study, we tested the ability of Gelam honey to scavenge peroxynitrites in LPS/IFN-γ-stimulated murine macrophages (RAW 264.7) *in vitro*, and in a rat model of inflammation *in vivo*. Therefore, in this study, we carefully examined the cytoprotective effects of Gelam honey under the cellular damage conditions induced by two doses of LPS. In parallel, the effect of NO on murine macrophage (RAW 264.7) cell viability was also examined.

## 2. Results

### 2.1. Effect of Gelam Honey on Untreated and LPS/IFN-γ-Stimulated Cells

In RAW 264.7 cells, none of the doses of honey resulted in cytotoxicity, and the concentrations of honey did not affect cell viability (Figure 1). However, the viability of cells stimulated with 1 µg/mL LPS and 35 ng/mL IFN-γ was <68% of the control (untreated) value.

In contrast, as shown in Figure 2A, pretreatment with honey had a protective effect on LPS/IFN-γ-stimulated cells, significantly increasing their viability to >76% ($p < 0.03$), whereas the viability of cells pretreated with 1400W was >90% ($p < 0.001$). Increasing the LPS concentration to 3 µg/mL significantly reduced the viability of the untreated cells to <50% of the control value, whereas the addition of honey increased viability to >69% ($p < 0.001$), and the addition of 1400W resulted in >79% viability ($p < 0.001$) (Figure 2B).

**Figure 1.** Effect of honey and the iNOS inhibitor 1400W on the viability of RAW 264.7 macrophages. Cells were treated or not treated (control) with the indicated concentrations of honey or 100 μM 1400W. Cell viability was determined by the mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Data are expressed as the mean ± SEM of five independent experiments performed in triplicate. The viability of untreated cells was defined as 100%.
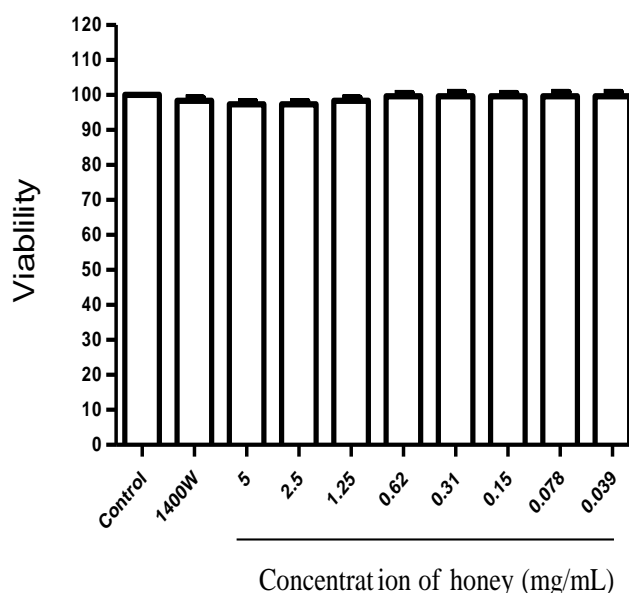


**Figure 2.** Cytoprotective effect of honey against LPS/IFN-γ-induced cytotoxicity. RAW 264.7 cells were incubated with either 1 μg/mL LPS and 35 ng/mL IFN-γ (**A**) or 3 μg/mL LPS and 35 ng/mL IFN-γ (**B**), and with various concentrations of honey or 100 μM of the iNOS inhibitor 1400W. The negative control was completely untreated (control), and the positive control was treated only with LPS/IFN-γ (LPS). After 24 h incubation, cell viability was determined using an MTT assay. *** $p < 0.001$ and ** $p < 0.003$ indicate significant differences compared with the LPS/IFN-γ group.
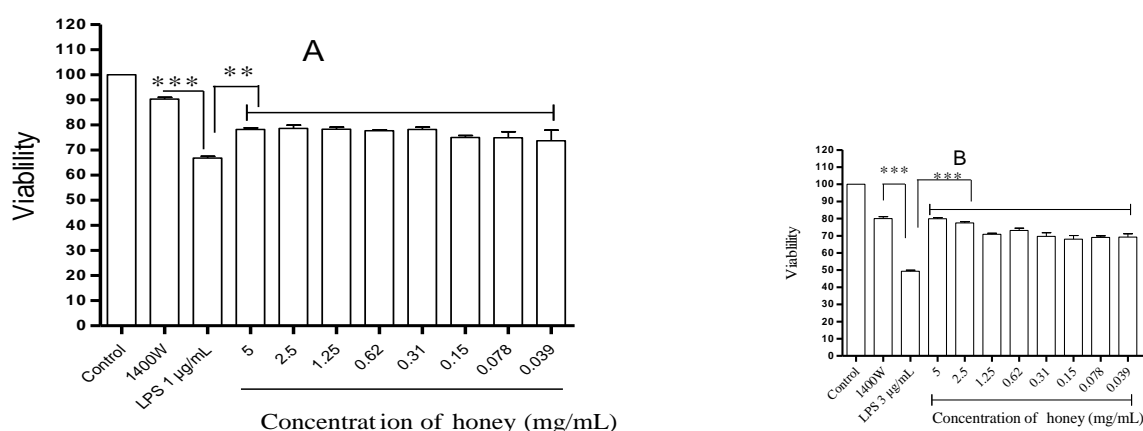


Honey also significantly inhibited NO formation in cells that were stimulated with the indicated concentration of LPS (1 μg/mL or 3 μg/mL). A dose-dependent effect was observed at higher concentrations of honey (Figure 3).

**Figure 3.** Effect of honey on NO production. NO production was estimated in RAW 264.7 macrophages pretreated for 1 h with the indicated concentrations of honey or the iNOS inhibitor 1400W (100 μM) and then exposed to 1 μg/mL LPS and 35 ng/mL IFN-γ (**A**) or 3 μg/mL LPS and 35 ng/mL IFN-γ (**B**) for 24 h. Nitrite accumulation in the supernatant was measured by the Griess reaction. All results are expressed as a percentage of the LPS/IFN-γ control (mean ± SEM of 5 independent experiments performed in duplicate). *** $p < 0.001$, ** $p < 0.003$, and * $p < 0.05$ indicate significant differences compared with the LPS/IFN-γ group.
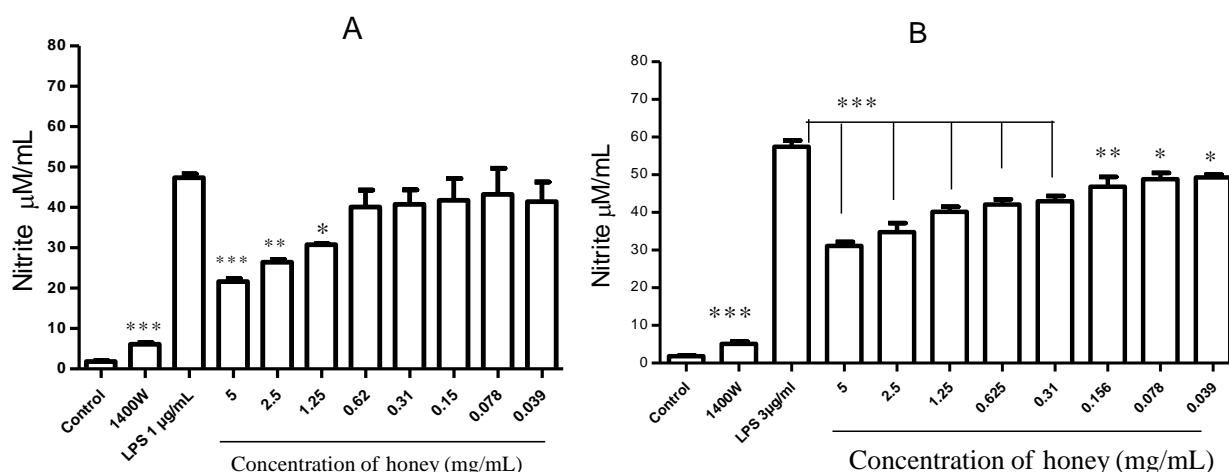


*2.2. Effect of Gelam Honey on Peroxynitrite* in vitro *and* in vivo

An increase in the oxidation of dihydrorhodamine 123 (DHR-123) is indicative of the presence of peroxynitrite. In the 3-morpholinosydnonimine (SIN-1) model, honey was found to be a potent scavenger of peroxynitrite, inhibiting the SIN-1–induced oxidation of DHR-123 to rhodamine 123 with a half-maximal inhibitory concentration (IC$_{50}$) of 0.148 mg/mL. Highly significant levels of oxidation were recorded in the presence of the iNOS inhibitor 1400W and in the (untreated) positive control (Figure 4A). To directly confirm the peroxynitrite scavenging activity of honey, peroxynitrite was incubated with or without different concentrations of honey (Figure 4B). In these experiments, honey also inhibited DHR-123 oxidation, presumably by scavenging peroxynitrite, with an IC$_{50}$ of 0.68 mg/mL. However, oxidation was not inhibited by 1400W because the level of rhodamine 123 fluorescence was the same as that in the untreated control (Figure 4B). The addition of Gelam honey to RAW 264.7 cells that were induced with LPS/IFN-γ for 24 h also completely attenuated peroxynitrite activity and had an IC$_{50}$ of 0.254 mg/mL (Figure 4C). In this experiment (unlike the previous 2 experiments), peroxynitrite synthesis was inhibited, and DHR-123 was not converted to its fluorescent product because cellular iNOS was blocked by 1400W (100 μM) (Figure 4C).

**Figure 4.** Effect of honey on the peroxynitrite-induced oxidation of DHR-123. (**A**) SIN-1, a peroxynitrite donor, was incubated for 2 h with different dilutions of honey (in PBS), 1400W (100 μM), and DHR-123, and the formation of rhodamine 123 was measured; (**B**) Honey (different dilutions in PBS) or 1400W (100 μM) were incubated for 15 min with DHR-123 and peroxynitrite, and the formation of rhodamine 123 was measured; (**C**) RAW 264.7 cells were incubated with honey (different dilutions in PBS), 1400W (100 μM), and DHR-123 for 60 min. Then, LPS/IFN-γ was added, and the cultures were incubated for an additional 24 h, after which the formation of rhodamine 123 was measured. Results are expressed as a percentage of the control (mean ± SEM of 3 independent experiments performed in triplicate). *** $p < 0.001$ and ** $p < 0.003$. Rhodamine 123 formation in the untreated control was defined as 100%.
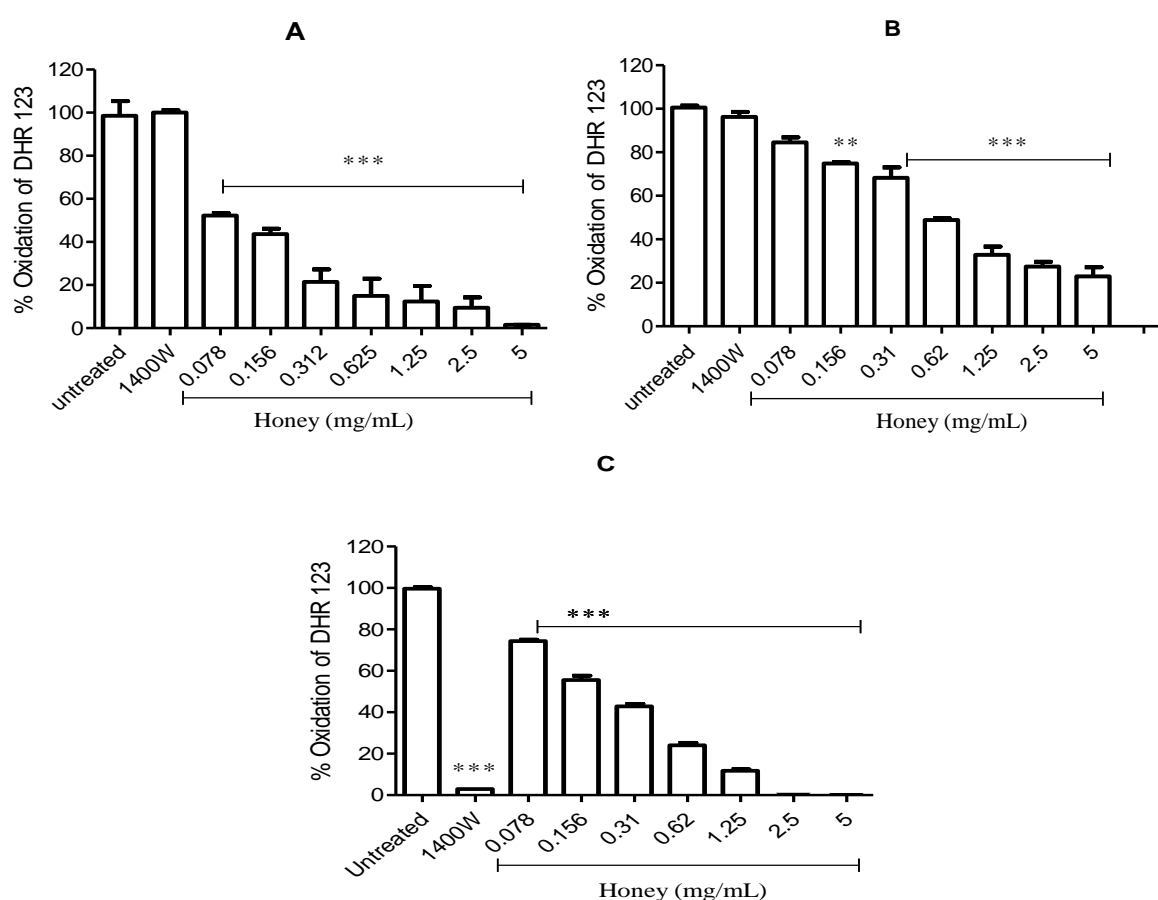


Pretreatment with 50 mg/kg or 500 mg/kg of honey also significantly inhibited peroxynitrite formation in rats, albeit not in a dose-dependent manner. Inhibition was determined by a reduction in 3-nitrotyrosine, which is an *in vitro* marker of peroxynitrite (Figure 5).

**Figure 5.** Effect of honey on the concentration of 3-nitrotyrosine in rat serum. Treated groups of animals were intravenously injected with either honey (50 or 500 mg/kg diluted in saline) or saline alone. One hour later, the treated animals were injected with LPS (5 mg/kg), and 4 h later, the treated and untreated rats were killed. The sera were collected and assayed for the presence of 3-nitrotyrosine. Data are expressed as the mean ± SEM. *** $p < 0.001$ and * $p < 0.05$ compared with the positive (LPS alone) control.
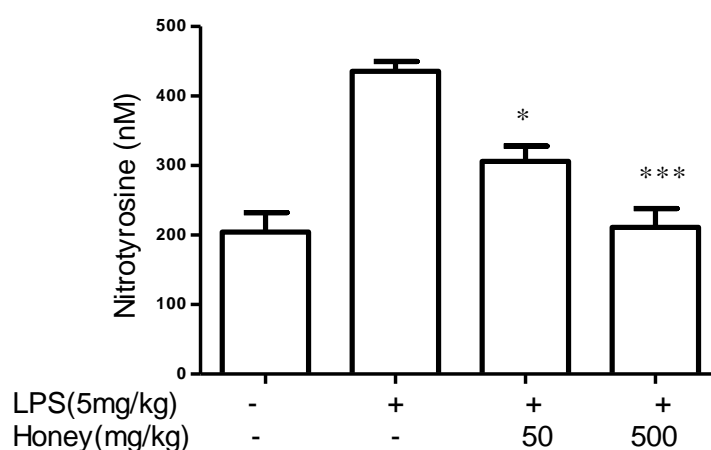


## 3. Discussion

This study investigated the ability of honey to modulate peroxynitrite-induced cell damage in LPS/IFN-γ-stimulated cultured macrophages and in a rat model of LPS-induced inflammation. The data presented here suggest that honey, through its antioxidant properties, is able to protect host immune cells from the inflammation-mediated cytotoxicity that develops in response to LPS stimulation. Gelam honey prevented the LPS-mediated decrease in RAW 264.7 cell viability caused by the high production of cytotoxic molecules such as cytokines, ROS, NO, and peroxynitrite [1]. Honey apparently scavenged peroxynitrite in RAW 264.7 cells induced with LPS/IFN-γ *in vitro*. Inhibitory effects were also observed upon incubation with the substrate SIN-1, a peroxynitrite donor, and with peroxynitrite itself. The ability of honey to scavenge peroxynitrite in tissues *in vivo* was demonstrated by the absence of 3-nitrotyrosine, a peroxynitrite marker, from rat serum.

Excess NO production is cytotoxic and has a broad spectrum of cellular effects. In RAW 264.7 macrophages, there was a pronounced increase in NO production and a significant decrease in cell viability after stimulation with LPS/IFN-γ, which is known to induce iNOS and thus increase cellular NO concentrations. This decrease in viability was much less pronounced in the presence of 1400W, suggesting that cytotoxicity was induced by iNOS and NO. In fact, NO production, measured as nitrite in the Griess reaction, was almost completely inhibited by iNOS inhibition.

The mechanism by which NO mediates toxicity includes the generation of reactive nitrogen derivatives such as peroxynitrite, which acts upon multiple cellular targets, including DNA and various proteins [44]. Thus, cellular exposure to high concentrations of peroxynitrite often leads to rapid, necrotic-type cell death due to acute and severe cellular energetic derangements [11,45,46]. In contrast, lower concentrations of peroxynitrite trigger delayed apoptosis that is mainly dependent on the activation of caspases 3, 2, 8, and 9, similar to other forms of oxidant/free radical-mediated apoptosis [47–50].

The effects of NO on cell viability are proportional to the cellular non-heme iron content. Thus, NO induces apoptosis in cells with a low non-heme iron level, such as RAW 264.7 macrophages, whereas it induces necrosis in cells with a high non-heme iron level, such as hepatocytes [51]. For example, NO induces both caspase-3 activation and cytochrome *c* release in apoptotic RAW 264.7 cells, and a caspase-3 inhibitor prevents NO-mediated RAW 264.7 apoptotic cell death [51]. NO readily reacts with non-heme iron to form iron-nitrosyl complexes [52,53], which are thought to protect cells from NO-induced toxicity by a scavenging mechanism [53]. Alternatively, NO may be converted to *S*-nitrosylating species, which act as potent regulatory molecules in a variety of cell types and cellular functions [54,55].

Recent studies show that peroxynitrite stimulates the release of the mitochondrial apoptosis-inducing factor, which subsequently triggers DNA fragmentation [56], release of mitochondrial pro-apoptotic factors, and cytochrome *c*-dependent apoptosis in the cytosol, through peroxynitrite-dependent oxidation of the mitochondrial permeability transition pore. The key role of peroxynitrite in promoting mitochondrial dysfunction is clearly exemplified in experimental sepsis, in which peroxynitrite production results in the inhibition of mitochondrial respiration in the diaphragm in a process associated with mitochondrial protein nitration. The latter is prevented by NO synthase inhibitors and Mn-porphyrin therapy [57]. Peroxynitrite-induced activation of the MLK/p38/JNK pathway also plays a crucial role in apoptosis [58–60]. This study shows that honey can inhibit NO production, and thus peroxynitrite formation, thereby reducing the effects of these cytotoxic compounds both *in vitro* and *in vivo*. Moreover, scavengers of peroxynitrite are known to be protective against tissue damage [9]. Some scavengers of peroxynitrite, such as uric acid, ebselen, mercaptoalkylguanidines, *N*-acetylcysteine, and dihydrolipoic acid, and some chemicals that work as decomposition catalysts of peroxynitrite, such as metalloporphyrins of iron and manganese, can attenuate the toxic effects of peroxynitrite *in vitro* and *in vivo* [11,16,61–68]. These compounds can reduce 3-nitrotyrosine immunoreactivity in various pathophysiological conditions and have beneficial effects in animal models of inflammation, sepsis, and reperfusion injury [57,68–77]. Many phenolic compounds, such as gallic acid, caffeic acid, kaempferol, ferulic acid, *p*-coumaric, and quercetin have been shown to inhibit peroxynitrite. Monohydroxylated phenolic compounds, such as ferulic acid and *p*-coumaric acid, act as peroxynitrite scavengers by nitration. On the other hand, compounds with a catechol moiety, such as caffeic acid and chlorogenic acid, reduce peroxynitrite by electron donation [66,78]. Interestingly, all of the above phenolic compounds were identified in Gelam honey [40]. Data from this study was in agreement with that of previous studies as described earlier. Moreover, a direct interaction through nitration and electron donation between honey and peroxynitrite is suggested by the results of the experiments involving SIN-1 and the direct incubation of honey and peroxynitrite. In addition, the presence of phenolic compounds in Gelam honey that act as potent scavengers of peroxynitrite, as described above, supports this hypothesis. In our previous studies, we demonstrated the anti-inflammatory activity of Gelam honey and its methanol and ethyl acetate extracts, on the basis of their abilities to suppress NO production in macrophages and rat inflammation models, inhibit the release of NO-induced cytokines (such as TNF-α, IL-1β, and IL-10) and high mobility group protein 1 (HMGB1), induces HO-1 in animal models, and protects organs from lethal doses of LPS that induce sepsis [39–41,79]. Gelam honey also inhibited iNOS protein expression in an animal model [80]. The results of this study support these previous findings, confirming the protective effects of honey mediated through the

inhibition of NO and its derivatives (ONOO⁻) and, thus, its ability to prevent inflammatory-type cytotoxicity both in cultured macrophages and in animals.

The antioxidant and radical-scavenging abilities of Gelam honey are attributable to its phenolic compounds, which were also identified and quantified in previous studies [38]. The potential of Gelam honey to reduce LPS-induced inflammation is also mediated by its ability to reduce the release of proinflammatory cytokines and prostaglandin (PG) $E_2$, both of which play a central role in inflammation [39,79]. In these studies, the reduced release of cytokines such as TNF-α, IL-1β, and IL-10 was quite dramatic in an endotoxemia model. This finding is particularly relevant to macrophages, where these mediators play a fundamental role in cell activation because they are released during the early stages of the inflammatory cascade [81]. In addition, honey may suppress NF-κB activation through other biologically active components, and consequently, inhibit iNOS induction [82,83]. Taken together, our work supports further investigations into the use of honey as a natural antioxidant, based on its ability to protect cells by inhibiting NO production, scavenging peroxynitrite, and modulating other inflammatory mediators such as $PGE_2$, HMGB1, HO-1, and cytokines. The involvement of mechanisms other than iNOS inhibition, NO production, and peroxynitrite scavenging in these processes is suggested because low doses of honey had no effect on NO synthesis but did increase the viability of cells treated with LPS.

## 4. Experimental Section

### 4.1. Preparation of Gelam Honey

Fresh Malaysian honey, *Apis mellifera* (Gelam), was obtained from the National Apiary (Department of Agriculture, Parit Botak, Johor, Malaysia) and sent to the Malaysian Nuclear Agency for sterilization using a cobalt-60 source (model JS10000). Before its use in the *in vitro* and *in vivo* experiments described below, the sterilized honey was passed through a 0.20-µm filter syringe. The following concentrations were tested: 0.039, 0.078, 0.15, 0.31, 0.62, 1.25, 2.5, and 5 mg/mL.

### 4.2. Animals

Male Sprague–Dawley rats weighing 300–350 g were housed in individual cages under standard conditions (12 h light and 12 h dark). The animals were fed a diet of Purina lab chow and given water *ad libitum.* The study was carried out in accordance with the guidelines for animal experimentation of the University of Malaya Animal Ethics Committee and the protocols were approved under the terms set out: project license ANES/14/07/2010/MKAK (R).

### 4.3. Cell Culture and Reagents

The murine macrophage cell line RAW 264.7 was maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% (*v/v*) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin in a humidified 37 °C, 5% $CO_2$ incubator. DMEM without phenol red, FBS, and antibiotics (penicillin, streptomycin) were purchased from Nacalai Tesque (Kyoto, Japan). LPS (*Escherichia coli* 0111 B4), iNOS inhibitor (1400W), and IFN-γ were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 4.4. LPS/IFN-γ Stimulation of RAW 264.7 Cells

RAW 264.7 cells were grown in 10% FBS-DMEM and seeded at a density of $2 \times 10^6$ in a 24-well plate, followed by incubation for 24 h at 37 °C. The cells were then washed with PBS and resuspended in fresh medium containing different concentrations of Gelam honey (0.039–5 mg/mL) or 100 µM of 1400W. Untreated cells were included as positive or negative controls in each experiment. One hour later, 1 or 3 µg/mL of LPS and 35 ng/mL of IFN-γ were added to the cultures, followed by incubation for 24 h at 37 °C and 5% $CO_2$ [84]. The cells were then processed for viability or NO or peroxynitrite detection as described below.

### 4.5. Measurement of Mitochondrial Respiration

The viability of RAW 264.7 macrophages was determined in cultures treated or not treated with LPS/IFN-γ and different concentrations of Gelam honey or 1400W. The viability was measured in terms of cellular respiration as assessed by the mitochondrial-dependent reduction of MTT to formazan. Cells were cultured, stimulated with LPS, and treated with honey or 1400W as described above, after which 100 µL of MTT (5 mg/mL) was added to each well, followed by 1 h incubation under the same conditions. The MTT solution was then removed and the cells were solubilized in 200 µL DMSO with shaking for 5 min. The absorbance was measured at 550 nm using a microplate reader (GloMax®-Multi Microplate detection; Promega, Madison, WI, USA) [85]. All experiments were repeated 5 times in triplicate.

### 4.6. Nitric Oxide Assay

Nitric oxide has a half-life of only a few seconds before it is quickly converted to nitrate and nitrite. These products can be measured using the colorimetric Griess reaction to indirectly determine the NO concentration. Therefore, RAW 264.7 cells were cultured and treated as described in Subsection 4.4 (LPS/IFN-γ stimulation of RAW 264.7 cells), after which 100 µL of the culture was placed in a 96-well plate, together with an equal amount of Griess reagent (50 µL of 1% sulfanilamide in 5% concentrated $H_3PO_4$ and 50 µL of 0.1% naphthylethylenediamine dihydrochloride in distilled water). The reaction between the Griess reagent and the nitrite present in the supernatant yields a pink derivative that can be spectrophotometrically quantified from a concentration curve prepared from a nitrite standard [40].

### 4.7. DHR-123 Oxidation Assay

4.7.1. DHR-123 Oxidation Using SIN-1

SIN-1 spontaneously releases NO and superoxide under physiological conditions. At pH 7.4, SIN-1 is converted to SIN-1A via base-catalyzed ring opening. During ring opening, the oxygen undergoes univalent reduction to $O_2^-$. SIN-1A then releases NO and is converted to the stable metabolite SIN-1C, whereas the $O_2^-$ radical reacts with NO to form peroxynitrite ($ONOO^-$). The oxidation of DHR-123 by $ONOO^-$ results in the formation of fluorescent rhodamine 123, the amount of which can be measured by fluorometric analysis (GloMax®-Multi Microplate) at an excitation wavelength of 460–530 nm and

an emission wavelength of 530–590 nm. In experiments examining the effects of honey on peroxynitrite scavenging, 100 μM of SIN-1 was used and the reactions were carried out in PBS, with the incubation of the samples for 2 h at 37 °C [84].

### 4.7.2. DHR-123 Oxidation Using Peroxynitrite

The ability of peroxynitrite to oxidize DHR-123, thus converting it to rhodamine 123, was also measured directly as previously described [9]. Briefly, 10 μM peroxynitrite was mixed in PBS containing 20 μM DHR-123, in the absence or presence of either honey (0–5 mg/mL) or 100 μM of 1400W. After a 15-min incubation period at room temperature, the fluorescence of the rhodamine 123 reaction product was measured (GloMax®-Multi Microplate) at an excitation wavelength of 460–530 nm and an emission wavelength of 530–590 nm.

### 4.7.3. DHR-123 Oxidation by LPS/IFN-γ-Treated RAW 264.7 Cells

Cells were cultured, stimulated with LPS, and treated with honey or 1400W as described in subsection 4.4 (LPS/IFN-γ stimulation of RAW 264.7 cells) in the presence of 10 μM DHR-123. After 24 h, 100 μL of the culture suspension was removed, and the amount of rhodamine 123 was determined fluorometrically.

### *4.8. Induction of an Immune Response in LPS-Stimulated Rats and the Effects of Honey*

Rats were divided into four groups of six animals each. An immune response was induced in the animals in three of the four groups by intravenous injection of 5 mg/kg LPS (0111B4; Sigma) diluted in saline. One of these groups served as the positive control (LPS only), whereas the other two groups were intravenously injected with 50 or 500 mg/kg honey diluted in saline. The fourth (negative control) group was given saline only. All doses of LPS and honey were prepared immediately before injection, and 0.5 mL of the preparations were injected. The blood was collected (by cardiac puncture) from rats 4 h after the immune response was induced. The levels of 3-nitrotyrosine in the sera were measured using an ELISA kit according to the manufacturer's protocol (Cell Biolabs Inc., San Diego, CA, USA).

### *4.9. Statistical Analysis*

Student's *t*-test and a non-parametric one-way ANOVA were used to determine the statistical significance of differences between the experimental and control groups, with $p \leq 0.05$ considered to be statistically significant.

### 5. Conclusion

In conclusion, our data showed that honey is a potent peroxynitrite scavenger *in vitro* and *in vivo* that has cytoprotective effects against peroxynitrite-mediated cellular injury and death. Moreover, the preservation of cellular viability from peroxynitrite-mediated damage is critical to any consideration of the potential therapeutic value of peroxynitrite scavengers in many diseases, including inflammation and sepsis. This finding suggests that honey has therapeutic applications for a wide range of inflammatory disorders.

**Acknowledgments**

**Conflict of Interest**

The authors declare no conflict of interest.

**References**

1. Sampath, V.; Radish, A.C.; Eis, A.L.; Broniowska, K.; Hogg, N.; Konduri, G.G. Attenuation of lipopolysaccharide-induced oxidative stress and apoptosis in fetal pulmonary artery endothelial cells by hypoxia. *Free Radical Biol. Med.* **2009**, *46*, 663–671.

2. Kedzierska, K.; Crowe, S.M. Cytokines and hiv-1: Interactions and clinical implications. *Antiviral Chem. Chemother.* **2001**, *12*, 133–150.

3. Hanna, M.G., Jr. Immunologic aspects of bcg-mediated regression of established tumors and metastases in guinea pigs. *Semin. Oncol.* **1974**, *1*, 319–335.

4. Sawa, T.; Ohshima, H. Nitrative DNA damage in inflammation and its possible role in carcinogenesis. *Nitric Oxide* **2006**, *14*, 91–100.

5. Kimura, H.; Hokari, R.; Miura, S.; Shigematsu, T.; Hirokawa, M.; Akiba, Y.; Kurose, I.; Higuchi, H.; Fujimori, H.; Tsuzuki, Y.; *et al.* Increased expression of an inducible isoform of nitric oxide synthase and the formation of peroxynitrite in colonic mucosa of patients with active ulcerative colitis. *Gut* **1998**, *42*, 180–187.

6. Han, K.Y.; Kwon, T.H.; Lee, T.H.; Lee, S.J.; Kim, S.H.; Kim, J. Suppressive effects of lithospermum erythrorhizon extracts on lipopolysaccharide-induced activation of ap-1 and nf-kappab via mitogen-activated protein kinase pathways in mouse macrophage cells. *BMB Rep.* **2008**, *41*, 328–333.

7. Dannenberg, A.J.; Altorki, N.K.; Boyle, J.O.; Dang, C.; Howe, L.R.; Weksler, B.B.; Subbaramaiah, K. Cyclo-oxygenase 2: A pharmacological target for the prevention of cancer. *Lancet Oncol.* **2001**, *2*, 544–551.

8. Petros, A.; Bennett, D.; Vallance, P. Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *Lancet* **1991**, *338*, 1557–1558.

9. Szabo, C.; Ischiropoulos, H.; Radi, R. Peroxynitrite: Biochemistry, pathophysiology and development of therapeutics. *Nat. Rev. Drug Discov.* **2007**, *6*, 662–680.

10. Zouki, C.; Jozsef, L.; Ouellet, S.; Paquette, Y.; Filep, J.G. Peroxynitrite mediates cytokine-induced il-8 gene expression and production by human leukocytes. *J. Leukoc. Biol.* **2001**, *69*, 815–824.

11. Szabo, C. Multiple pathways of peroxynitrite cytotoxicity. *Toxicol. Lett.* **2003**, *140–141*, 105–112.

12. Lanone, S.; Manivet, P.; Callebert, J.; Launay, J.M.; Payen, D.; Aubier, M.; Boczkowski, J.; Mebazaa, A. Inducible nitric oxide synthase (nos2) expressed in septic patients is nitrated on selected tyrosine residues: Implications for enzymic activity. *Biochem. J.* **2002**, *366*, 399–404.

13. Whiteman, M.; Ketsawatsakul, U.; Halliwell, B. A reassessment of the peroxynitrite scavenging activity of uric acid. *Ann. N. Y. Acad. Sci.* **2002**, *962*, 242–259.

14. Scott, G.S.; Spitsin, S.V.; Kean, R.B.; Mikheeva, T.; Koprowski, H.; Hooper, D.C. Therapeutic intervention in experimental allergic encephalomyelitis by administration of uric acid precursors. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 16303–16308.

15. Robinson, K.M.; Morre, J.T.; Beckman, J.S. Triuret: A novel product of peroxynitrite-mediated oxidation of urate. *Arch. Biochem. Biophys.* **2004**, *423*, 213–217.

16. Scott, G.S.; Cuzzocrea, S.; Genovese, T.; Koprowski, H.; Hooper, D.C. Uric acid protects against secondary damage after spinal cord injury. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 3483–3488.

17. Sanz, M.L.; Sanz, J.; Martinez-Castro, I. Gas chromatographic-mass spectrometric method for the qualitative and quantitative determination of disaccharides and trisaccharides in honey. *J. Chromatogr. A* **2004**, *1059*, 143–148.

18. Erejuwa, O.O.; Sulaiman, S.A.; Wahab, M.S. Honey—A novel antidiabetic agent. *Int. J. Biol. Sci.* **2012**, *8*, 913–934.

19. Munstedt, K.; Bohme, M.; Hauenschild, A.; Hrgovic, I. Consumption of rapeseed honey leads to higher serum fructose levels compared with analogue glucose/fructose solutions. *Eur. J. Clin. Nutr.* **2011**, *65*, 77–80.

20. Erejuwa, O.O.; Sulaiman, S.A.; Wahab, M.S. Fructose might contribute to the hypoglycemic effect of honey. *Molecules* **2012**, *17*, 1900–1915.

21. Munstedt, K.; Sheybani, B.; Hauenschild, A.; Bruggmann, D.; Bretzel, R.G.; Winter, D. Effects of basswood honey, honey-comparable glucose-fructose solution, and oral glucose tolerance test solution on serum insulin, glucose, and c-peptide concentrations in healthy subjects. *J. Med. Food* **2008**, *11*, 424–428.

22. Gomes, T.; Feas, X.; Iglesias, A.; Estevinho, L.M. Study of organic honey from the northeast portugal. *Molecules* **2011**, *16*, 5374–5386.

23. Martos, I.; Ferreres, F.; Yao, L.; D'Arcy, B.; Caffin, N.; Tomas-Barberan, F.A. Flavonoids in monospecific eucalyptus honeys from australia. *J. Agric. Food Chem.* **2000**, *48*, 4744–4748.

24. Bertoncelj, J.; Dobersek, U.; Jamnik, M.; Golob, T. Evaluation of the phenolic content, antioxidant activity and colour of slovenian honey. *Food Chem.* **2007**, *105*, 822–828.

25. Mohamed, M.; Sulaiman, S.A.; Jaafar, H.; Sirajudeen, K.N. Antioxidant protective effect of honey in cigarette smoke-induced testicular damage in rats. *Int. J. Mol. Sci.* **2011**, *12*, 5508–5521.

26. Moskaug, J.O.; Carlsen, H.; Myhrstad, M.C.; Blomhoff, R. Polyphenols and glutathione synthesis regulation. *Am. J. Clin. Nutr.* **2005**, *81*, 277S–283S.

27. Morais, M.; Moreira, L.; Feas, X.; Estevinho, L.M. Honeybee-collected pollen from five portuguese natural parks: Palynological origin, phenolic content, antioxidant properties and antimicrobial activity. *Food Chem. Toxicol.* **2011**, *49*, 1096–1101.

28. Hung, H.C.; Joshipura, K.J.; Jiang, R.; Hu, F.B.; Hunter, D.; Smith-Warner, S.A.; Colditz, G.A.; Rosner, B.; Spiegelman, D.; Willett, W.C. Fruit and vegetable intake and risk of major chronic disease. *J. Natl. Cancer Inst.* **2004**, *96*, 1577–1584.

29. Schramm, D.D.; Karim, M.; Schrader, H.R.; Holt, R.R.; Cardetti, M.; Keen, C.L. Honey with high levels of antioxidants can provide protection to healthy human subjects. *J. Agric. Food Chem.* **2003**, *51*, 1732–1735.

30. Tartibian, B.; Maleki, B.H. The effects of honey supplementation on seminal plasma cytokines, oxidative stress biomarkers, and antioxidants during 8 weeks of intensive cycling training. *J. Androl.* **2012**, *33*, 449–461.

31. Estevinho, L.; Pereira, A.P.; Moreira, L.; Dias, L.G.; Pereira, E. Antioxidant and antimicrobial effects of phenolic compounds extracts of northeast portugal honey. *Food Chem. Toxicol.* **2008**, *46*, 3774–3779.

32. Chaudhuri, S.; Banerjee, A.; Basu, K.; Sengupta, B.; Sengupta, P.K. Interaction of flavonoids with red blood cell membrane lipids and proteins: Antioxidant and antihemolytic effects. *Int. J. Biol. Macromol.* **2007**, *41*, 42–48.

33. Pawlikowska-Pawlega, B.; Gruszecki, W.I.; Misiak, L.E.; Gawron, A. The study of the quercetin action on human erythrocyte membranes. *Biochem. Pharmacol.* **2003**, *66*, 605–612.

34. Mendes, L.; de Freitas, V.; Baptista, P.; Carvalho, M. Comparative antihemolytic and radical scavenging activities of strawberry tree (arbutus unedo l.) leaf and fruit. *Food Chem. Toxicol.* **2011**, *49*, 2285–2291.

35. Valente, M.J.; Baltazar, A.F.; Henrique, R.; Estevinho, L.; Carvalho, M. Biological activities of portuguese propolis: Protection against free radical-induced erythrocyte damage and inhibition of human renal cancer cell growth *in vitro*. *Food Chem. Toxicol.* **2011**, *49*, 86–92.

36. Beretta, G.; Orioli, M.; Facino, R.M. Antioxidant and radical scavenging activity of honey in endothelial cell cultures (ea.Hy926). *Planta Med.* **2007**, *73*, 1182–1189.

37. Blasa, M.; Candiracci, M.; Accorsi, A.; Piacentini, M.P.; Albertini, M.C.; Piatti, E. Raw millefiori honey is packed full of antioxidants. *Food Chem.* **2006**, *97*, 217–222.

38. Aljadi, A.M.; Kamaruddin, M.Y. Evaluation of the phenolic contents and antioxidant capacities of two malaysian floral honeys. *Food Chem.* **2004**, *85*, 513–518.

39. Kassim, M.; Achoui, M.; Mansor, M.; Yusoff, K.M. The inhibitory effects of gelam honey and its extracts on nitric oxide and prostaglandin e(2) in inflammatory tissues. *Fitoterapia* **2010**, *81*, 1196–1201.

40. Kassim, M.; Achoui, M.; Mustafa, M.R.; Mohd, M.A.; Yusoff, K.M. Ellagic acid, phenolic acids, and flavonoids in malaysian honey extracts demonstrate *in vitro* anti-inflammatory activity. *Nutr. Res.* **2010**, *30*, 650–659.

41. Kassim, M.; Yusoff, K.M.; Ong, G.; Sekaran, S.; Yusof, M.Y.; Mansor, M. Gelam honey inhibits lipopolysaccharide-induced endotoxemia in rats through the induction of heme oxygenase-1 and the inhibition of cytokines, nitric oxide, and high-mobility group protein b1. *Fitoterapia* **2012**, *83*, 1054–1059.

42. Kassim, M.; Mansor, M.; Al-Abd, N.; Yusoff, K.M. Gelam honey has a protective effect against lipopolysaccharide (lps)-induced organ failure. *Int. J. Mol. Sci.* **2012**, *13*, 6370–6381.

43. Yao, L.K.; Abdul Razak, S.L.; Nazhirah, I.; Ng, C.F.; Asyraf, M.H.; Asgar, M.; Nursyahirah, M.S.; Aan, G.J.; Jubri, Z. Malaysian gelam honey reduces oxidative damage and modulates antioxidant enzyme activities in young and middle aged rats. *J. Med. Plant Res.* **2011**, *5*, 5618–5625.

44. Fang, F.C. Perspectives series: Host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *J. Clin. Investig.* **1997**, *99*, 2818–2825.

45. Jagtap, P.; Szabo, C. Poly(adp-ribose) polymerase and the therapeutic effects of its inhibitors. *Nat. Rev. Drug Discov.* **2005**, *4*, 421–440.

46. Virag, L.; Szabo, E.; Gergely, P.; Szabo, C. Peroxynitrite-induced cytotoxicity: Mechanism and opportunities for intervention. *Toxicol. Lett.* **2003**, *140–141*, 113–124.
47. Shacka, J.J.; Sahawneh, M.A.; Gonzalez, J.D.; Ye, Y.Z.; D'Alessandro, T.L.; Estevez, A.G. Two distinct signaling pathways regulate peroxynitrite-induced apoptosis in pc12 cells. *Cell Death Diff.* **2006**, *13*, 1506–1514.
48. Virag, L.; Marmer, D.J.; Szabo, C. Crucial role of apopain in the peroxynitrite-induced apoptotic DNA fragmentation. *Free Radic Biol. Med.* **1998**, *25*, 1075–1082.
49. Zhuang, S.; Simon, G. Peroxynitrite-induced apoptosis involves activation of multiple caspases in hl-60 cells. *Am. J. Physiol. Cell Physiol.* **2000**, *279*, C341–C351.
50. Vicente, S.; Perez-Rodriguez, R.; Olivan, A.M.; Martinez Palacian, A.; Gonzalez, M.P.; Oset-Gasque, M.J. Nitric oxide and peroxynitrite induce cellular death in bovine chromaffin cells: Evidence for a mixed necrotic and apoptotic mechanism with caspases activation. *J. Neurosci. Res.* **2006**, *84*, 78–96.
51. Kim, Y.M.; Chung, H.T.; Simmons, R.L.; Billiar, T.R. Cellular non-heme iron content is a determinant of nitric oxide-mediated apoptosis, necrosis, and caspase inhibition. *J. Biol. Chem.* **2000**, *275*, 10954–10961.
52. Stadler, J.; Bergonia, H.A.; Di Silvio, M.; Sweetland, M.A.; Billiar, T.R.; Simmons, R.L.; Lancaster, J.R., Jr. Nonheme iron-nitrosyl complex formation in rat hepatocytes: Detection by electron paramagnetic resonance spectroscopy. *Arch. Biochem. Biophys.* **1993**, *302*, 4–11.
53. Kazmierski, W.M.; Wolberg, G.; Wilson, J.G.; Smith, S.R.; Williams, D.S.; Thorp, H.H.; Molina, L. Iron chelates bind nitric oxide and decrease mortality in an experimental model of septic shock. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 9138–9141.
54. Kim, Y.M.; Chung, H.T.; Kim, S.S.; Han, J.A.; Yoo, Y.M.; Kim, K.M.; Lee, G.H.; Yun, H.Y.; Green, A.; Li, J.; *et al.* Nitric oxide protects pc12 cells from serum deprivation-induced apoptosis by cgmp-dependent inhibition of caspase signaling. *J. Neurosci.* **1999**, *19*, 6740–6747.
55. Boese, M.; Mordvintcev, P.I.; Vanin, A.F.; Busse, R.; Mulsch, A. *S*-nitrosation of serum albumin by dinitrosyl-iron complex. *J. Biol. Chem.* **1995**, *270*, 29244–29249.
56. Zhang, X.; Chen, J.; Graham, S.H.; Du, L.; Kochanek, P.M.; Draviam, R.; Guo, F.; Nathaniel, P.D.; Szabo, C.; Watkins, S.C.; *et al.* Intranuclear localization of apoptosis-inducing factor (aif) and large scale DNA fragmentation after traumatic brain injury in rats and in neuronal cultures exposed to peroxynitrite. *J. Neurochem.* **2002**, *82*, 181–191.
57. Nin, N.; Cassina, A.; Boggia, J.; Alfonso, E.; Botti, H.; Peluffo, G.; Trostchansky, A.; Batthyany, C.; Radi, R.; Rubbo, H.; *et al.* Septic diaphragmatic dysfunction is prevented by mn(iii)porphyrin therapy and inducible nitric oxide synthase inhibition. *Intensive Care Med.* **2004**, *30*, 2271–2278.
58. Trujillo, M.; Budde, H.; Pineyro, M.D.; Stehr, M.; Robello, C.; Flohe, L.; Radi, R. Trypanosoma brucei and trypanosoma cruzi tryparedoxin peroxidases catalytically detoxify peroxynitrite via oxidation of fast reacting thiols. *J. Biol. Chem.* **2004**, *279*, 34175–34182.
59. Dubuisson, M.; Vander Stricht, D.; Clippe, A.; Etienne, F.; Nauser, T.; Kissner, R.; Koppenol, W.H.; Rees, J.F.; Knoops, B. Human peroxiredoxin 5 is a peroxynitrite reductase. *FEBS Lett.* **2004**, *571*, 161–165.

60. Rhee, S.G.; Chae, H.Z.; Kim, K. Peroxiredoxins: A historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic Biol. Med.* **2005**, *38*, 1543–1552.

61. Spitsin, S.V.; Scott, G.S.; Kean, R.B.; Mikheeva, T.; Hooper, D.C. Protection of myelin basic protein immunized mice from free-radical mediated inflammatory cell invasion of the central nervous system by the natural peroxynitrite scavenger uric acid. *Neurosci. Lett.* **2000**, *292*, 137–141.

62. Hooper, D.C.; Spitsin, S.; Kean, R.B.; Champion, J.M.; Dickson, G.M.; Chaudhry, I.; Koprowski, H. Uric acid, a natural scavenger of peroxynitrite, in experimental allergic encephalomyelitis and multiple sclerosis. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 675–680.

63. Szabo, C.; Ferrer-Sueta, G.; Zingarelli, B.; Southan, G.J.; Salzman, A.L.; Radi, R. Mercaptoethylguanidine and guanidine inhibitors of nitric-oxide synthase react with peroxynitrite and protect against peroxynitrite-induced oxidative damage. *J. Biol. Chem.* **1997**, *272*, 9030–9036.

64. Ploner, F.; Radermacher, P.; Theisen, M.; Tugtekin, I.F.; Matejovic, M.; Stehr, A.; Szabo, C.; Southan, G.J.; Georgieff, M.; Bruckner, U.B.; *et al.* Effects of combined selective inos inhibition and peroxynitrite blockade during endotoxemia in pigs. *Shock* **2001**, *16*, 130–136.

65. Lancel, S.; Tissier, S.; Mordon, S.; Marechal, X.; Depontieu, F.; Scherpereel, A.; Chopin, C.; Neviere, R. Peroxynitrite decomposition catalysts prevent myocardial dysfunction and inflammation in endotoxemic rats. *J. Am. Coll. Cardiol.* **2004**, *43*, 2348–2358.

66. Klotz, L.O.; Sies, H. Defenses against peroxynitrite: Selenocompounds and flavonoids. *Toxicol. Lett.* **2003**, *140–141*, 125–132.

67. Daiber, A.; Zou, M.H.; Bachschmid, M.; Ullrich, V. Ebselen as a peroxynitrite scavenger *in vitro* and *ex vivo*. *Biochem. Pharmacol.* **2000**, *59*, 153–160.

68. Noiri, E.; Nakao, A.; Uchida, K.; Tsukahara, H.; Ohno, M.; Fujita, T.; Brodsky, S.; Goligorsky, M.S. Oxidative and nitrosative stress in acute renal ischemia. *Am. J. Physiol. Renal Physiol.* **2001**, *281*, F948–F957.

69. Crow, J.P. Peroxynitrite scavenging by metalloporphyrins and thiolates. *Free Radic Biol. Med.* **2000**, *28*, 1487–1494.

70. Briviba, K.; Roussyn, I.; Sharov, V.S.; Sies, H. Attenuation of oxidation and nitration reactions of peroxynitrite by selenomethionine, selenocystine and ebselen. *Biochem. J.* **1996**, *319*, 13–15.

71. Tabuchi, Y.; Sugiyama, N.; Horiuchi, T.; Furusawa, M.; Furuhama, K. Ebselen, a seleno-organic compound, protects against ethanol-induced murine gastric mucosal injury in both *in vivo* and *in vitro* systems. *Eur. J. Pharmacol.* **1995**, *272*, 195–201.

72. Batinic-Haberle, I.; Spasojevic, I.; Stevens, R.D.; Hambright, P.; Neta, P.; Okado-Matsumoto, A.; Fridovich, I. New class of potent catalysts of O2.-dismutation. Mn(iii) ortho-methoxyethylpyridyl- and di-ortho-methoxyethylimidazolylporphyrins. *Dalton Trans.* **2004**, 1696–1702.

73. Wang, W.; Jittikanont, S.; Falk, S.A.; Li, P.; Feng, L.; Gengaro, P.E.; Poole, B.D.; Bowler, R.P.; Day, B.J.; Crapo, J.D.; *et al.* Interaction among nitric oxide, reactive oxygen species, and antioxidants during endotoxemia-related acute renal failure. *Am. J. Physiol. Renal Physiol.* **2003**, *284*, F532–F537.

74. Brodsky, S.V.; Gealekman, O.; Chen, J.; Zhang, F.; Togashi, N.; Crabtree, M.; Gross, S.S.; Nasjletti, A.; Goligorsky, M.S. Prevention and reversal of premature endothelial cell senescence and vasculopathy in obesity-induced diabetes by ebselen. *Circ. Res.* **2004**, *94*, 377–384.

75. Gealekman, O.; Brodsky, S.V.; Zhang, F.; Chander, P.N.; Friedli, C.; Nasjletti, A.; Goligorsky, M.S. Endothelial dysfunction as a modifier of angiogenic response in zucker diabetic fat rat: Amelioration with ebselen. *Kidney Int.* **2004**, *66*, 2337–2347.

76. Crow, J.P. Catalytic antioxidants to treat amyotropic lateral sclerosis. *Expert Opin. Investig. Drugs* **2006**, *15*, 1383–1393.

77. Beckman, J.S. Oxidative damage and tyrosine nitration from peroxynitrite. *Chem. Res. Toxicol.* **1996**, *9*, 836–844.

78. Pannala, A.S.; Razaq, R.; Halliwell, B.; Singh, S.; Rice-Evans, C.A. Inhibition of peroxynitrite dependent tyrosine nitration by hydroxycinnamates: Nitration or electron donation? *Free Radic Biol. Med.* **1998**, *24*, 594–606.

79. Kassim, M.; Mansor, M.; Achoui, M.; Ong, G.S.Y.; Sekaran, S.D.; Yusoff, K.M. Honey as an immunomodulator during sepsis in animal models. *Crit. Care J.* **2009**, *13*, P40.

80. Hussein, S.Z.; Mohd Yusoff, K.; Makpol, S.; Mohd Yusof, Y.A. Gelam honey inhibits the production of proinflammatory, mediators no, pge2, tnf-&#x3b1;, and il-6 in carrageenan-induced acute paw edema in rats. *Evid.-Based Complement. Altern. Med.* **2012**, *2012*, 13.

81. Laskin, D.L.; Pendino, K.J. Macrophages and inflammatory mediators in tissue injury. *Annu. Rev. Pharmacol. Toxicol.* **1995**, *35*, 655–677.

82. Cheng, Y.W.; Chang, C.Y.; Lin, K.L.; Hu, C.M.; Lin, C.H.; Kang, J.J. Shikonin derivatives inhibited lps-induced nos in raw 264.7 cells via downregulation of mapk/nf-kappab signaling. *J. Ethnopharmacol.* **2008**, *120*, 264–271.

83. Sandur, S.K.; Ichikawa, H.; Sethi, G.; Ahn, K.S.; Aggarwal, B.B. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) suppresses nf-kappab activation and nf-kappab-regulated gene products through modulation of p65 and ikappabalpha kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agents. *J. Biol. Chem.* **2006**, *281*, 17023–17033.

84. Muijsers, R.B.; van Den Worm, E.; Folkerts, G.; Beukelman, C.J.; Koster, A.S.; Postma, D.S.; Nijkamp, F.P. Apocynin inhibits peroxynitrite formation by murine macrophages. *Br. J. Pharmacol.* **2000**, *130*, 932–936.

85. Achoui, M.; Appleton, D.; Abdulla, M.A.; Awang, K.; Mohd, M.A.; Mustafa, M.R. *In vitro* and *in vivo* anti-inflammatory activity of 17-*O*-acetylacuminolide through the inhibition of cytokines, nf-kappab translocation and ikkbeta activity. *PLoS One* **2010**, *5*, e15105.

# CHAPTER 4. GENERAL DISCUSION AND CONCLUSION

## 4.1 Discussion

Although HPLC did not provide information about some compounds and their derivatives and conjugates, the identification of some phenolic compounds and their derivatives, such as ellagic acid and ellagitannin and their conjugates was possible with LC-MS. The inhibition of inflammatory mediators, such as TNF and NO, which were explored in this study, is one of the important steps in controlling inflammation. Reactive oxygen species (ROS) play a critical role in mediating TNF-α-induced cytotoxicity (Kunnumakkara *et al.*, 2008). It was shown that such cytotoxicity can be blocked by specific free radical scavengers (Goossens *et al.*, 1995). Our findings show that both types of the honey extracts had a dose-dependent protective effect in TNF-α-mediated cytotoxicity. Previous research has reported that Malaysian honey has free radical scavenging activity. Although the concentrations of the phenolics identified were higher in HME, the in vitro anti-inflammatory activity seemed to be better for HEAE. This could be explained by the fact that the concentrations were reported for every 100 g of honey extracted. The dry extract yield ratio of the HEAE to the HME had been 1:5 for every 100 g of honey, hence over-representing the concentrations of the phenolic compounds in the methanol extract. Phenolic compounds in Gelam honey and its extracts do appear to have anti-inflammatory effects against the inflammatory mediators NO and PGE$_2$ in tissues. Effects on NO and PGE$_2$ correspond with the reduction in paw edema volume and the inhibition of pain. Honey and its extracts are, therefore, potentially useful for treating inflammatory conditions. The immune model (LPS) showed a faster development of edema, with the largest edema volume being recorded at 3 hrs. On the other hand, the non-immune model (carrageenan) induced a larger edema volume, and the development of edema occurred over a longer period with

the largest edema volume being recorded at 6 hrs. The effects of honey and its extracts were significant in both models but were more pronounced in the carrageenan model. This may be attributed to the fact that carrageenan is known to destroy macrophages (Ogata *et al.*, 1999). In this model, a decrease in paw withdrawal latency to radiant heat and withdrawal threshold was observed throughout the 30 min - 7 hrs time period after induction of the paws by carrageenan. The results depict that honey and its extracts showed potent anti-nociceptive activity which is caused by the inhibition of $PGE_2$ and NO. The extracts were more significant supporting the above suggestion for the involvement of phenolic compounds in this activity.

Nitric oxide (NO) is known to be an important mediator of acute and chronic inflammation. The results show that honey and it extracts inhibited NO in inflammatory tissues in both models. In the carrageenan model, the inhibition was more pronounced than that of the LPS model. The major phenolic compounds in the methanol and ethyl acetate extracts were gallic acid, ellagic acid, caffeic acid, luteolin, chrysin and quercetin. The anti-inflammatory activity correlates positively with the radical-scavenging activity and total phenolic content (Terra *et al.*, 2007). Prostaglandin is a very important mediator of all types of inflammation. Previous studies have shown that COX-2 is responsible for increased prostaglandin production in inflamed tissue (DeWitt, 1991). The results indicate that honey and its extracts inhibit the $PGE_2$ in inflammatory tissues of both inflammation models. Specific inhibition of COX-2 expression at the transcriptional level is a potent mechanism in the treatment of inflammatory disease (O'Banion *et al.*, 1992). It is possible that honey and its extracts are selective inhibitors of COX-2 because honey has no side effects on the gastrointestinal system. Different extraction methods and solvents yield different concentrations of phenolic compounds in honey. In addition the study's findings resulted in Malaysian honey displaying varying anti-inflammatory activities in the two

in vitro models of inflammation used. This bioactivity may be attributed, at least in part, to the phenolic compounds within the extracts, thus, highlighting the nutritional value of this food. In addition to its well-known properties as a natural sweetener, honey has many anti-inflammatory properties. These include the ability to stimulate HO-1 production and to inhibit the release of both pro- and anti-inflammatory cytokines (TNF-α, IL-1, IL-10), HMGB1, and NO. Together, these effects suggest a mechanism by which honey is able to protect animals from the lethal effects of LPS-induced endotoxemia. Therefore, honey should be further explored with respect to its anti-inflammatory and immunomodulatory properties for use in the treatment of inflammatory diseases. Intravenous injection of honey into LPS-treated rats inhibited cytokine production, including that of TNF-α, IL-1, and IL-10, as well as HMGB1 and NO release, while at the same time inducing *HO-1*. Thus, consistent with *in vitro* studies demonstrating the immunomodulatory effects of gelam honey on cytokines and NO released in L929 and RAW 264.7(Kassim *et al.*, 2010), our results show that Gelam honey inhibits cytokines, NO and protects rats from endotoxemia. Upregulation of *HO-1* inhibits the release of cytokines, HMGB1 and NO. The enzyme HO-1 protects animals from severe inflammation, and a clear relationship has been determined between HO-1 activation and decreased HMGB1 levels which, in turn, protects animals from endotoxemia. Our results have been shown that honey inhibited HMGB1 while inducing HO-1 and increasing the survival of LPS-treated rats. Gelam honey protects organs from lethal doses of LPS by improving organ functions, reducing infiltration by PMNs that cause tissue damage, reducing MPO activity and increasing the survival rate. When immune responses are insufficient, infections can lead to sepsis (Molan, 2001). Sepsis-induced acute lung injury is a major clinical problem with significant morbidity and mortality (Luce, 1998; Vincent *et al.*, 2003; Bernard *et al.*, 1994). The present study is in agreement with previous studies showing that sepsis induces changes in pulmonary

microvascular neutrophil sequestration and alveolar neutrophil infiltration, (Doerschuk, 2001; Brown *et al.*, 1995; Skoutelis *et al.*, 2000; Razavi *et al.*, 2004) as clearly shown in the untreated group but not in the honey-treated group. The reductions in RBC, WBC, and platelet counts observed in the untreated group confirm those seen in earlier reports (Aoki *et al.*, 2000; Chiou *et al.*, 2002). Treatment with honey significantly attenuated the severe reductions in blood counts (WBC and RBC) and thrombocytopenia, suggesting that honey has a protective role against sepsis-induced disseminated intravascular coagulation. Our results confirm that sepsis caused liver failure, as shown by significantly elevated serum levels of AST, ALP, and γ-GT in the untreated group; honey inhibited these increases. Improved liver function tests after honey treatment indicate that honey may potentially protect liver cells from sepsis. Lipid profiles showed that cholesterol, triglycerides, and LDL levels were significantly increased in the LPS-induced untreated sepsis group but not in the honey-treated group. However, the HDL levels were significantly lower in the untreated group. Injection of LPS into animals induces renal dysfunction characterized by increased blood urea nitrogen and plasma creatinine levels (Chen *et al.*, 1999; Wellings *et al.*, 1995). Urea nitrogen and plasma creatinine levels were increased by LPS injection, but were lower in the honey-treated group than in the untreated group. Both our previous studies and the above results show that LPS increased the levels of hepatic damage markers, modified lipid metabolism, and increased lipid profiles, hematological values, and renal dysfunction (Memon *et al.*, 1993). Our data showed that honey is a potent peroxynitrite scavenger *in vitro* and *in vivo* that has cytoprotective effects against peroxynitrite-mediated cellular injury and death. Moreover, the preservation of cellular viability from peroxynitrite-mediated damage is critical to any consideration of the potential therapeutic value of peroxynitrite scavengers in many diseases, including inflammation and sepsis. This finding suggests that honey has therapeutic applications for a wide range of inflammatory disorders. The

data presented here suggest that honey, through its antioxidant properties, is able to protect host immune cells from the inflammation-mediated cytotoxicity that develops in response to LPS stimulation. Excess NO production is cytotoxic and has a broad spectrum of cellular effects. In RAW 264.7 macrophages, there was a pronounced increase in NO production and a significant decrease in cell viability after stimulation with LPS/IFN-$\gamma$, which is known to induce iNOS and thus increase cellular NO concentrations. This decrease in viability was much less pronounced in the presence of 1400W, suggesting that cytotoxicity was induced by iNOS and NO. In fact, NO production, measured as nitritein the Griess reaction, was almost completely inhibited by iNOS inhibition. Earlier studies show that peroxynitrite stimulates the release of the mitochondrial apoptosis-inducing factor, which subsequently triggers DNA fragmentation (Zhang *et al.*, 2002), release of mitochondrial pro-apoptotic factors, and cytochrome *c*-dependent apoptosis in the cytosol through peroxynitrite-dependent oxidation of the mitochondrial permeability transition pore. This study shows that honey can inhibit NO production, and thus peroxynitrite formation, thereby reducing the effects of these cytotoxic compounds both *in vitro* and *in vivo*. Moreover, scavengers of peroxynitrite are known to be protective against tissue damage (Szabo *et al.*, 2007). These compounds can reduce 3-nitrotyrosine immune reactivity in various pathophysiological conditions and have beneficial effects in animal models of inflammation, sepsis, and reperfusion injury (Crow, 2000Crow, 2000; Briviba *et al.*, 1996; Tabuchi *et al.*, 1995; Batinic-Haberle *et al.*, 2004; Noiri *et al.*, 2001; Wang *et al.*, 2003; Brodsky *et al.*, 2004; Gealekman *et al.*, 2004; Nin *et al.*, 2004; Crow, 2006; Beckman, 1996).

### 4.2 Conclusion

In conclusion, this study indicated that different extraction methods and solvents will yield different concentrations of phenolic compounds in honey. Additionally, this study's findings also supported our hypothesis that Gelam honey extracts would display varying anti-inflammatory activities in the two in vitro models of inflammation utilized. This bioactivity may be attributed, at least in part, to the phenolic compounds within the extracts. As such, this study has made a contribution to the elucidation of the potential therapeutic value of honey and its extracts in inflammatory conditions, thus highlighting the nutritional value of this food. Phenolic compounds in Gelam honey and its extracts do appear to have anti-inflammatory effects against the inflammatory mediators NO and $PGE_2$ in tissues. Effects on NO and $PGE_2$ correspond with the reduction in paw edema volume and the inhibition of pain. In addition to its well-known properties as a natural sweetener, honey has many anti-inflammatory properties. These include the ability to stimulate HO-1 production and to inhibit the release of both pro- and anti-inflammatory cytokines (TNF-$\alpha$, IL-1, IL-10), HMGB1, and NO. Together, these effects suggest a mechanism by which honey is able to protect animals from the lethal effects of LPS-induced endotoxemia. Gelam honey protects organs from lethal doses of LPS by improving organ functions, reducing infiltration by PMNs that cause tissue damage, reducing MPO activity and increasing the survival rate. Honey inhibited peroxynitrite synthesis in vitro and vivo (endotoxemia). Thus, by suppressing the production of cytotoxic molecules such as NO and peroxynitrite, honey may attenuate the inflammatory responses that lead to cell damage and, potentially, to cell death, suggesting it has therapeutic applications for a wide range of inflammatory disorders. Suggestion, honey should be further explored with respect to its anti-inflammatory and immunomodulatory properties for use in the treatment of inflammatory diseases.

# REFERENCES

Agnello, D., Wang, H., Yang, H., Tracey, K. J., & Ghezzi, P. (2002). HMGB-1, a DNA-binding protein with cytokine activity, induces brain TNF and IL-6 production, and mediates anorexia and taste aversion. *Cytokine, 18*(4), 231-236. doi: S1043466602908902 [pii]

Alissandrakis, E., Kibaris, A. C., Tarantilis, P. A., Harizanis, P. C., & Polissiou, M. (2005). Flavour compounds of Greek cotton honey. *Journal of the Science of Food and Agriculture, 85, 1444-1452.*

Aljadi, A., & Kamaruddin, M. (2004). Evaluation of the phenolic contents and antioxidant capacities of two malaysian floral honeys. *Food Chem, 85*, 513–518.

Aljadi A. M. , K. M. Y. (2003). Isolation and Identification of Phenolic Acids in Malaysian Honey with Antibacterial Property. *Turkish Journal of Medical Sciences, 33, 229-236.*

Alkharfy, K. M., Al-Daghri, N. M., Al-Attas, O. S., & Alokail, M. S. (2011). The protective effect of thymoquinone against sepsis syndrome morbidity and mortality in mice. [Research Support, Non-U.S. Gov't]. *Int Immunopharmacol, 11*(2), 250-254. doi: 10.1016/j.intimp.2010.11.032

Alves-Filho, J. C., Tavares-Murta, B. M., Barja-Fidalgo, C., Benjamim, C. F., Basile-Filho, A., Arraes, S. M., & Cunha, F. Q. (2006). Neutrophil function in severe sepsis. [Research Support, Non-U.S. Gov't Review]. *Endocr Metab Immune Disord Drug Targets, 6*(2), 151-158.

Amann, R., Schuligoi, R., Lanz, I., & Donnerer, J. (1995). Histamine-induced edema in the rat paw--effect of capsaicin denervation and a CGRP receptor antagonist. [Research Support, Non-U.S. Gov't]. *European Journal of Pharmacology, 279*(2-3), 227-231.

Anderson, R. J. (2012). Acute kidney injury with sepsis: Is nitric oxide a friend, foe, both, or neither?*. *Crit Care Med, 40*(8), 2511-2512. doi: 10.1097/CCM.0b013e318255d7b4

Andersson, U., Wang, H., Palmblad, K., Aveberger, A. C., Bloom, O., Erlandsson-Harris, H., . . . Tracey, K. J. (2000). High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. *J Exp Med, 192*(4), 565-570.

Andrade, P., F. Ferreres, M.I. Gil, and F.A. Tomás-Barberán ( 1997b). Determination of phenolic compounds in honeys with different floral origin by capillary zone electrophoresis. *Food Chemistry, 60(1), 79-84.*

Andrade, P., F. Ferreres, M.T. Amaral   (1997a). Analysis of honey phenolic acids by HPLC, its application to honey botanical characterization. *J. Liq. Chrom. Rel. Technol, 20(14), 2281-2288.*

Angus, D. C., Linde-Zwirble, W. T., Lidicker, J., Clermont, G., Carcillo, J., & Pinsky, M. R. (2001). Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med, 29*(7), 1303-1310.

Anklam, E. (1998). A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chemistry 63(4), 549-562.*

Anklam, E. B. R. (2001). Suitable analytical methods for determining the origin of European honey. *Am. Lab, 5, 60-64.*

Aoki, Y., Ota, M., Katsuura, Y., Komoriya, K., & Nakagaki, T. (2000). Effect of activated human protein C on disseminated intravascular coagulation induced by lipopolysaccharide in rats. *Arzneimittelforschung, 50*(9), 809-815.

AT, A. (1991). Prevention of ethanol-induced gastric lesions in rats by natural honey and its possible mechanism of action. *Scand J Gastroenterol 26:281-288.*

AW, S. (2006). How superoxide production by neutrophil leukocytes kills microbes. *Novartis.Found.Symp, 279:92-8.*

Ayala, A., & Chaudry, I. H. (1996). Immune dysfunction in murine polymicrobial sepsis: mediators, macrophages, lymphocytes and apoptosis. *Shock, 6 Suppl 1*, S27-38.

Bankova, V., A. Dyulgerov, S. Popov, and N. Marekov. (1987). A GC/MS study of the propolis phenolic constituents. *Zeitsch. f. Naturfors, 42C, 147-151.*

Batinic-Haberle, I., Spasojevic, I., Stevens, R. D., Hambright, P., Neta, P., Okado-Matsumoto, A., & Fridovich, I. (2004). New class of potent catalysts of O2.-dismutation. Mn(III) ortho-methoxyethylpyridyl- and di-ortho-methoxyethylimidazolylporphyrins. *Dalton Trans*(11), 1696-1702. doi: 10.1039/b400818a

Beaulieu, J., Dupont, C., & Lemieux, P. (2007). Anti-inflammatory potential of a malleable matrix composed of fermented whey proteins and lactic acid bacteria in an atopic dermatitis model. *J Inflamm (Lond), 4*, 6. doi: 10.1186/1476-9255-4-6

Beckman, J. S. (1996). Oxidative damage and tyrosine nitration from peroxynitrite. *Chem Res Toxicol, 9*(5), 836-844. doi: 10.1021/tx9501445

Bellezza, I., Tucci, A., Galli, F., Grottelli, S., Mierla, A. L., Pilolli, F., & Minelli, A. (2012). Inhibition of NF-kappaB nuclear translocation via HO-1 activation underlies alpha-tocopheryl succinate toxicity. *J Nutr Biochem.* doi: 10.1016/j.jnutbio.2011.10.012

Bergenfelz, C., Medrek, C., Ekstrom, E., Jirstrom, K., Janols, H., Wullt, M., . . . Leandersson, K. (2012). Wnt5a induces a tolerogenic phenotype of macrophages in sepsis and breast cancer patients. [Research Support, Non-U.S. Gov't]. *J Immunol, 188*(11), 5448-5458. doi: 10.4049/jimmunol.1103378

Bhatia, M., & Moochhala, S. (2004). Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. [Research Support, Non-U.S. Gov't Review]. *J Pathol, 202*(2), 145-156. doi: 10.1002/path.1491

Blonska, M., Bronikowska, J., Pietsz, G., Czuba, Z. P., Scheller, S., & Krol, W. (2004). Effects of ethanol extract of propolis (EEP) and its flavones on inducible gene expression in J774A.1 macrophages. *J Ethnopharmacol, 91*(1), 25-30. doi: 10.1016/j.jep.2003.11.011

Bogdanov, S. (1989). Determination of pinocembrin in honey using HPLC. *J. Apic. Res, 28(1), 55-57.*

Bortscher, S., Chang, J., Vilz, T. O., Schafer, N., Sommer, N., Wehner, S., . . . Overhaus, M. (2012). Hemin induction of HO-1 protects against LPS-induced septic ileus. *J Surg Res*. doi: 10.1016/j.jss.2012.07.064

Boudourova-Krasteva, G., V.S. Bankova, J.M. Sforcin, N. Nikolova, and S.S. Popov. (1997). Phenolics from Brazilian propolis. *Zeitschrift f. Naturforschung, 52, 676-679.*

Boveris A, A. S., Navarro A (2002). The role of mitochondrial nitric oxide synthase in inflammation and septic shock. *Free Radic Biol Med, 33: 1186-1193.*

Brennan FM, C. D., Jackson A, Maini RN. (1989). Inhibitory effect of TNF-α antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. *Feldomann M. Lancet, 334:244-7.*

Briviba, K., Roussyn, I., Sharov, V. S., & Sies, H. (1996). Attenuation of oxidation and nitration reactions of peroxynitrite by selenomethionine, selenocystine and ebselen. *Biochem J, 319 ( Pt 1)*, 13-15.

Brodsky, S. V., Gealekman, O., Chen, J., Zhang, F., Togashi, N., Crabtree, M., . . . Goligorsky, M. S. (2004). Prevention and reversal of premature endothelial cell senescence and vasculopathy in obesity-induced diabetes by ebselen. *Circ Res, 94*(3), 377-384. doi: 10.1161/01.RES.0000111802.09964.EF

Brouard, S., Berberat, P. O., Tobiasch, E., Seldon, M. P., Bach, F. H., & Soares, M. P. (2002). Heme oxygenase-1-derived carbon monoxide requires the activation of transcription factor NF-kappa B to protect endothelial cells from tumor necrosis factor-alpha-mediated apoptosis. *J Biol Chem, 277*(20), 17950-17961. doi: 10.1074/jbc.M108317200

Brown, D. M., Drost, E., Donaldson, K., & MacNee, W. (1995). Deformability and CD11/CD18 expression of sequestered neutrophils in normal and inflamed lungs. [Research Support, Non-U.S. Gov't]. *Am J Respir Cell Mol Biol, 13*(5), 531-539.

Brudecki, L., Ferguson, D. A., McCall, C. E., & El Gazzar, M. (2012). Adoptive transfer of CD34(+) cells during murine sepsis rebalances macrophage lipopolysaccharide responses. *Immunol Cell Biol*. doi: 10.1038/icb.2012.32

Bulman, M. W. (1953). Honey as a surgical dressing. *Middx. Hosp. J, 55: 188-189.*

Buras, J. A., Holzmann, B., & Sitkovsky, M. (2005). Animal models of sepsis: setting the stage. *Nat Rev Drug Discov, 4*(10), 854-865. doi: 10.1038/nrd1854

C, S. (2003). Multiple pathways of peroxynitrite cytotoxicity. *Toxicol. Lett, **140-141**, 105-112.*

CA, D. ( 2000). Proinflammatory cytokines. *Chest, 118(2): 503-8.*

Carchman, E. H., Rao, J., Loughran, P. A., Rosengart, M. R., & Zuckerbraun, B. S. (2011). Heme oxygenase-1-mediated autophagy protects against hepatocyte cell death and hepatic injury from infection/sepsis in mice. *Hepatology, 53*(6), 2053-2062. doi: 10.1002/hep.24324

Carrillo Esper, R., Pena Perez, C., Mucino Bermejo, J., Carrillo Cordova, J. R., & Carrillo Cordova, L. D. (2011). [Severe sepsis, septic shock and secondary multiple organ dysfunction in infection by Kluyvera ascorbata]. [Case Reports]. *Gac Med Mex, 147*(4), 355-360.

Cassatella, M., L. Meda, S. Bonora, M. Ceska, and G. Constantin. (1993). Interleukin10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes: evidence for an autocrine role of tumor necrosis factor and IL-1b in mediating the production of IL-8 triggered by lipopolysaccharide. *J. Exp. Med, 178:2207*.

Casteels, P., C. Ampe, F. Jacobs, P. Tempst. (1993). Functional and chemical characterisation of hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honey-bee (Apis mellifera). *Journal of Biological Chemistry, 268: 7044-7054*.

Castell JV, A. T., Kunz D, Heinrich PC. (1989). Interleukin-6. The major regulator of acute-phase protein synthesis in man and rat. *Ann.N.Y.Acad.Sci, 557:87-99*.

Cernada, M., Badia, N., Modesto, V., Alonso, R., Mejias, A., Golombek, S., & Vento, M. (2012). Cord blood interleukin-6 as a predictor of early-onset neonatal sepsis. *Acta Paediatr, 101*(5), e203-207. doi: 10.1111/j.1651-2227.2011.02577.x

Chaplin, M. F. C. B. (1990). Enzyme Technology. *Cambridge University Press*.

Chavan, S. S., Huerta, P. T., Robbiati, S., Valdes-Ferrer, S. I., Ochani, M., Dancho, M., . . . Diamond, B. (2012). HMGB1 Mediates Cognitive Impairment in Sepsis Survivors. *Mol Med*. doi: 10.2119/molmed.2012.00195

Chen, C. P., Yokozawa, T., & Kitani, K. (1999). Beneficial effects of sanguisorbae radix in renal dysfunction caused by endotoxin in vivo. [Research Support, Non-U.S. Gov't]. *Biol Pharm Bull, 22*(12), 1327-1330.

Chiou, W. F., Ko, H. C., Chen, C. F., & Chou, C. J. (2002). Evodia rutaecarpa protects against circulation failure and organ dysfunction in endotoxaemic rats through modulating nitric oxide release. *J Pharm Pharmacol, 54*(10), 1399-1405. doi: 10.1211/002235702760345491

Cho, S. Y., Park, S. J., Kwon, M. J., Jeong, T. S., Bok, S. H., Choi, W. Y., . . . Jeong, K. S. (2003). Quercetin suppresses proinflammatory cytokines production through MAP kinases andNF-kappaB pathway in lipopolysaccharide-stimulated macrophage. [Research Support, Non-U.S. Gov't]. *Mol Cell Biochem, 243*(1-2), 153-160.

Cohen, J. (2002). The immunopathogenesis of sepsis. [Review]. *Nature, 420*(6917), 885-891. doi: 10.1038/nature01326

Crane, E. (1979). The past and present importance of bee products to man in bee production: properties, application and apitherarpy. *(A. Mizrahi & Y. Lensky) New York Plenum Press, 1-13*.

Crow, J. P. (2000). Peroxynitrite scavenging by metalloporphyrins and thiolates. *Free Radic Biol Med, 28*(10), 1487-1494.

Crow, J. P. (2006). Catalytic antioxidants to treat amyotropic lateral sclerosis. *Expert Opin Investig Drugs, 15*(11), 1383-1393.

Daiber, A., Zou, M. H., Bachschmid, M., & Ullrich, V. (2000). Ebselen as a peroxynitrite scavenger in vitro and ex vivo. *Biochem Pharmacol, 59*(2), 153-160.

Daniele, G., Maitre, D., & Casabianca, H. (2012). Identification, quantification and carbon stable isotopes determinations of organic acids in monofloral honeys. A powerful tool for botanical and authenticity control. *Rapid Commun Mass Spectrom, 26*(17), 1993-1998. doi: 10.1002/rcm.6310

de Montmollin, E., & Annane, D. (2011). Year in review 2010: Critical Care--Multiple organ dysfunction and sepsis. *Crit Care, 15*(6), 236. doi: 10.1186/cc10359

de Vries, J. E. (1995). Immunosuppressive and anti-inflammatory properties of interleukin 10. *Ann Med, 27*(5), 537-541. doi: 10.3109/07853899509002465

Dejager, L., Pinheiro, I., Dejonckheere, E., & Libert, C. (2011). Cecal ligation and puncture: the gold standard model for polymicrobial sepsis? *Trends Microbiol, 19*(4), 198-208. doi: 10.1016/j.tim.2011.01.001

Del Boccio, P., & Rotilio, D. (2004). Quantitative analysis of caffeic acid phenethyl ester in crude propolis by liquid chromatography-electrospray ionization mass spectrometry. [Research Support, Non-U.S. Gov't]. *J Sep Sci, 27*(7-8), 619-623.

Deng, J., Wang, X., Qian, F., Vogel, S., Xiao, L., Ranjan, R., . . . Christman, J. W. (2012). Protective role of reactive oxygen species in endotoxin-induced lung inflammation through modulation of IL-10 expression. *J Immunol, 188*(11), 5734-5740.

DeWitt, D. L. (1991). Prostaglandin endoperoxide synthase: regulation of enzyme expression. *Biochim Biophys Acta, 1083*(2), 121-134.

Dinarello, C. A. (2011a). Blocking interleukin-1beta in acute and chronic autoinflammatory diseases. *J Intern Med, 269*(1), 16-28.

Dinarello, C. A. (2011b). Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood, 117*(14), 3720-3732.

Dubuisson, M., Vander Stricht, D., Clippe, A., Etienne, F., Nauser, T., Kissner, R., . . . Knoops, B. (2004). Human peroxiredoxin 5 is a peroxynitrite reductase. [Research Support, Non-U.S. Gov't]. *FEBS Lett, 571*(1-3), 161-165. doi: 10.1016/j.febslet.2004.06.080

Dugas, B., Debre, P., & Moncada, S. (1995). Nitric oxide, a vital poison inside the immune and inflammatory network. [Review]. *Res Immunol, 146*(9), 664-670.

E, C. (1990). Bees and beekeeping: science, practice and world resources. *Heinemann Newnes, Oxford, 614*.

Emura, I., & Usuda, H. (2010). Histopathological and cytological examination of autopsy cases with multiple organ dysfunction syndromes. *Pathol Int, 60*(6), 443-451. doi: 10.1111/j.1440-1827.2010.02539.x

Enkhbaatar, P., Lange, M., Nakano, Y., Hamahata, A., Jonkam, C., Wang, J., . . . Traber, D. (2009). Role of neuronal nitric oxide synthase in ovine sepsis model. *Shock, 32*(3), 253-257. doi: 10.1097/SHK.0b013e318193e2ba

Fan, J., Li, Y., Levy, R. M., Fan, J. J., Hackam, D. J., Vodovotz, Y., . . . Wilson, M. A. (2007). Hemorrhagic shock induces NAD(P)H oxidase activation in neutrophils: role of HMGB1-TLR4 signaling. *J Immunol, 178*(10), 6573-6580.

Fernandes, E. S., Liang, L., Smillie, S. J., Kaiser, F., Purcell, R., Rivett, D. W., . . . Brain, S. D. (2012). TRPV1 deletion enhances local inflammation and accelerates the onset of systemic inflammatory response syndrome. [Research Support, Non-U.S. Gov't]. *J Immunol, 188*(11), 5741-5751. doi: 10.4049/jimmunol.1102147

Ferreres, F., A. Ortiz, C. Silva, C. Garcia-Viguera, F.A. Tomás-Barberán, F. Tomás-Lorente. (1992). Flavonoids of "La Alcarria" honey - A study of their botanical origin. *Zeitschrift f. Lebensmittel-Untersuchung und -Forschung 194: 139-143*.

Ferreres, F., F.A. Tomás-Barberán, C. Soler, C. García-Viguera, A. Ortiz, F. Tomás-Lorente ( 1994c). A simple extractive technique for honey flavonoid HPLC analysis. *Apidologie, 25(1): 21-30.*

Ferreres, F., F.A. Tomás-Barberán, M.I. Gil, and F. Tomás-Lorente. ( 1991). An HPLC technique for flavonoid analysis in honey. *J. Sci. Food Agric, 56: 49-56.*

Fidan, H., Sahin, O., Yavuz, Y., Kilbas, A., Cetinkaya, Z., Ela, Y., . . . Altuntas, I. (2007). Caffeic acid phenethyl ester reduces mortality and sepsis-induced lung injury in rats. *Crit Care Med, 35*(12), 2822-2829.

Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M., & O'Garra, A. (1991). IL-10 inhibits cytokine production by activated macrophages. *J Immunol, 147*(11), 3815-3822.

Frankel, S., G.E. Robinson, M.R. Berenbaum. ( 1998). Antioxidant capacity and correlated characteristics of 14 unifloral honeys. *Journal of Apicultural Research 37(1): 27-52.*

Freels, J. L. *e. a.* (2002). Enhanced activity of human IL-10 after nitration in reducing human IL-1 production by stimulated peripheral blood mononuclear cells. *J. Immunol, **169**, 4568-4571*

Fujiwara, S., J. Imai, M. Fujiwara, T. Yaeshima, T. Kawashima, K. Kobayashi (1990). A potent antibacterial protein in royal jelly. *Journal of Biological Chemistry, 265, 11333-11337.*

Furchgott, R. F., J.V. Zawadzki. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature, 288: 373.*

Gerard, C., C. Bruyns, A. Marchant, D. Abramowicz, P. Vandenabeele, A. Delvaux, W. Fiers, M. Goldman, and T. Velu (1993). Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. *J. Exp. Med 177:547.*

Gheldof, H., X. Wang, N.J. Engeseth. ( 2002a). Identification and quantification of antioxidant component of honeys from various floral sources. *Journal of Agricultural and Food Chemistry, 50(21): 5870-5877.*

Gheldof, H., X. Wang, N.J. Engeseth. ( 2002b). Identification and quantification of antioxidant component of honeys from various floral sources. *Journal of Agricultural and Food Chemistry, 50(21): 5870-5877.*

Gheldof, H. N. J. E. (2002c). Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples. *J. of Agricultural and Food Chemistry, 50(10): 3050-3055.*

Gheldof, N., Wang, X. H., & Engeseth, N. J. (2003). Buckwheat honey increases serum antioxidant capacity in humans. *J Agric Food Chem, 51*(5), 1500-1505. doi: 10.1021/jf025897t

Goode, H. F., & Webster, N. R. (1993). Free radicals and antioxidants in sepsis. *Crit Care Med, 21*(11), 1770-1776.

Goossens, V., Grooten, J., De Vos, K., & Fiers, W. (1995). Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. [Research Support, Non-U.S. Gov't]. *Proc Natl Acad Sci U S A, 92*(18), 8115-8119.

Goris, R. J. (1996). MODS/SIRS: result of an overwhelming inflammatory response? [Review]. *World J Surg, 20*(4), 418-421.

Gouel-Cheron, A., Allaouchiche, B., Guignant, C., Davin, F., Floccard, B., Monneret, G., & AzuRea, G. (2012). Early interleukin-6 and slope of monocyte human leukocyte antigen-DR: a powerful association to predict the development of sepsis after major trauma. [Research Support, Non-U.S. Gov't]. *PLoS One, 7*(3), e33095. doi: 10.1371/journal.pone.0033095

Grimble, R. F. (1994). Nutritional antioxidants and the modulation of inflammation: theory and practice. [Review]. *New Horiz, 2*(2), 175-185.

Griswold, D. E., Martin, L. D., Badger, A. M., Breton, J., & Chabot-Fletcher, M. (1998). Evaluation of the cutaneous anti-inflammatory activity of azaspiranes. *Inflamm Res, 47*(2), 56-61.

Grommes, J., & Soehnlein, O. (2011). Contribution of neutrophils to acute lung injury. [Review]. *Mol Med, 17*(3-4), 293-307. doi: 10.2119/molmed.2010.00138

Gutierrez-Venegas, G., Jimenez-Estrada, M., & Maldonado, S. (2007). The effect of flavonoids on transduction mechanisms in lipopolysaccharide-treated human gingival fibroblasts. *Int Immunopharmacol, 7*(9), 1199-1210. doi: S1567-5769(07)00134-8 [pii] 10.1016/j.intimp.2007.05.004

Habtemariam, S. (2000). Natural inhibitors of tumour necrosis factor-alpha production, secretion and function. [Review]. *Planta Med, 66*(4), 303-313. doi: 10.1055/s-2000-8660

Hamalainen, M., Nieminen, R., Vuorela, P., Heinonen, M., & Moilanen, E. (2007). Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-kappaB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-kappaB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. [Research Support, Non-U.S. Gov't]. *Mediators Inflamm, 2007*, 45673. doi: 10.1155/2007/45673

Hao, L. N., Zhang, X. D., Wang, M., Yang, T., & He, S. Z. (2011). Peroxynitrite-induced expression of inducible nitric oxide synthase and activated apoptosis via nuclear factor-kappa B pathway in retinal pigment epithelial cells and antagonism of cholecystokinin octapeptide-8 in vitro. *Int J Ophthalmol, 4*(5), 474-479. doi: 10.3980/j.issn.2222-3959.2011.05.03

Hayashi, F., Means, T.K. & Luster, A.D. (2003). Toll-like receptors stimulate human neutrophil function. *Blood, 102, 2660-2669*.

Hickey, M. J., Issekutz, A. C., Reinhardt, P. H., Fedorak, R. N., & Kubes, P. (1998). Endogenous interleukin-10 regulates hemodynamic parameters, leukocyte-endothelial cell interactions, and microvascular permeability during endotoxemia. [Research Support, Non-U.S. Gov't]. *Circ Res, 83*(11), 1124-1131.

Hildebrand, D., Sahr, A., Wolfle, S. J., Heeg, K., & Kubatzky, K. F. (2012). Regulation of Toll-like receptor 4-mediated immune responses through Pasteurella multocida toxin-induced G protein signalling. *Cell Commun Signal, 10*(1), 22. doi: 10.1186/1478-811X-10-22

Hoffmann, J. A., Kafatos, F. C., Janeway, C. A., & Ezekowitz, R. A. (1999). Phylogenetic perspectives in innate immunity. [Review]. *Science, 284*(5418), 1313-1318.

Hooper, D. C., Spitsin, S., Kean, R. B., Champion, J. M., Dickson, G. M., Chaudhry, I., & Koprowski, H. (1998). Uric acid, a natural scavenger of peroxynitrite, in experimental allergic encephalomyelitis and multiple sclerosis. [Research Support, Non-U.S. Gov't]. *Proc Natl Acad Sci U S A, 95*(2), 675-680.

Hotchkiss, R. S., & Karl, I. E. (2003). The pathophysiology and treatment of sepsis. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review]. *N Engl J Med, 348*(2), 138-150. doi: 10.1056/NEJMra021333

Howard, M., T. Muchamuel, S. Andrade, and S. Menon. (1993). Interleukin 10 protects mice from lethal endotoxemia. *J. Exp. Med, 177:1205*.

Hultmark, D. (1996). Insect lysozymes, in P. Jollès Lysozymes: Model enzymes in biochemistry and biology. *Experientia Supplement, 75, 87-102*.

Ishikawa, K., Calzavacca, P., Bellomo, R., Bailey, M., & May, C. N. (2012). Effect of selective inhibition of renal inducible nitric oxide synthase on renal blood flow and function in experimental hyperdynamic sepsis*. *Crit Care Med, 40*(8), 2368-2375. doi: 10.1097/CCM.0b013e3182514be9

Iskit, A. B., & Guc, O. (2003). Effects of endothelin and nitric oxide on organ injury, mesenteric ischemia, and survival in experimental models of septic shock. [Research Support, Non-U.S. Gov't Review]. *Acta Pharmacol Sin, 24*(10), 953-957.

Jackson, D. L., C.B. Graff, J.D. Richardson and K.M. Hargreaves. (1995). Glutamate participates in the peripheral modulation of thermal hyperalgesia in rats. *Eur. J. Pharmacol., 284 : 321*.

Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W., . . . Pezzuto, J. M. (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. [Research Support, U.S. Gov't, P.H.S.]. *Science, 275*(5297), 218-220.

Jeddar, A., A. Kharsany, U.G. Ramsaroop, A. Bhamjee, I.E. Haffejee, A. Moosa. (1985). The antibacterial action of honey: an *in vitro* study. *S. Afr. Med. J, 67: 257-258*.

Jedinak, A., Dudhgaonkar, S., Wu, Q. L., Simon, J., & Sliva, D. (2011). Anti-inflammatory activity of edible oyster mushroom is mediated through the inhibition of NF-kappaB and AP-1 signaling. [Research Support, Non-U.S. Gov't]. *Nutr J, 10*, 52. doi: 10.1186/1475-2891-10-52

Jeong, J. B., & Jeong, H. J. (2010). Rheosmin, a naturally occurring phenolic compound inhibits LPS-induced iNOS and COX-2 expression in RAW264.7 cells by blocking NF-kappaB activation pathway. [Research Support, Non-U.S. Gov't]. *Food Chem Toxicol, 48*(8-9), 2148-2153. doi: 10.1016/j.fct.2010.05.020

Joerg, E. (1996). Phenolic acids and phenolic esters in various types of honey: determination with HPLC and coulometric multielectrode detection. *Dissert. Abs. Int, 57(3): 929*.

Joerg, E. G. S. (1992). Determination of phenolic acids in honey by HPLC using coulometric dual electrode detection. *Deutsshe Lebensmittel Rundschau, 80(6): 179-183*.

Joerg, E. G. S. (1993). Multichannel coulometric detection coupled with liquid chromatography for determination of phenolic esters in honey. *J. Chrom, 635: 137-142*.

Joo, S. Y., Song, Y. A., Park, Y. L., Myung, E., Chung, C. Y., Park, K. J., . . . Joo, Y. E. (2012). Epigallocatechin-3-gallate Inhibits LPS-Induced NF-kappaB and MAPK Signaling Pathways in Bone Marrow-Derived Macrophages. *Gut Liver, 6*(2), 188-196. doi: 10.5009/gnl.2012.6.2.188

Jung, E., Perrone, E. E., Liang, Z., Breed, E. R., Dominguez, J. A., Clark, A. T., . . . Coopersmith, C. M. (2012). Cecal ligation and puncture followed by methicillin-resistant Staphylococcus aureus pneumonia increases mortality in mice and blunts production of local and systemic cytokines. [Research Support, N.I.H., Extramural]. *Shock, 37*(1), 85-94. doi: 10.1097/SHK.0b013e3182360faf

Kandil , M., El-Banby, M., Abdel-Wahed, K., Abdel-Gawwad, M., & Fayez, M. (1987a). Curative properties of true floral and false non-floral honeys on induced gastric ulcers. *Journal of Drug Research., 17* (1-2), 103-106.

Kandil , M., El-Banby, M., Abdel-Wahed, K., Abdel-Gawwad, M., & Fayez, M. (1987b). Curative properties of true floral and false non-floral honeys on induced gastric ulcers. *Journal of Drug Research, 17 (1-2)*, 103-106.

Kanters, E., Pasparakis, M., Gijbels, M. J., Vergouwe, M. N., Partouns-Hendriks, I., Fijneman, R. J., . . . de Winther, M. P. (2003). Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. [Research Support, Non-U.S. Gov't]. *J Clin Invest, 112*(8), 1176-1185. doi: 10.1172/JCI18580

Kasama, T., R. M. Strieter, N. W. Lukacs, M. D. Burdick, and S. L. Kunkel. (1994). Regulation of neutrophil-derived chemokine expression by IL-10. *J. Immunol, 152:3559*.

Kassim, M., Achoui, M., Mustafa, M. R., Mohd, M. A., & Yusoff, K. M. (2010). Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity. [Research Support, Non-U.S. Gov't]. *Nutr Res, 30*(9), 650-659. doi: 10.1016/j.nutres.2010.08.008

Kawahara, K., Hashiguchi, T., Masuda, K., Saniabadi, A. R., Kikuchi, K., Tancharoen, S., . . . Maruyama, I. (2009). Mechanism of HMGB1 release inhibition from RAW264.7 cells by oleanolic acid in Prunus mume Sieb. et Zucc. [Research Support, Non-U.S. Gov't]. *Int J Mol Med, 23*(5), 615-620.

Kibe, S., Adams, K., & Barlow, G. (2011). Diagnostic and prognostic biomarkers of sepsis in critical care. [Review]. *J Antimicrob Chemother, 66 Suppl 2*, ii33-40. doi: 10.1093/jac/dkq523

Kindt, G. C., Gadek, J. E., & Weiland, J. E. (1991). Initial recruitment of neutrophils to alveolar structures in acute lung injury. [Research Support, Non-U.S. Gov't]. *J Appl Physiol, 70*(4), 1575-1585.

Kleiman, A., Hubner, S., Rodriguez Parkitna, J. M., Neumann, A., Hofer, S., Weigand, M. A., . . . Tuckermann, J. P. (2012). Glucocorticoid receptor dimerization is required for survival in septic shock via suppression of interleukin-1 in macrophages. [Research Support, Non-U.S. Gov't]. *FASEB J, 26*(2), 722-729. doi: 10.1096/fj.11-192112

Klotz, L. O., & Sies, H. (2003). Defenses against peroxynitrite: selenocompounds and flavonoids. [Research Support, Non-U.S. Gov't  Review]. *Toxicol Lett, 140-141*, 125-132.

Kolgazi, M., Sener, G., Cetinel, S., Gedik, N., & Alican, I. (2006). Resveratrol reduces renal and lung injury caused by sepsis in rats. *J Surg Res, 134*(2), 315-321. doi: 10.1016/j.jss.2005.12.027

Kooy, N. W., Royall, J. A., Ischiropoulos, H. & Beckman, J. S (1994). Peroxynitrite-mediated oxidation of dihydrorhodamine 123. *Free Radic. Biol. Med, **16**,149-156*

Kou, J., Ni, Y., Li, N., Wang, J., Liu, L., & Jiang, Z. H. (2005). Analgesic and anti-inflammatory activities of total extract and individual fractions of Chinese medicinal ants Polyrhachis lamellidens. [Comparative Study Research Support, Non-U.S. Gov't]. *Biol Pharm Bull, 28*(1), 176-180.

Kruttgen, A., & Rose-John, S. (2012). Interleukin-6 in sepsis and capillary leakage syndrome. [Research Support, Non-U.S. Gov't Review]. *J Interferon Cytokine Res, 32*(2), 60-65. doi: 10.1089/jir.2011.0062

Kumar, P. P., & Kuttan, G. (2009). Vernonia cinerea L. scavenges free radicals and regulates nitric oxide and proinflammatory cytokines profile in carrageenan induced paw edema model. *Immunopharmacol Immunotoxicol, 31*(1), 94-102. doi: 10.1080/08923970802438391

Kunnumakkara, A. B., Anand, P., & Aggarwal, B. B. (2008). Curcumin inhibits proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell signaling proteins. [Research Support, Non-U.S. Gov't Review]. *Cancer Lett, 269*(2), 199-225. doi: 10.1016/j.canlet.2008.03.009

Kuper, C., Fraek, M. L., Muller, H. H., Beck, F. X., & Neuhofer, W. (2012). Sepsis-induced urinary concentration defect is related to nitric oxide-dependent inactivation of TonEBP/NFAT5, which downregulates renal medullary solute transport proteins and aquaporin-2. [Research Support, Non-U.S. Gov't]. *Crit Care Med, 40*(6), 1887-1895. doi: 10.1097/CCM.0b013e31824e1186

Laichalk, L. L., J. Danforth, T. J. Standiford. (1996). Interleukin-10 inhibits neutrophil phagocytic and bactericidal activity. *FEMS Immunol. Med. Microbiol, 15:181.*

Lancel, S., Tissier, S., Mordon, S., Marechal, X., Depontieu, F., Scherpereel, A., . . . Neviere, R. (2004). Peroxynitrite decomposition catalysts prevent myocardial dysfunction and inflammation in endotoxemic rats. [Comparative StudyEvaluation Studies Research Support, Non-U.S. Gov't]. *J Am Coll Cardiol, 43*(12), 2348-2358. doi: 10.1016/j.jacc.2004.01.047

Landry, D. W., & Oliver, J. A. (2001). The pathogenesis of vasodilatory shock. [Review]. *N Engl J Med, 345*(8), 588-595. doi: 10.1056/NEJMra002709

Lanone, S. *e. a.* (2002). Inducible NO synthase (NOS2) expressed in septic patients is nitrated on selected tyrosine residues: implications for enzymic activity. *Biochem. J, **366**, 399-404*

Latifi SQ, O. R. M., Levine AD. (2002). Interleukin-10 controls the onset of irreversible septic shock. *Infect Immun, 70(8):4441-6.*

Lazaridou, A., Biliaderis, C. G., Bacandritsos, N., & Sabatini, A. G. (2004). Composition, thermal and rheological behaviour of selected Greek Honeys. *Journal of Food Engineering, 64*, 9-21.

Li, W., Ashok, M., Li, J., Yang, H., Sama, A. E., & Wang, H. (2007). A major ingredient of green tea rescues mice from lethal sepsis partly by inhibiting HMGB1. [Research Support, N.I.H., Extramural]. *PLoS One, 2*(11), e1153. doi: 10.1371/journal.pone.0001153

Li, Y., Sun, J. F., Cui, X., Mani, H., Danner, R. L., Li, X., . . . Eichacker, P. Q. (2011). The effect of heparin administration in animal models of sepsis: a prospective study in Escherichia coli-challenged mice and a systematic review and metaregression analysis of published studies. [Comparative Study Meta-Analysis Research Support, N.I.H., Intramural Review]. *Crit Care Med, 39*(5), 1104-1112. doi: 10.1097/CCM.0b013e31820eb718

Lin, C. M., Shyu, K. G., Wang, B. W., Chang, H., Chen, Y. H., & Chiu, J. H. (2010). Chrysin suppresses IL-6-induced angiogenesis via down-regulation of JAK1/STAT3 and VEGF: an in vitro and in ovo approach. [In Vitro Research Support, Non-U.S. Gov't]. *J Agric Food Chem, 58*(11), 7082-7087. doi: 10.1021/jf100421w

Liu, J., Li, X., Yue, Y., Li, J., He, T., & He, Y. (2005). The inhibitory effect of quercetin on IL-6 production by LPS-stimulated neutrophils. *Cell Mol Immunol, 2*(6), 455-460.

Luce, J. M. (1998). Acute lung injury and the acute respiratory distress syndrome. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review]. *Crit Care Med, 26*(2), 369-376.

Luo, T. H., Wang, Y., Lu, Z. M., Zhou, H., Xue, X. C., Bi, J. W., . . . Fang, G. E. (2009). The change and effect of endothelial progenitor cells in pig with multiple organ dysfunction syndromes. [Research Support, Non-U.S. Gov't]. *Crit Care, 13*(4), R118. doi: 10.1186/cc7968

MacGarvey, N. C., Suliman, H. B., Bartz, R. R., Fu, P., Withers, C. M., Welty-Wolf, K. E., & Piantadosi, C. A. (2012). Activation of mitochondrial biogenesis by heme oxygenase-1-mediated NF-E2-related factor-2 induction rescues mice from lethal Staphylococcus aureus sepsis. [Comparative Study Research Support, N.I.H., Extramural]. *Am J Respir Crit Care Med, 185*(8), 851-861. doi: 10.1164/rccm.201106-1152OC

Martos, I., F. Ferreres, L.H. Yao, B.R. D'Arcy, N. Caffin, F.A. Tomás-Barberán. (2000b). Flavonoids in monospecific Eucalyptus honeys from Australia. *J. of Agricultural and Food Chemistry, 48(10): 4744-4748.*

Martos, I., M. Cossentini, F. Ferreres, F.A. Tomás-Barberán. ( 1997). Flavonoid composition of Tunisian honeys and propolis. . *J. of Agricultural and Food Chemistry, 45(8): 2824-2829.*

Matsuda N, H. Y. (2006). Systemic inflammatory response syndrome (SIRS): molecular pathophysiology and gene therapy. *J Pharmacol Sci, 101(3):189-98.*

Memon, R. A., Grunfeld, C., Moser, A. H., & Feingold, K. R. (1993). Tumor necrosis factor mediates the effects of endotoxin on cholesterol and triglyceride metabolism in mice. *Endocrinology, 132*(5), 2246-2253.

Merrill, J. C., You, J., Constable, C., Leeman, S. E., & Amar, S. (2011). Whole-body deletion of LPS-induced TNF-alpha factor (LITAF) markedly improves experimental endotoxic shock and inflammatory arthritis. [Research Support, N.I.H., Extramural]. *Proc Natl Acad Sci U S A, 108*(52), 21247-21252. doi: 10.1073/pnas.1111492108

Middleton, E., Jr., Kandaswami, C., & Theoharides, T. C. (2000). The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. [Research Support, Non-U.S. Gov't Review]. *Pharmacol Rev, 52*(4), 673-751.

Milam, J. E., Erb-Downward, J. R., Chen, G. H., Osuchowski, M. F., McDonald, R., Chensue, S. W., . . . Olszewski, M. A. (2010). CD11c+ cells are required to prevent progression from local acute lung injury to multiple organ failure and death. [Research Support, N.I.H., Extramural Research Support, U.S. Gov't, Non-P.H.S.]. *Am J Pathol, 176*(1), 218-226. doi: 10.2353/ajpath.2010.081027

Mira, J. P., Cariou, A., Grall, F., Delclaux, C., Losser, M. R., Heshmati, F., . . . Dhainaut, J. F. (1999). Association of TNF2, a TNF-alpha promoter polymorphism, with septic shock susceptibility and mortality: a multicenter study. [Multicenter Study Research Support, Non-U.S. Gov't]. *JAMA, 282*(6), 561-568.

Misko, T. P., Moore, W. M., Kasten, T. P., Nickols, G. A., Corbett, J. A., Tilton, R. G., . . . Currie, M. G. (1993). Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. *Eur J Pharmacol, 233*(1), 119-125.

Mittal, R., Gonzalez-Gomez, I., Panigrahy, A., Goth, K., Bonnet, R., & Prasadarao, N. V. (2010). IL-10 administration reduces PGE-2 levels and promotes CR3-mediated clearance of Escherichia coli K1 by phagocytes in meningitis. [Research Support, N.I.H., Extramural]. *J Exp Med, 207*(6), 1307-1319. doi: 10.1084/jem.20092265

Molan, P. C. (2001). The potential of honey to promote oral wellness. *Gen Dent, 49*(6), 584-589.

Murta, V., & Ferrari, C. C. (2012). Influence of Peripheral inflammation on the progression of multiple sclerosis: Evidence from the clinic and experimental animal models. *Mol Cell Neurosci*. doi: 10.1016/j.mcn.2012.06.004

Nijsten, M. W., Hack, C. E., Helle, M., ten Duis, H. J., Klasen, H. J., & Aarden, L. A. (1991). Interleukin-6 and its relation to the humoral immune response and clinical parameters in burned patients. *Surgery, 109*(6), 761-767.

Nikota, J. K., & Stampfli, M. R. (2012). Cigarette smoke-induced inflammation and respiratory host defense: Insights from animal models. *Pulm Pharmacol Ther, 25*(4), 257-262. doi: 10.1016/j.pupt.2012.05.005

Nin, N., Cassina, A., Boggia, J., Alfonso, E., Botti, H., Peluffo, G., . . . Hurtado, F. J. (2004). Septic diaphragmatic dysfunction is prevented by Mn(III)porphyrin therapy and inducible nitric oxide synthase inhibition. [Research Support, Non-U.S. Gov't]. *Intensive Care Med, 30*(12), 2271-2278. doi: 10.1007/s00134-004-2427-x

Nin, N., El-Assar, M., Sanchez, C., Ferruelo, A., Sanchez-Ferrer, A., Martinez-Caro, L., . . . Lorente, J. A. (2011). Vascular dysfunction in sepsis: effects of the peroxynitrite decomposition catalyst MnTMPyP. [Research Support, Non-U.S. Gov't]. *Shock, 36*(2), 156-161. doi: 10.1097/SHK.0b013e31821e50de

Noiri, E., Nakao, A., Uchida, K., Tsukahara, H., Ohno, M., Fujita, T., . . . Goligorsky, M. S. (2001). Oxidative and nitrosative stress in acute renal ischemia. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. *Am J Physiol Renal Physiol, 281*(5), F948-957.

Novelli, G. P. (1997). Role of free radicals in septic shock. *J Physiol Pharmacol, 48*(4), 517-527.

Nuttall, S. L., Kendall, M. J., & Martin, U. (1999). Antioxidant therapy for the prevention of cardiovascular disease. [Review]. *QJM, 92*(5), 239-244.

O'Banion, M. K., Winn, V. D., & Young, D. A. (1992). cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. *Proc Natl Acad Sci U S A, 89*(11), 4888-4892.

O'Sullivan, S. T., J. A. Lederer, A. F. Horgan, D. H. L. Chin, J. A. Mannick, and M. L. Roderick. (1995). Major injury leads to predominance of the T helper-2 lymphocyte phenotype and diminished interleukin-12 production associated with decreased resistance. *Ann. Surg, 4:482.*

Ocuin, L. M., Bamboat, Z. M., Balachandran, V. P., Cavnar, M. J., Obaid, H., Plitas, G., & DeMatteo, R. P. (2011). Neutrophil IL-10 suppresses peritoneal inflammatory monocytes during polymicrobial sepsis. [Research Support, N.I.H., Extramural]. *J Leukoc Biol, 89*(3), 423-432. doi: 10.1189/jlb.0810479

Ogata, M., Matsui, T., Kita, T., & Shigematsu, A. (1999). Carrageenan primes leukocytes to enhance lipopolysaccharide-induced tumor necrosis factor alpha production. [Research Support, Non-U.S. Gov't]. *Infect Immun, 67*(7), 3284-3289.

Ogino, H., Yamada, K., Yuhara, M., Tsuchida, S., Maezawa, K., Kizu, J., & Hori, S. (2009). Fluoroquinolones reduce carrageenan-induced edema in rats and the involvement of the glucocorticoid receptor system. *J Infect Chemother, 15*(2), 113-117. doi: 10.1007/s10156-009-0675-y

Orman, M. A., Ierapetritou, M. G., Berthiaume, F., & Androulakis, I. P. (2011). The dynamics of the early inflammatory response in double-hit burn and sepsis animal models. [Research Support, N.I.H., Extramural]. *Cytokine, 56*(2), 494-502. doi: 10.1016/j.cyto.2011.07.001

Orman, M. A., Ierapetritou, M. G., Berthiaume, F., & Androulakis, I. P. (2012). Long-term dynamic profiling of inflammatory mediators in double-hit burn and sepsis animal models. [Research Support, N.I.H., Extramural]. *Cytokine, 58*(2), 307-315. doi: 10.1016/j.cyto.2012.01.017

Oswald, I., T. Wynn, A. Sher (1992). Interleukin 10 inhibits macrophage microbicidal activity by blocking endogenous production of tumor necrosis factor α required as a costimulatory factor for interferon-γ-induced activation. *Proc. Natl. Acad. Sci.*

Panda, S. K., Kumar, S., Tupperwar, N. C., Vaidya, T., George, A., Rath, S., . . . Ravindran, B. (2012). Chitohexaose activates macrophages by alternate pathway through TLR4 and blocks endotoxemia. [Research Support, Non-U.S. Gov't]. *PLoS Pathog, 8*(5), e1002717. doi: 10.1371/journal.ppat.1002717

Pannala, A. S., Razaq, R., Halliwell, B., Singh, S., & Rice-Evans, C. A. (1998). Inhibition of peroxynitrite dependent tyrosine nitration by hydroxycinnamates: nitration or electron donation? [Research Support, Non-U.S. Gov't]. *Free Radic Biol Med, 24*(4), 594-606.

Parker, A. J., Arango, M., Abderrahmane, S., Lambert, E., Tourette, C., Catoire, H., & Neri, C. (2005). [Resveratrol rescues mutant polyglutamine cytotoxicity in nematode and mammalian neurons]. [News]. *Med Sci (Paris), 21*(5), 556-557. doi: 10.1051/medsci/2005215556

Parker, s., J., & Watkins, P., E. (2001). Experimental models of gram-negative sepsis. [Review]. *Br J Surg, 88*(1), 22-30. doi: 10.1046/j.1365-2168.2001.01632.x

Phillipson, M., & Kubes, P. (2011). The neutrophil in vascular inflammation. [Research Support, Non-U.S. Gov't Review]. *Nat Med, 17*(11), 1381-1390. doi: 10.1038/nm.2514

Pils, M. C., Pisano, F., Fasnacht, N., Heinrich, J. M., Groebe, L., Schippers, A., . . . Muller, W. (2010). Monocytes/macrophages and/or neutrophils are the target of IL-10 in the LPS endotoxemia model. [Research Support, Non-U.S. Gov't]. *Eur J Immunol, 40*(2), 443-448. doi: 10.1002/eji.200939592

Ploner, F., Radermacher, P., Theisen, M., Tugtekin, I. F., Matejovic, M., Stehr, A., . . . Trager, K. (2001). Effects of combined selective iNOS inhibition and peroxynitrite blockade during endotoxemia in pigs. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S.]. *Shock, 16*(2), 130-136.

Postmes, T. J., M.M.C. Bosch, R. Dutrieux, J. van Baare, M.J. Hoekstra. (1997). Speeding up the healing of burns with honey. In A. Mizrahi & Y. Lensky (eds). *Bee Products: Properties, applications and apitherapy. New York: Plenum Press, 27-37.*

Qiu, P., Cui, X., Barochia, A., Li, Y., Natanson, C., & Eichacker, P. Q. (2011). The evolving experience with therapeutic TNF inhibition in sepsis: considering the potential influence of risk of death. [Research Support, N.I.H., Intramural Review]. *Expert Opin Investig Drugs, 20*(11), 1555-1564. doi: 10.1517/13543784.2011.623125

Qiu, Z. L., Ye, Y. P., & Zhang, N. (2012). [Clinical efficacy of shenfu injection in treating severe sepsis and its effects on serum levels of interleukin-6 and interleukin-10]. *Zhongguo Zhong Xi Yi Jie He Za Zhi, 32*(3), 348-351.

Razavi, H. M., Wang le, F., Weicker, S., Rohan, M., Law, C., McCormack, D. G., & Mehta, S. (2004). Pulmonary neutrophil infiltration in murine sepsis: role of inducible nitric oxide synthase. [Research Support, Non-U.S. Gov't]. *Am J Respir Crit Care Med, 170*(3), 227-233. doi: 10.1164/rccm.200306-846OC

Rhee, S. G., Chae, H. Z., & Kim, K. (2005). Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. [Review]. *Free Radic Biol Med, 38*(12), 1543-1552. doi: 10.1016/j.freeradbiomed.2005.02.026

Riedemann, N. C., Guo, R. F., & Ward, P. A. (2003). The enigma of sepsis. [Research Support, Non-U.S. Gov't Review]. *J Clin Invest, 112*(4), 460-467. doi: 10.1172/JCI19523

Risco, A., Del Fresno, C., Mambol, A., Alsina-Beauchamp, D., Mackenzie, K. F., Yang, H. T., . . . Cuenda, A. (2012). p38gamma and p38delta kinases regulate the Toll-like receptor 4 (TLR4)-induced cytokine production by controlling ERK1/2 protein kinase pathway activation. *Proc Natl Acad Sci U S A, 109*(28), 11200-11205. doi: 10.1073/pnas.1207290109

Rizelio, V. M., Tenfen, L., da Silveira, R., Gonzaga, L. V., Costa, A. C., & Fett, R. (2012). Development of a fast capillary electrophoresis method for determination of carbohydrates in honey samples. [Research Support, Non-U.S. Gov't]. *Talanta, 93*, 62-66. doi: 10.1016/j.talanta.2012.01.034

RJ, G. (1996). MODS/SIRS: result of an overwhelming inflammatory response? . *World J Surg, 20(4):418-21.*

Robinson, K. M., Morre, J. T. & Beckman, J. S. Triuret. (2004). a novel product of peroxynitrite-mediated oxidation of urate. *Arch. Biochem. Biophys, 423, 213-217.*

Rolph, P., I. Nakainz, A. Sampson-Johannes, S. Fong, D. Lowe, H. Min, . (1992). IL-10, T lymphocyte inhibitor of human blood cell production of IL-10. *J. Immunol, 148:808.*

Romay, C., Ledon, N., & Gonzalez, R. (1998). Further studies on anti-inflammatory activity of phycocyanin in some animal models of inflammation. *Inflamm Res, 47*(8), 334-338.

Romier, B., Van De Walle, J., During, A., Larondelle, Y., & Schneider, Y. J. (2008). Modulation of signalling nuclear factor-kappaB activation pathway by polyphenols in human intestinal Caco-2 cells. *Br J Nutr, 100*(3), 542-551. doi: S0007114508966666 [pii] 10.1017/S0007114508966666

Ryter, S. W., Alam, J., & Choi, A. M. (2006). Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov' Review]. *Physiol Rev, 86*(2), 583-650. doi: 10.1152/physrev.00011.2005

S.E, E. (1993). Recent advances in the management of Fournier's gangrene: preliminary observations. *Surgery, 113: 200-204.*

Sabatier, S., M.J. Amiot, M. Tacchini, S. Aubert. ( 1992a). Identification of flavonoids in sunflower honey. *J. of Food Science, 57(3): 773-775.*

Sabatier, S., M.J. Amiot, M. Tacchini, S. Aubert. ( 1992b). Identification of flavonoids in sunflower honey. *J. of Food Science 57(3): 773-775.*

Sadowitz, B., Roy, S., Gatto, L. A., Habashi, N., & Nieman, G. (2011). Lung injury induced by sepsis: lessons learned from large animal models and future directions for treatment. [Review]. *Expert Rev Anti Infect Ther, 9*(12), 1169-1178. doi: 10.1586/eri.11.141

Salem, S. N. (1982). Honey regimen in gastrointestinal disorders. *Bulletin. of Islamic Medicine, 2(5): 422-425.*

Sanz S., G. G., Jiemeno F., Perez C., Juan T (1994). Fermentation Problem in Spanish North-Coast Honey. *Journal of Food Protection, 58(5), 515-518.*

Scapagnini, G., Foresti, R., Calabrese, V., Giuffrida Stella, A. M., Green, C. J., & Motterlini, R. (2002). Caffeic acid phenethyl ester and curcumin: a novel class of heme oxygenase-1 inducers. [Research Support, Non-U.S. Gov't]. *Mol Pharmacol, 61*(3), 554-561.

Scott, G. S., Cuzzocrea, S., Genovese, T., Koprowski, H., & Hooper, D. C. (2005). Uric acid protects against secondary damage after spinal cord injury. [Research Support, Non-U.S. Gov't]. *Proc Natl Acad Sci U S A, 102*(9), 3483-3488. doi: 10.1073/pnas.0500307102

Scott, G. S. *e. a.* (2002). Therapeutic intervention in experimental allergic encephalomyelitis by administration of uric acid precursors. *Proc. Natl Acad. Sci. USA, 99, 16303-16308.*

Scott, G. S. *e. a.* (2005). Uric acid protects against secondary damage after spinal cord injury. *Proc. Natl Acad. Sci.USA 102, 3483-3488*

Seija, M., Baccino, C., Nin, N., Sanchez-Rodriguez, C., Granados, R., Ferruelo, A., . . . Lorente, J. A. (2012). Role of Peroxynitrite in Sepsis- Induced Acute Kidney Injury in an Experimental Model of Sepsis in Rats. *Shock.* doi: 10.1097/SHK.0b013e31826660f2

Sereia, M. J., Alves, E. M., Toledo, V. A., Marchini, L. C., Serine, E. S., Faquinello, P., . . . Moreti, A. C. (2011). Physicochemical characteristics and pollen spectra of organic and non-organic honey samples of Apis mellifera L. [Research Support, Non-U.S. Gov't]. *An Acad Bras Cienc, 83*(3), 1077-1090.

Seth, A. K., Geringer, M. R., Gurjala, A. N., Abercrombie, J. A., Chen, P., You, T., . . . Leung, K. P. (2012). Understanding the host inflammatory response to wound infection: an in vivo study of Klebsiella pneumoniae in a rabbit ear wound model. [Research Support, U.S. Gov't, Non-P.H.S.]. *Wound Repair Regen, 20*(2), 214-225. doi: 10.1111/j.1524-475X.2012.00764.x

Setoguchi, D., Yatsuki, H., Sadahiro, T., Nakamura, M., Hirayama, Y., Watanabe, E., . . . Oda, S. (2012). Effects of a peripheral cholinesterase inhibitor on cytokine production and autonomic nervous activity in a rat model of sepsis. [Research Support, Non-U.S. Gov't]. *Cytokine, 57*(2), 238-244. doi: 10.1016/j.cyto.2011.11.003

Sgambato, A., Ardito, R., Faraglia, B., Boninsegna, A., Wolf, F. I., & Cittadini, A. (2001). Resveratrol, a natural phenolic compound, inhibits cell proliferation and prevents oxidative DNA damage. [Research Support, Non-U.S. Gov't]. *Mutat Res, 496*(1-2), 171-180.

Shacka, J. J. *e. a.* (2006). Two distinct signaling pathways regulate peroxynitrite-induced apoptosis in PC12 cells. *Cell Death Differ, 13, 1506-1514*

Shah, T. J., Leik, C. E., & Walsh, S. W. (2010). Neutrophil infiltration and systemic vascular inflammation in obese women. [Research Support, N.I.H., Extramural]. *Reprod Sci, 17*(2), 116-124. doi: 10.1177/1933719109348252

Shahavi, V. M., & Desai, S. K. (2008). Anti-inflammatory activity of Butea monosperma flowers. *Fitoterapia, 79*(2), 82-85. doi: 10.1016/j.fitote.2007.06.014

Shahidi, F. a. W., P.K.J.P.D. (1992). Phenolic antioxidants. *CRC Crit. Rev. Food Sci. Nutr, 32,67-103.*

Shibuya, E. K., Morris, J., Rapp, U.R., and Ruderman, J.V. (1996). Activation of the Xenopus oocyte MAP kinase pathway is independent of Raf. *Cell Growth & Differen, 7: 235-241.*

Skoutelis, A. T., Kaleridis, V., Athanassiou, G. M., Kokkinis, K. I., Missirlis, Y. F., & Bassaris, H. P. (2000). Neutrophil deformability in patients with sepsis, septic shock, and adult respiratory distress syndrome. [Comparative Study Research Support, Non-U.S. Gov't]. *Crit Care Med, 28*(7), 2355-2359.

Song, Y. S., Park, E. H., Hur, G. M., Ryu, Y. S., Lee, Y. S., Lee, J. Y., . . . Jin, C. (2002). Caffeic acid phenethyl ester inhibits nitric oxide synthase gene expression and enzyme activity. [Research Support, Non-U.S. Gov't]. *Cancer Lett, 175*(1), 53-61.

Soriano, F. G., Lorigados, C. B., Pacher, P., & Szabo, C. (2011). Effects of a potent peroxynitrite decomposition catalyst in murine models of endotoxemia and sepsis. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. *Shock, 35*(6), 560-566. doi: 10.1097/SHK.0b013e31820fe5d5

Spitsin, S. V., Scott, G. S., Kean, R. B., Mikheeva, T., & Hooper, D. C. (2000). Protection of myelin basic protein immunized mice from free-radical mediated inflammatory cell invasion of the central nervous system by the natural peroxynitrite scavenger uric acid. [Research Support, Non-U.S. Gov't]. *Neurosci Lett, 292*(2), 137-141.

Standiford, T. J., R. M. Strieter, N. W. Lukacs, and S. L. Kunkel. (1995). Neutralization of IL-10 increases lethality in endotoxemia: cooperative effects of macrophage inflammatory protein-2 and tumor necrosis factor. *J. Immunol, 155:2222*.

Steinhauser, M. L., Hogaboam, C. M., Kunkel, S. L., Lukacs, N. W., Strieter, R. M., & Standiford, T. J. (1999). IL-10 is a major mediator of sepsis-induced impairment in lung antibacterial host defense. [Research Support, U.S. Gov't, P.H.S.]. *J Immunol, 162*(1), 392-399.

Subrahmanyam, M. (1996). Honey dressing versus boiled potato peel in the treatment of burns: a prospective randomised study. *Burns, 22(6): 491-493*.

Subrahmanyam, M. (1998). A prospective randomised clinical and histological study of superficial burn wound healing with honey and silver sulfadiazine. *Burns, 24: 157-161*.

Szabo, C. (2003). Multiple pathways of peroxynitrite cytotoxicity. [Review]. *Toxicol Lett, 140-141*, 105-112.

Szabo, C., Ferrer-Sueta, G., Zingarelli, B., Southan, G. J., Salzman, A. L., & Radi, R. (1997). Mercaptoethylguanidine and guanidine inhibitors of nitric-oxide synthase react with peroxynitrite and protect against peroxynitrite-induced oxidative damage. [Research Support, Non-U.S. Gov't]. *J Biol Chem, 272*(14), 9030-9036.

Szabo, C., Ischiropoulos, H., & Radi, R. (2007). Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Review]. *Nat Rev Drug Discov, 6*(8), 662-680. doi: 10.1038/nrd2222

Tabuchi, Y., Sugiyama, N., Horiuchi, T., Furusawa, M., & Furuhama, K. (1995). Ebselen, a seleno-organic compound, protects against ethanol-induced murine gastric mucosal injury in both in vivo and in vitro systems. *European Journal of Pharmacology, 272*(2-3), 195-201.

Takaesu, G., Kishida, S., Hiyama, A., Yamaguchi, K., Shibuya, H., Irie, K., . . . Matsumoto, K. (2000). TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. [Research Support, Non-U.S. Gov't]. *Mol Cell, 5*(4), 649-658.

Takamiya, R., Hung, C. C., Hall, S. R., Fukunaga, K., Nagaishi, T., Maeno, T., . . . Perrella, M. A. (2009). High-mobility group box 1 contributes to lethality of endotoxemia in heme oxygenase-1-deficient mice. *Am J Respir Cell Mol Biol, 41*(2), 129-135. doi: 2008-0331OC [pii] 10.1165/rcmb.2008-0331OC

Tate, R. M., & Repine, J. E. (1983). Neutrophils and the adult respiratory distress syndrome. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review]. *Am Rev Respir Dis, 128*(3), 552-559.

Taylor, P. R., Li, B., Dawsey, S. M., Li, J. Y., Yang, C. S., Guo, W., & Blot, W. J. (1994). Prevention of esophageal cancer: the nutrition intervention trials in Linxian, China. Linxian Nutrition Intervention Trials Study Group. [Clinical Trial Comparative Study Randomized Controlled Trial]. *Cancer Res, 54*(7 Suppl), 2029s-2031s.

Tazawa, S., T. Warashina, T. Noro. (1999). Studies on the constituents of Brazilian propolis. II. Chem. Pharm. *Bull, 47(10): 1388-1392*.

Terra, X., Valls, J., Vitrac, X., Merrillon, J. M., Arola, L., Ardevol, A., . . . Blay, M. (2007). Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. [Research Support, Non-U.S. Gov't]. *J Agric Food Chem, 55*(11), 4357-4365. doi: 10.1021/jf0633185

Tomás-Barberán, F. A., F. Tomás-Lorente, F. Ferreres, and C. Garcia-Viguera. (1989). Flavonoids as biochemical markers of the plant origin of bee pollen. *J. Sci. Food Agric, 47: 337-340*.

Tomás-Barberán, F. A., Ferreres, F., Garcia-Viguera, C. & Tomás-Lorente, F (1993a). Flavonoids in honey of different geographical origin. *Z. Lebensm. Unters. Forsch, 196, 38-44*.

Tomás-Barberán, F. A., I. Martos, F. Ferreres, B.S. Radovic and E. Anklam. (2001). HPLC flavonoid profiles as markers for the botanical origin of European unifloral honeys. *J. of Science, Food and Agriculture 81(5): 485-496*.

Tonks, A. J., Cooper, R. A., Jones, K. P., Blair, S., Parton, J., & Tonks, A. (2003). Honey stimulates inflammatory cytokine production from monocytes. *Cytokine, 21*(5), 242-247.

Trujillo, M., Budde, H., Pineyro, M. D., Stehr, M., Robello, C., Flohe, L., & Radi, R. (2004). Trypanosoma brucei and Trypanosoma cruzi tryparedoxin peroxidases catalytically detoxify peroxynitrite via oxidation of fast reacting thiols. [Research Support, Non-U.S. Gov't]. *J Biol Chem, 279*(33), 34175-34182. doi: 10.1074/jbc.M404317200

Tsuchiya, Y., Nozu, T., Kumei, S., Ohhira, M., & Okumura, T. (2012). IL-1 Receptor Antagonist Blocks the Lipopolysaccharide-Induced Inhibition of Gastric Motility in Freely Moving Conscious Rats. *Dig Dis Sci*. doi: 10.1007/s10620-012-2210-8

Tweedie, D., Ferguson, R. A., Fishman, K., Frankola, K. A., Van Praag, H., Holloway, H. W., . . . Rosi, S. (2012). Tumor necrosis factor-alpha synthesis inhibitor 3,6'-dithiothalidomide attenuates markers of inflammation, Alzheimer pathology and behavioral deficits in animal models of neuroinflammation and Alzheimer's disease. *J Neuroinflammation, 9*, 106. doi: 10.1186/1742-2094-9-106

Uhlig, H. (1998). Application of technical enzyme preparations. In *Industrial enzymes and their applications*. *New York: John Willey and Sons Inc.Vanderbilt Medical Centre. Infant Botulism*.

Underhill, D. M., Ozinsky, A., Hajjar, A.M., Stevens, A., Wilson, C.B., Bassetti, M., and Aderem, A (1999). The Tolllike receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature, **401**: 811-815*.

Urbonas, V., Eidukaite, A., & Tamuliene, I. (2012). Increased interleukin-10 levels correlate with bacteremia and sepsis in febrile neutropenia pediatric oncology patients. [Research Support, Non-U.S. Gov't]. *Cytokine, 57*(3), 313-315. doi: 10.1016/j.cyto.2011.11.012

Van Amersfoort, E. S., Van Berkel, T. J., & Kuiper, J. (2003). Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. [Review]. *Clin Microbiol Rev, 16*(3), 379-414.

Vandevyver, S., Dejager, L., Van Bogaert, T., Kleyman, A., Liu, Y., Tuckermann, J., & Libert, C. (2012). Glucocorticoid receptor dimerization induces MKP1 to protect against TNF-induced inflammation. [Research Support, Non-U.S. Gov't]. *J Clin Invest, 122*(6), 2130-2140. doi: 10.1172/JCI60006

Vincent, J. L., Sakr, Y., & Ranieri, V. M. (2003). Epidemiology and outcome of acute respiratory failure in intensive care unit patients. [Review]. *Crit Care Med, 31*(4 Suppl), S296-299. doi: 10.1097/01.CCM.0000057906.89552.8F

Wang H, B. O., Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ. . (1999). HMG-1 as a late mediator of endotoxin lethality in mice. *Science, 285: 248-251*.

Wang, H., O. Bloom, M. Zhang, J.M. Vishnubhakat, M. Ombrellino, J. Che, A. Frazier, H. Yang, S. Ivanova, L. Borovikova, et al. (1999). HMG-1 as a late mediator of endotoxin lethality in mice. *Science, 285:248-251*.

Wang, L., Taneja, R., Razavi, H. M., Law, C., Gillis, C., & Mehta, S. (2012). Specific role of neutrophil inducible nitric oxide synthase in murine sepsis-induced lung injury in vivo. [Research Support, Non-U.S. Gov't]. *Shock, 37*(5), 539-547. doi: 10.1097/SHK.0b013e31824dcb5a

Wang, W., Jittikanont, S., Falk, S. A., Li, P., Feng, L., Gengaro, P. E., . . . Schrier, R. W. (2003). Interaction among nitric oxide, reactive oxygen species, and antioxidants during endotoxemia-related acute renal failure. [Research Support, U.S. Gov't, P.H.S.]. *Am J Physiol Renal Physiol, 284*(3), F532-537. doi: 10.1152/ajprenal.00323.2002

Wellings, R. P., Corder, R., & Vane, J. R. (1995). Lack of effect of ET antibody or SB 209670 on endotoxin-induced renal failure. *J Cardiovasc Pharmacol, 26 Suppl 3*, S476-478.

Weston, R. J. (2000). the contribution of catalase and other natural products to the antibacterial activity of honey: a review. *Food Chemistry, 71(2): 235-239*.

Weston, R. J., & Brocklebank, L. K. (1999). The oligosaccharide composition of some New Zealand honeys. *Food Chemistry, Volume 64*(Issue 1), 33-37.

Wheeler-Aceto, H., Porreca, F., & Cowan, A. (1990). The rat paw formalin test: comparison of noxious agents. [Research Support, U.S. Gov't, P.H.S.]. *Pain, 40*(2), 229-238.

Wheeler, D. S., Catravas, J. D., Odoms, K., Denenberg, A., Malhotra, V., & Wong, H. R. (2004). Epigallocatechin-3-gallate, a green tea-derived polyphenol, inhibits IL-1 beta-dependent proinflammatory signal transduction in cultured respiratory epithelial cells. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. *J Nutr, 134*(5), 1039-1044.

White, J. W. (1978a). Honey. *Advances in Food Research, 24: 288-375.*

White, J. W. (1978b). Honey. *Advances in Food Research, 24: 288-375.*

White, J. W., M.H. Subers, and A.I. Schepartz (1963). The identification of inhibine: the antibacterial factor in honey as hydrogen peroxidese and its origin in a honey glucose oxidase system. *Biochem. Biophys Act, 73: 57-70.*

White, J. W. J. (1980). Detection of Honey Adulteration by Carbohydrate Analysis JAOAC. *63(1):11-18.*

Whiteman, M., Ketsawatsakul, U. & Halliwell, B (2002). Areassessment of the peroxynitrite scavenging activity of uric acid. *Ann. N. Y. Acad. Sci, 962, 242-259*

Whittle, B. A. (1964). The Use of Changes in Capillary Permeability in Mice to Distinguish between Narcotic and Nonnarcotic Alalgesics. *Br J Pharmacol Chemother, 22*, 246-253.

Wilson, P. G., Manji, M., & Neoptolemos, J. P. (1998). Acute pancreatitis as a model of sepsis. [Review]. *J Antimicrob Chemother, 41 Suppl A*, 51-63.

Wink, D. A., Miranda, K. M., Espey, M. G., Pluta, R. M., Hewett, S. J., Colton, C., . . . Grisham, M. B. (2001). Mechanisms of the antioxidant effects of nitric oxide. [Review]. *Antioxid Redox Signal, 3*(2), 203-213.

Woo, K. J., Jeong, Y. J., Inoue, H., Park, J. W., & Kwon, T. K. (2005). Chrysin suppresses lipopolysaccharide-induced cyclooxygenase-2 expression through the inhibition of nuclear factor for IL-6 (NF-IL6) DNA-binding activity. [Research Support, Non-U.S. Gov't]. *FEBS Lett, 579*(3), 705-711. doi: 10.1016/j.febslet.2004.12.048

Wu, H. M., Jin, M., & Marsh, C. B. (2005). Toward functional proteomics of alveolar macrophages. [Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S Review]. *Am J Physiol Lung Cell Mol Physiol, 288*(4), L585-595. doi: 10.1152/ajplung.00305.2004

Yamanishi, Y., Takahashi, M., Izawa, K., Isobe, M., Ito, S., Tsuchiya, A., . . . Kitaura, J. (2012). A Soluble Form of LMIR5/CD300b Amplifies Lipopolysaccharide-Induced Lethal Inflammation in Sepsis. *J Immunol, 189*(4), 1773-1779. doi: 10.4049/jimmunol.1201139

Yang, C. S., & Landau, J. M. (2000). Effects of tea consumption on nutrition and health. [Review]. *J Nutr, 130*(10), 2409-2412.

Yang, H., M. Ochani, J. Li, X. Qiang, M. Tanovic, H.E. Harris, S.M. Susarla, L. Ulloa, H.C. Wang, R. DiRaimo, et al. (2004). Reversing established sepsis with antagonists of endogenous HMGB1. *Proc. Natl. Acad. Sci. USA, 101:296-301.*

Yang, M. H., Yoon, K. D., Chin, Y. W., Park, J. H., & Kim, J. (2009). Phenolic compounds with radical scavenging and cyclooxygenase-2 (COX-2) inhibitory activities from Dioscorea opposita. [Research Support, Non-U.S. Gov't]. *Bioorg Med Chem, 17*(7), 2689-2694. doi: 10.1016/j.bmc.2009.02.057

Ye, H., Arron, J. R., Lamothe, B., Cirilli, M., Kobayashi, T., Shevde, N. K., Segal, D., Dzivenu, O. K., Vologodskaia, M., Yim, M. et al (2002). Distinct molecular mechanism for initiating TRAF6 signalling. *Nature, **418**, 443-447.*

Yoon, S. H. (2012). Concerns of the anesthesiologist: anesthetic induction in severe sepsis or septic shock patients. *Korean J Anesthesiol, 63*(1), 3-10. doi: 10.4097/kjae.2012.63.1.3

Yoza, B. K., & McCall, C. E. (2011). Facultative heterochromatin formation at the IL-1 beta promoter in LPS tolerance and sepsis. [Research Support, N.I.H., Extramural]. *Cytokine, 53*(2), 145-152. doi: 10.1016/j.cyto.2010.10.007

Yu, J. B., & Yao, S. L. (2008). Protective effects of hemin pretreatment combined with ulinastatin on septic shock in rats. [Research Support, Non-U.S. Gov't]. *Chin Med J (Engl), 121*(1), 49-55.

Yu, Y. M., Wang, Z. H., Liu, C. H., & Chen, C. S. (2007). Ellagic acid inhibits IL-1beta-induced cell adhesion molecule expression in human umbilical vein endothelial cells. [Research Support, Non-U.S. Gov't]. *Br J Nutr, 97*(4), 692-698. doi: 10.1017/S0007114507666409

Zhang, X., Chen, J., Graham, S. H., Du, L., Kochanek, P. M., Draviam, R., . . . Clark, R. S. (2002). Intranuclear localization of apoptosis-inducing factor (AIF) and large scale DNA fragmentation after traumatic brain injury in rats and in neuronal cultures exposed to peroxynitrite. [Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S.]. *J Neurochem, 82*(1), 181-191.

Zhang, X. *e. a.* (2002). Intranuclear localization of apoptosisinducing factor (AIF) and large scale DNA fragmentation after traumatic brain injury in rats and in neuronal cultures exposed to peroxynitrite. *J. Neurochem, **82**, 181-191*

Zhong, M., Cheng, G. F., Wang, W. J., Guo, Y., Zhu, X. Y., & Zhang, J. T. (1999). Inhibitory effect of resveratrol on interleukin 6 release by stimulated peritoneal macrophages of mice. *Phytomedicine, 6*(2), 79-84.

Zouki, C., Jozsef, L., Ouellet, S., Paquette, Y. & Filep,J. G. (2001). Peroxynitrite mediates cytokine-induced *IL-8* gene expression and production by human leukocytes. *J. Leukoc. Biol, **69**, 815-824.*
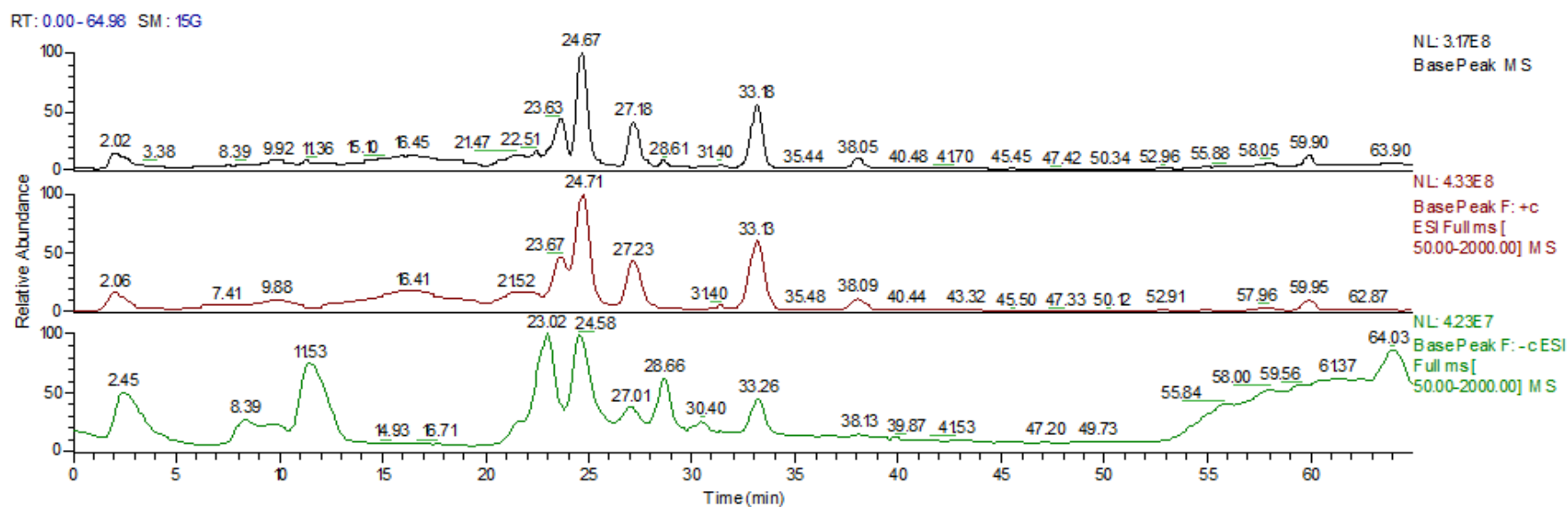
## APPENDIX:

**Appendix A: Supplementary of 3.3**



Figure S1: Chromatograms for the phenolic compounds of gelam honey using electrospray ionisation-mass spectra (ESI-MS), the based peaks (black colour), positive ionisation [+ESI-MS] (red colour) and negative ionisation [-ESI-MS] (green colour). gellic acid at 2.02 min , ferulic acid at 11.53 min , quercetin at23.64 min , ellagic acid at 24,58 min, Hesperetin at 27.23 min , and chrysin at 33.26 min.
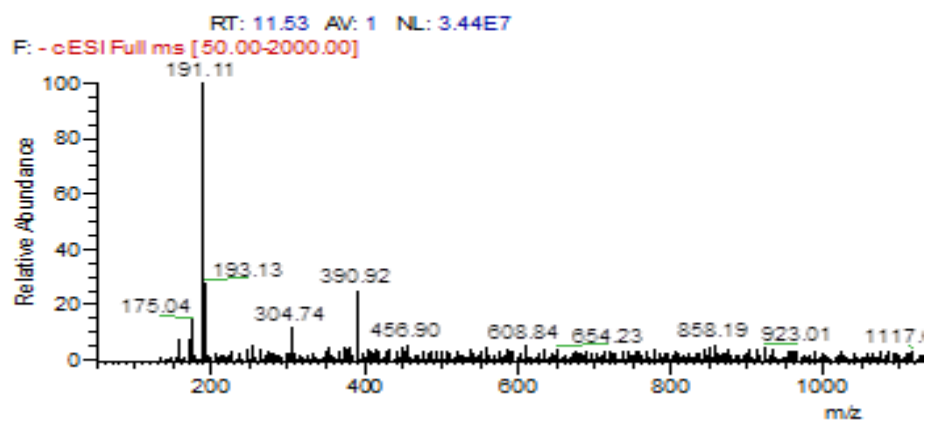
Figure S2: Electrospray ionisation-mass spectra negative ionisation (-ESI-MS) for ferulic acid , MW =Molecular weight (MW= 194 g/mol), ESI-MS [M-H-H$_2$O] = 193.
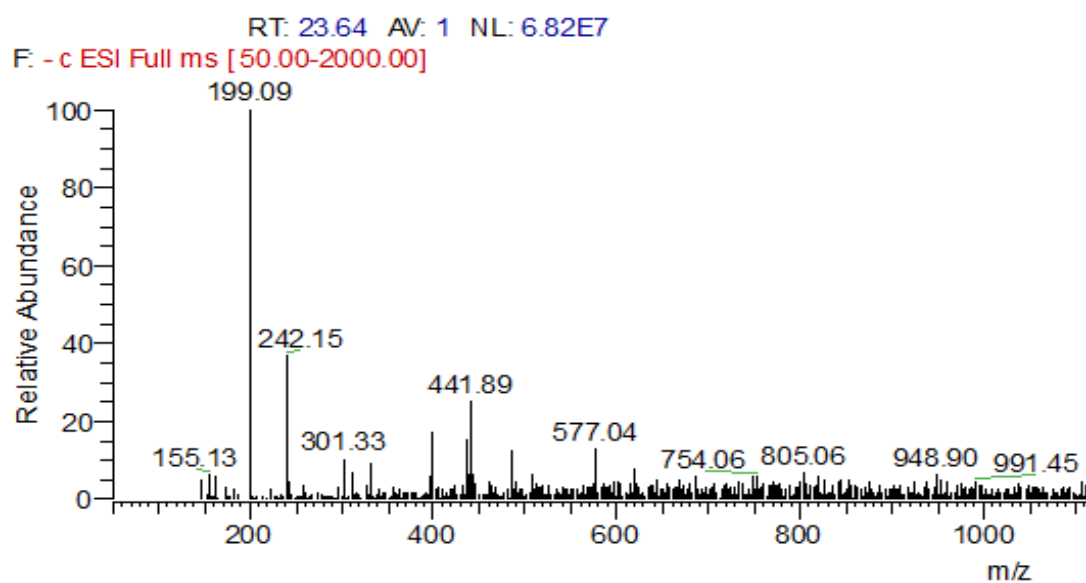
Figure S3: Electrospray ionisation-mass spectra (ESI-MS) negative ionisation for quercetin, MW =Molecular weight (MW=302 g/mol), ESI-MS [M-H] = 301
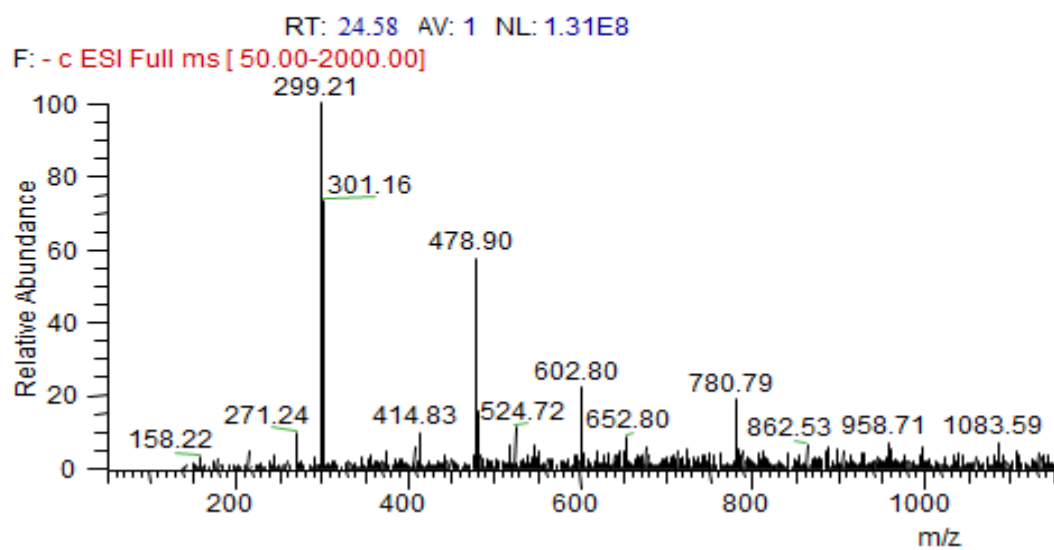
Figure S4: Electrospray ionisation-mass spectra (ESI-MS) negative ionisation for ellagic acid, MW =Molecular weight (MW=302 g/mol), ESI-MS [M-H] = 301.
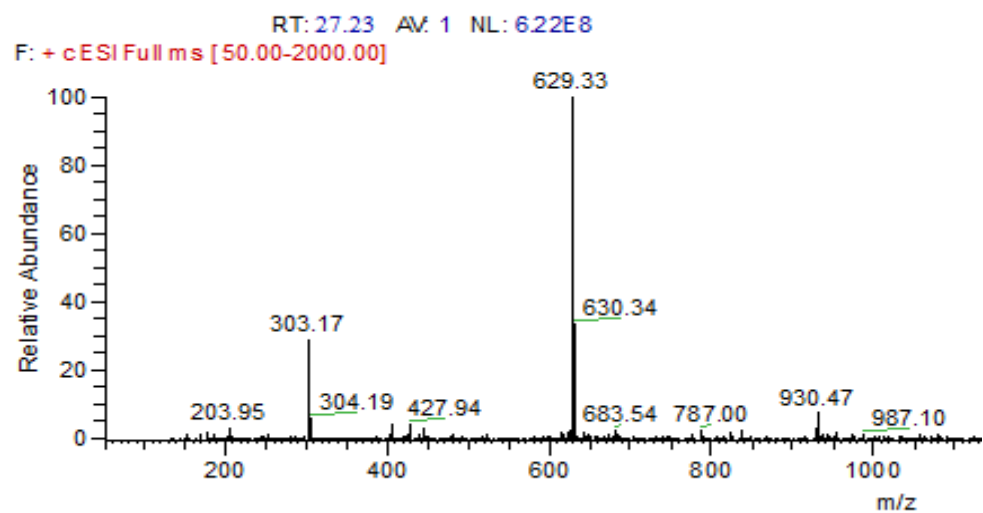
Figure S5: Electrospray ionisation-mass spectra (ESI-MS) posittive ionisation for Hesperetin, MW =Molecular weight (MW=302 g/mol), ESI-MS [M+H] = 303
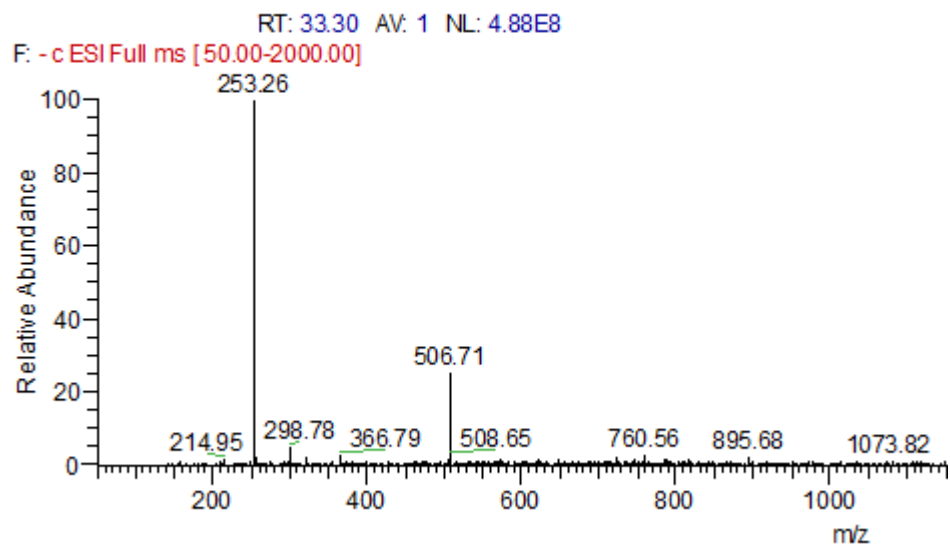
Figure S6: Electrospray ionisation-mass spectra (ESI-MS) negative ionisation for chrysin, MW =Molecular weight (MW=254.242g/mol), ESI-MS [M-H] = 253.28.A, P. S. (2002). procedure to identify a honey type. *Food Chemistry, 79, 401-406*.