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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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)-α 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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Keywords:
Honey
Endotoxemia
Cytokines
High-mobility group box 1
Nitric oxide
Heme oxygenase-1

Article info
Article history:
Received 17 March 2012
Received in revised form 10 May 2012
Accepted 14 May 2012
Available online 22 May 2012

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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-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. These results support the further examination of honey as a natural compound for the treatment of a wide range of inflammatory diseases.
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 N2; the dry residues were redissolved in methanol, and then filtered through a membrane (45 μml). 20 μl 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, liruric 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

![Graphs showing cytokine levels](image_url)
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 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
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 endoxemia, 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 NFκB. A previous study shows that NFκB 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 endotoxemia [30,32]. High circulating levels of IL-10 are important mediator of endotoxemia-induced immunosuppression and endotoxemia-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 NFκB. A previous study shows that NFκB 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]. 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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.

Acknowledgments and funding

This work was supported in part by grants PV009/2011B, RG031/09HTM, and RG225/10HTM from the University of Malaya.

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.


