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–\(\alpha\) (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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Keywords: Honey extracts; Chromatography, High-performance liquid chromatography (HPLC); Electrospray ionization mass spectrometry (ESI mass spectrometry); Tumor necrosis factor–\(\alpha\) (TNF); Nitric oxide (NO); Reactive oxygen species (ROS)

Abbreviations: DMEM, Dulbecco modified Eagle medium; EC\textsubscript{50}, median maximal effective concentrations; ESI-MS, electrospray ionization mass spectrometry; HEAE, honey ethyl acetate extract; HME, honey methanol extract; HO-1, heme oxygenase-1; HPLC, high-performance liquid chromatography; IFN-\(\gamma\), interferon-\(\gamma\); iNOS, inducible nitric oxide synthase; LC-MS, liquid chromatography–mass spectrometry; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MW, molecular weight; NO, nitric oxide; OD, optical density; TNF–\(\alpha\)/TNF, tumor necrosis factor–\(\alpha\).

1. Introduction

Honey, which is consumed worldwide, is increasingly being used as a substitute for granulated sugar [1]. In
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–α (TNF-α) 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 important mediator of acute and chronic inflammation [8]. It is hoped that, via empirical evidence of its effects [11,12], this is the first time, to the best of our knowledge, that the extracts will exhibit anti-inflammatory effects in vitro and the resin was washed at a rate of 10 mL/min with 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 μg/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-μm membrane filter before LC-MS analysis.

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-μm 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 μg/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-μm 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 × 250 mm; particle size, 5 μm) (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 the phenolic compounds in the extracts [21]. The calibration curves of quercetin and ellagic acid at 340 nm were used for calculate phenolic acids concentrations, whereas calibration curves of 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 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 × 4.6 mm; particle size, 5 μm; 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 μL/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 μL per well. Cell density was 1 × 10^4 (L929) or 1 × 10^5 (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 μL of fresh DMEM and 20 μL of 5 mg/mL MTT and incubated for 1 hour. Subsequently, the cell medium was aspirated; and 100 μL 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)) × 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 μg/mL) and actinomycin D (1 μg/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 × 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 μg/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 μg/mL) or with the inducible nitric oxide synthase (iNOS) inhibitor aminoxyguanidine at 1 mmol/L as a positive control; untreated cells were used as negative controls. The final
volume per well was 100 μL. The plates were then incubated for 16 to 20 hours at 37°C, 5% CO2.

Following incubation, NO inhibition was assessed by quantifying nitrite (NO2−) 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:

\[\text{% Inhibition} = 100 \times \left(\frac{[\text{NO}_2^-]_{\text{control}} - [\text{NO}_2^-]_{\text{sample}}}{[\text{NO}_2^-]_{\text{control}}}\right)\]

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 (EC50) 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 μg/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.](image)
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 − H2O] 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 MSn 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.](image)

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

<table>
<thead>
<tr>
<th>Compounds</th>
<th>μg/100 g honey at 290/340 nm ethyl acetate extract</th>
<th>μg/100 g honey at 290/340 nm methanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>341.01 ± 28</td>
<td></td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>153.65 ± 12.5</td>
<td></td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>158.39 ± 17.4</td>
<td></td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>80.68 ± 7.6</td>
<td></td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>239.08 ± 19.4</td>
<td></td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>3295.83 ± 38.6</td>
<td></td>
</tr>
<tr>
<td>Myricetin</td>
<td>223.57 ± 27.1</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>66.50 ± 7.2</td>
<td></td>
</tr>
<tr>
<td>Hesperetin</td>
<td>109.27 ± 13.5</td>
<td></td>
</tr>
<tr>
<td>Luteolin</td>
<td>33.61 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>Kaempferol</td>
<td>16.12 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Chrysin</td>
<td>69.01 ± 8.7</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>MW</th>
<th>MS</th>
<th>ESI-MS [M ± H]</th>
<th>UV band (nm)</th>
<th>Fragment ions</th>
<th>Compound names</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.39</td>
<td>194</td>
<td>175</td>
<td></td>
<td>296, 324</td>
<td>175</td>
<td>Ferulic acid</td>
</tr>
<tr>
<td>11.53</td>
<td>194</td>
<td>193</td>
<td></td>
<td>296, 324</td>
<td>242, 172, 158</td>
<td>Ferulic acid</td>
</tr>
<tr>
<td>21.35</td>
<td>299</td>
<td>298</td>
<td></td>
<td></td>
<td>361, 303, 199, 172</td>
<td>Ellagic-glucoside</td>
</tr>
<tr>
<td>21.66</td>
<td>464</td>
<td>463</td>
<td></td>
<td>235, 260, 350, 385</td>
<td>161, 261, 303</td>
<td>Ellagic acid</td>
</tr>
<tr>
<td>22.64</td>
<td>242</td>
<td>243</td>
<td>+</td>
<td>235, 285</td>
<td>242, 199, 155</td>
<td>Quercetin</td>
</tr>
<tr>
<td>24.63</td>
<td>302</td>
<td>301</td>
<td></td>
<td>235, 280, 235</td>
<td>263, 203</td>
<td>Ellagic acid</td>
</tr>
<tr>
<td>27.23</td>
<td>302</td>
<td>303</td>
<td>+</td>
<td>270,290</td>
<td>249, 188</td>
<td>Hesperetin</td>
</tr>
<tr>
<td>29.35</td>
<td>299</td>
<td>298</td>
<td></td>
<td>235, 280, 235</td>
<td>263, 203</td>
<td>Ellagic acid</td>
</tr>
<tr>
<td>29.39</td>
<td>299</td>
<td>300</td>
<td>+</td>
<td>270,290</td>
<td>249, 188</td>
<td>Hesperetin</td>
</tr>
<tr>
<td>33.26</td>
<td>254</td>
<td>253</td>
<td></td>
<td></td>
<td>260, 295, 320, 335</td>
<td>Chrysin</td>
</tr>
<tr>
<td>38.09</td>
<td>462</td>
<td>463</td>
<td>+</td>
<td></td>
<td>303, 271, 165</td>
<td>Quercetin-3-O-glucoside</td>
</tr>
<tr>
<td>59.95</td>
<td>418</td>
<td>272</td>
<td>+</td>
<td></td>
<td>273, 204</td>
<td>Rhamnoloy naringenin</td>
</tr>
</tbody>
</table>

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.

**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 μg/mL) in L929 cells and (3.125-100 μg/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](image_url)

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.)
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. 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).

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.
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 EC50 for NO inhibition by HEAE and HME were 37.5 and 271.7 μg/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-α-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-α-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, transactivation 1, another important transcription factor for 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.

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References

[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-κB [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.


