Identification of phenolic compound in manuka honey as specific superoxide anion radical scavenger using electron spin resonance (ESR) and liquid chromatography with coulometric array detection

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Abstract: Apitherapy has become the focus of attention as a form of folk and preventive medicine for treating certain conditions and diseases as well as promoting overall health and well-being. In apitherapy, honey is the therapeutic agent used for dressing surgical wounds, burns or skin ulcers, as well as dyspepsia, peptic ulcer, etc., because of its antioxidant activity. Therefore, it is important to determine the antioxidants in honey by analytical techniques. In the present study, the antioxidant activities of honeys from different floral sources were investigated by electron spin resonance (1,1-diphenyl-2-picrylhydrazyl (DPPH) and H2O2/NaOH/DMSO scavenging systems), liquid chromatography with coulometric array detection (LC-ED), and liquid chromatography with electrospray mass spectrometry (LC-MS). The antioxidant activities of some unifloral honeys (acacia, Chinese milk vetch, buckwheat and manuka) were evaluated using the radical scavenging systems. It was shown that DPPH radical scavenging activity was significantly different among the honeys, with buckwheat and manuka honeys having significantly higher scavenging activity than acacia honey. In addition, only manuka honey had specific scavenging activity for superoxide anion radicals. The compound responsible for this activity in manuka honey was identified by LC-ED and LC-MS. Careful examination of the LC-ED chromatographic patterns of manuka and other honey samples revealed a distinct peak in the chromatogram of manuka honey to be methyl syringate (MSYR). The radical scavenging activity of MSYR was specific for superoxide anion radicals, similar to the case of manuka honey.

Keywords: honey; manuka; antioxidant activity; electron spin resonance (ESR); LC-ED; methyl syringate

INTRODUCTION

Recently, apitherapy has become the focus of attention as a form of folk and preventive medicine for treating certain conditions and diseases as well as promoting overall health and well-being. In apitherapy, honey is the therapeutic agent used for dressing surgical wounds, burns or skin ulcers, as well as dyspepsia, peptic ulcer, etc., because of its antibacterial activity.1 Honey rapidly reduces inflammation, swelling and pain caused by many diseases. Furthermore, it was found that some honeys have antibacterial activity due to the presence of hydrogen peroxide. The antibacterial effects of various honeys were found to be due to both the physical property of osmosis and the generation of hydrogen peroxide by glucose oxidase. Many studies have been conducted on the 'non-peroxide' antibacterial activity of some honeys.2,3 In addition, honey is a rich source of phenolic compounds.4–6 Phenolic compounds such as aromatic acids and flavonoids are widely distributed in foods of plant origin and are regarded as having effective antioxidant and radical scavenging activities.7,8 Foods containing phenolic compounds are used not only as nourishment but also in the prevention of cancer, cardiovascular diseases and diabetes. Such chronic diseases are thought to be caused by oxidative stress,
which is defined by the imbalance between free radical production and the antioxidant defense system.\(^9\) Several studies have focused on the beneficial effects of natural antioxidants.\(^10–12\) The phenolic compounds in honey have been shown to suppress oxidative degradative reaction.\(^13,14\) However, studies on the antioxidant activity of honey are few and far between. It is therefore important to determine the antioxidant in honey by physical and analytical techniques.

In the present study, by means of electron spin resonance (ESR), we evaluated the antioxidant activities of honeys from different floral sources with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and free radical (methyl, hydroxyl and superoxide anions) scavenging systems. We also determined the antioxidant in the honeys by liquid chromatography with electro spray mass spectrometry (LCMS).

### EXPERIMENTAL

#### Chemicals

DPPH was purchased from Sigma Aldrich (St Louis, MO, USA). 5,5-Dimethyl-1-pyrrole-N-oxide (DMPO) was purchased from Labotec Co (Tokyo, Japan). Distilled water was purified with a Milli-Q gradient A 10 Elix system with an EDS polisher (Millipore, Bedford, MA, USA). Dimethyl sulfoxide (DMSO), methanol, sodium hydroxide (NaOH) and hydrogen peroxide (H\(_2\)O\(_2\)) were purchased from Wako Pure Chemical Industries Ltd (Tokyo, Japan). Folin–Ciocalteu reagent was purchased from Kanto Chemical Industries Ltd (Tokyo, Japan). Gallic acid and methyl 3,5-dimethoxy-4-hydroxybenzoate (MSYR) were purchased from Acros Organics (New Jersey, USA).

#### Honey samples

Honey samples were purchased at supermarkets in Tokyo and Kanagawa Prefecture, Japan. The honey samples originated in four floral sources, namely, acacia, Chinese milk vetch, buckwheat and manuka, and the number of honey samples was four for each floral source (Table 1).

#### Control solution for honey

Honey is composed of almost 80% glucose–fructose (1:1; w/w).\(^15,16\) Therefore, a control solution of 80% glucose–fructose (1:1; w/w) was used as blank, and the effects of the reducing sugars on the results obtained using the Folin–Ciocalteu method were determined. Free radicals were found to be generated in alkaline solution by reducing agents.\(^15–18\)

#### Evaluation of total phenolic content in honeys

Total phenolic content in honey samples was evaluated using the Folin–Ciocalteu method.\(^15\) By determining quantitatively well-known phenolic compounds per gram of gallic acid, we were able to determine the constant for use in calculating the total phenolic content in honey.\(^18\)

### Table 1. List of honey samples used in the present study

<table>
<thead>
<tr>
<th>Floral source</th>
<th>Place of origin</th>
<th>Year purchased</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acacia 1</td>
<td>Japan</td>
<td>2001</td>
</tr>
<tr>
<td>Acacia 2</td>
<td>Hungary</td>
<td>2001</td>
</tr>
<tr>
<td>Acacia 3</td>
<td>China</td>
<td>2001</td>
</tr>
<tr>
<td>Acacia 4</td>
<td>China</td>
<td>2000</td>
</tr>
<tr>
<td>Chinese milk vetch 1</td>
<td>Japan</td>
<td>2001</td>
</tr>
<tr>
<td>Chinese milk vetch 2</td>
<td>China</td>
<td>2001</td>
</tr>
<tr>
<td>Chinese milk vetch 3</td>
<td>China</td>
<td>2000</td>
</tr>
<tr>
<td>Chinese milk vetch 4</td>
<td>China</td>
<td>2000</td>
</tr>
<tr>
<td>Buckwheat 1</td>
<td>Japan</td>
<td>2001</td>
</tr>
<tr>
<td>Buckwheat 2</td>
<td>Japan</td>
<td>2001</td>
</tr>
<tr>
<td>Buckwheat 3</td>
<td>China</td>
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<td>2001</td>
</tr>
<tr>
<td>Manuka 1</td>
<td>New Zealand</td>
<td>2002</td>
</tr>
<tr>
<td>Manuka 2</td>
<td>New Zealand</td>
<td>2001</td>
</tr>
<tr>
<td>Manuka 3</td>
<td>New Zealand</td>
<td>2001</td>
</tr>
<tr>
<td>Manuka 4</td>
<td>New Zealand</td>
<td>2001</td>
</tr>
</tbody>
</table>

### ESR apparatus and instrument conditions

ESR experiments were performed using a JES-RE-1X system from Jeol Co (Tokyo, Japan). This spectrometer has the function of normalizing all spectra for accurate calculation using manganese oxide (MnO) as the internal standard. The following ESR conditions were used: magnetic field, 336.5 ± 5 mT; power, 8.0 mW; modulation frequency, 100 kHz; frequency, 9.425 GHz; modulation amplitude, 0.063 mT; gain, 200; and time constant, 0.03 s. All measurements were performed at room temperature. The intensity of the ESR signal indicates the scavenging efficiency of the added samples.

### DPPH radical scavenging activity

DPPH is one of the most stable free radicals and is frequently used in the evaluation of radical scavengers in natural foods.\(^5\) Free radical scavenging activity was tested using a methanolic solution of 300 \(\mu\)mol l\(^{-1}\) DPPH. DPPH and honey samples were mixed in a test tube for 30 min. Then, the mixture was transferred to a flat quartz cell and the ESR spectrum of the DPPH spin adducts was measured.

### Methyl, hydroxyl and superoxide anion radical (\(-\text{CH}_3, -\text{OH and O}_2^−\)) scavenging activity

Methyl, hydroxyl and superoxide anion radicals (\(-\text{CH}_3, -\text{OH and O}_2^−\)) were generated in an \(\text{H}_2\text{O}_2–\text{NaOH–DMSO system.}\(^19\) DMSO (130 \(\mu\)l), 25 \(\mu\)mol l\(^{-1}\) NaOH (130 \(\mu\)l), sample (100 \(\mu\)l), DMPO (10 \(\mu\)l) and \(\text{H}_2\text{O}_2\) (130 \(\mu\)l) were mixed in a test tube, and then the mixture was quickly transferred to a flat quartz cell. The ESR spectra of the DPPH–\(-\text{CH}_3\), DMPO–\(-\text{OH}\) and DMPO–\(-\text{OOH}\) spin adducts were measured.
Sample preparation for liquid chromatography
Solid-phase extraction (SPE) was used for preconditioning prior to liquid chromatography. A GL-Pak PLS-2 cartridge (270 mg/6 ml, GL Sciences, Tokyo, Japan) was used. The solid-phase material, polystyrene divinyl benzene polymer, has a higher recovery rate for hydrophilic compounds than conventional C18 cartridges. Because major phenolic compounds are hydrophilic, we used this cartridge for preconditioning. Ten grams of a honey sample were dissolved in 50 ml of distilled water. The sample solution was transferred into an SPE cartridge preconditioned with 5 ml of methanol and 5 ml of distilled water. Then, washing with 10 ml of water was carried out, followed by elution with 5 ml of methanol. The solutions were evaporated to dryness under a stream of nitrogen at 40 °C. The samples were adjusted by adding 10 ml of methanol, and the obtained samples were subjected to LC systems.

LC-ED apparatus and conditions
The LC system consisted of pumps, an autosampler (model 542), a column oven, a multichannel electrochemical detector containing cells from Coul Array model 6210, and the database from Coul Array System Win 32 volume 1.0 (ESA, Chelmsford, MA, USA). The analytical column was a Discovery RP Amide C16 (4.6 × 150 mm, 5 µm) from Supelco Co (Bellfonte, PA, USA), and column temperature was set at 40 °C throughout the analysis. The cell potentials of the seven electrodes consisted of an increasing array (C1–C7; from 50 to 600 mV). The injection volume was 5 µl.

Identification of phenolic compound by LC-mass spectrometry
The liquid chromatography–electrospray mass spectrometry (LC-MS) system for the determination of standards and samples was an Agilent 1100 MSD-SL system (Agilent Technologies, Palo Alto, CA, USA). Each fraction was collected for 2 min by LC-UV (wavelength 225 nm) under the same conditions as for LC-ED. A 5 µl aliquot of the fraction was injected into the LC and separation was carried out in the gradient mode using 0.5% acetic acid in water (mobile phase A) and 0.5% acetic acid in methanol (mobile phase B) was applied at a flow rate of 0.5 ml min⁻¹: 0–30 min at 20% solution B, and 30–60 min at 20–100% B.

RESULTS

DPPH radical scavenging activity
Based on the results of measurement of total phenolic content in various honeys, buckwheat and manuka honeys were focused on from the standpoint of radical scavenging activity. Figure 1 shows the DPPH radical scavenging activities of manuka, buckwheat, acacia and Chinese milk vetch honeys. As an index of potency, the IC50 value (the concentration in micrograms of honey per milliliter of DPPH solution at which honey sample caused 50% inhibition) was determined by graphical interpolation of each cumulative concentration–dependent response curve (CCRC), constructed and expressed as mean value for buckwheat (n = 4), manuka (n = 4), Chinese milk vetch (n = 4) and acacia (n = 3). The DPPH radical scavenging activity is expressed as 1/IC50 in Fig 2.

Free radical generation in H2O2–NaOH–DMSO system
The ability of the honeys to reduce methyl, hydroxyl and superoxide anion radicals (·CH3, ·OH and O2⁻) was measured by this system. The ESR spectrum of the control solution is shown in Fig 3. The mechanism of the radical-generating reaction is speculated as follows. Superoxide anion and hydroxyl radicals are generated from the degradation of H2O2, and the ·OH generates ·CH3 from DMSO. Therefore, this system can be used for evaluating the antioxidant activity of those three free radicals at the same time.

Methyl, hydroxyl, and superoxide anion radical scavenging activity
Manuka and buckwheat honeys showed strong scavenging activity for ·CH3, ·OH and O2⁻ in the CCRCs (data not shown). The IC50 value of each honey was determined by graphical interpolation of each CCRC constructed and expressed as mean value. Buckwheat honey showed the highest scavenging activity for hydroxyl radical. Other honeys showed lower activity, although their tendencies were the same as that of buckwheat. In contrast, manuka honey showed the highest scavenging activity for superoxide anion radical (Fig 4). Therefore, we conducted further investigations to identify the compound responsible for these results.
Identification of specific superoxide anion radical scavenger

Figure 1. DPPH radical scavenging activities of honeys from various floral sources \((n = 4)\). (A) Buckwheat and manuka honeys; (B) acacia and Chinese milk vetch honeys.

Figure 2. \(1/IC_{50}\) of DPPH radical scavenging activity of honeys. **\(p < 0.01\), indicates significant difference from acacia value. Buckwheat, manuka and Chinese milk vetch, \(n = 4\); acacia, \(n = 3\).

Figure 3. ESR spectrum of trapped free radicals (CH\(_3\), OH and O\(_2^-\)) with DMPO.

Figure 4. \(1/IC_{50}\) of buckwheat and manuka honeys in \(H_2O_2/NaOH/DMSO\) system (■, buckwheat; □, manuka).

honey indicated that it had the highest content of polar compounds under the LC-ED conditions used in this study. Note that the chromatogram of manuka honey exhibited a single peak with a retention time of approximately 22 min (Fig 5 (D)).

**Identification of specific phenolic compound in manuka honey**

The fraction (eluted for 1 min) containing the specific phenolic compound in manuka honey was collected by LC-UV and subjected to LC-ED. One peak was detected by LC-ED, its retention time being 22 min. Then this fraction was subjected to LC-MS. The mass spectrum of manuka honey shown in Fig 6 (A) was identical to that of methyl syringate (MSYR) from...
Figure 5. LC-ED chromatograms of honeys from (A) acacia; (B) Chinese milk vetch; (C) buckwheat; and (D) manuka.

Figure 6. Mass spectra of (A) specific phenolic compound in manuka honey and (B) MSYR.
Radical scavenging activity of MSYR

Next, we evaluated the radical scavenging activity of MSYR using the H$_2$O$_2$–NaOH–DMSO system. MSYR showed specific scavenging activity for superoxide anion radicals (Fig 7).

DISCUSSION

Apitherapy involves the therapeutic use of honeybee products, including honey, pollen, propolis, royal jelly and bee venom. A recent study has indicated that pure honey is widely available in most communities, although the biological mechanism underlying its use in preventive medicine remains unknown and requires further investigation.

Buckwheat honey is known to contain a large amount of phenolics and therefore has high radical scavenging activity. In the present study, we found that buckwheat honey has the highest scavenging activity for DPPH and hydroxyl radicals. In the human body, hydroxyl radicals directly attack DNA and proteins, etc., thereby causing cell injury due to oxidative stress.

The radical scavenging activity of buckwheat honey is due to the large amount of phenolic compounds in it. LC-ED chromatograms revealed that the redox-active compounds including phenolics (retention time 2–12 min) in all the honey samples investigated exhibited peak responses, with those from buckwheat honey having the highest intensity. Therefore, these compounds were found to be related to the hydroxyl radical scavenging activity of the honeys, based on the present results. We should take note, however, that the estimation of only the DPPH radical scavenging may be insufficient to accurately determine the antioxidant effect of honey.

On the other hand, the LC-ED chromatogram and the radical scavenging effect of manuka honey were different from those honey samples (Figs 4 and 5), and indicated the ability of manuka honey to specifically scavenge superoxide anion radicals. Thus, this particular activity of manuka honey was investigated by LC-ED, which has extremely high sensitivity and unequalled selectivity for the analysis of electro-active compounds. The LC-ED chromatogram of manuka honey exhibited a characteristic peak at the retention time of 22 min (Fig 5). This peak was identified to be that of MSYR based on LC-MS data and the literature.

By determining the specific antioxidant activity of MSYR in manuka honey, we found that it specifically scavenge superoxide anion radicals (Fig 7).

Manuka honey is known to have exceptionally high antibacterial activity and has been used for the prevention of secondary infection in surgical wounds since ancient times in New Zealand. In addition, manuka honey has high antibacterial activity against Helicobacter pylori, which causes stomach and duodenal cancers. In the present study, we proved that manuka honey can specifically scavenge superoxide anion radicals, due to the presence of MSYR. For these reasons, manuka honey can be used not only as a health food, but also in medicine because of its superoxide anion radical scavenging activity.

REFERENCES

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