Antihypertensive Effects of Flavonoids Isolated from Brazilian Green Propolis in Spontaneously Hypertensive Rats

Hiroe Maruyama, Yoshiki Sumitou, Takashi Sakamoto, Yoko Araki, and Hideaki Hara

Received November 27, 2008; accepted May 9, 2009; published online May 11, 2009

Propolis, a honeybee product, has become popular as a food and alternative medicine. Its constituents have been shown to exert pharmacological effects, such as anticancer, antimicrobial, and anti-inflammatory effects. The present study was performed to investigate whether Brazilian green propolis exerts antihypertensive effects in spontaneously hypertensive rats (SHR) and which constituents are involved in its effects. Brazilian green propolis was extracted with ethanol and subjected to LH-20 column chromatography eluted with ethanol. The ethanol-eluted fractions at 10 mg/kg were administered orally to SHR for 14 d. Significant decreases in blood pressure were observed in fractions 6 and 7. The active constituents were purified and identified to be four flavonoids: dihydrokaempferide and isosakuranetin in fraction 6 and betuletol and kaempferide in fraction 7. These flavonoids at 10 mg/kg were administered orally to SHR for 28 d, and as a result, isosakuranetin, dihydrokaempferide and betuletol produced significant decrease in blood pressure, especially marked were the effects observed in the group that received isosakuranetin. Brazilian green propolis, fractions 6 and 7, and the 4 active constituents relaxed isolated SHR aorta in a concentration-dependent manner. Therefore, these finding suggest that the vasodilating action may be partly involved in the mechanism of antihypertensive effect. Hence, the ethanol extract of Brazilian green propolis and its main constituents may be useful for prevention of hypertension.

Key words Brazilian green propolis; antihypertensive effect; flavonoid; spontaneously hypertensive rat

MATERIALS AND METHODS

Materials Brazilian green propolis was collected in Minas Gerais state, Brazil. The main botanical source was Baccharis dracunculifolia Dc. Propolis was extracted with 95% ethanol, and stirred for 16 h at room temperature. The insoluble part was removed by filtration, and adjusted to about 20% solid was designed EEP-B20 (Api Co., Ltd., Gifu, Japan). Isosakuranetin and kaempferide were purchased from Funakoshi, Ltd. (Tokyo, Japan). Dihydrokaempferide and betuletol were purified as follows. Amlodipine was purchased from Pfizer (Norvasc® OD Tablets; Tokyo, Japan).

Fractionated Propolis Ethanol-extracted propolis (EEP-B20; Api Co., Ltd.) was applied to Sephadex™ LH-20 (Amersham Biosciences, Piscataway, NJ, U.S.A.) column chromatography (φ62 mm×270 mm) eluted with ethanol. Ten fractions (each 200 ml) were collected and their composition monitored by TLC (solvents: CHCl3–MeOH in 9:1 or CHCl3 only) and those with similar TLC profiles were combined into 8 major fractions denoted as Fr. 1 to Fr. 8 (Fig. 1). Each fraction was collected and dried.

Isolation Fraction 6 was further purified by repeated silica gel column chromatography and eluted with CHCl3–MeOH (gradually replaced with MeOH 9:1 or CHCl3 only) and those with similar TLC profiles were combined into 8 major fractions denoted as Fr. 1 to Fr. 8 (Fig. 1). Each fraction was collected and dried.

Identification of Flavonoids Flavonoids were identified by NMR (Varian, MERCURY plus 300 MHz; Varian Tech-
Ethanol extract of propolis was subjected to Sephadex TM LH-20 column chromatography and eluted with ethanol. Isolation of flavonoids from the ethanol extract of propolis: LH-20 column chromatography fractions were further purified by repeated silica gel column chromatography, finally yielding purified 4 compounds.

Isolation of Four Flavonoids for Animal Experiments

Ethanol extract of propolis (EEP-B20; Api Co., Ltd.) was applied to a column of LH-20 gel, and eluted with ethanol four times. Fractions 6 and 7 were concentrated under reduced pressure to afford a yellow material (10 g). A portion of the 10 g extract was pre-adsorbed onto silica gel, applied to a silica gel column, and eluted with CHCl₃–MeOH (1% increment of MeOH), and finally with MeOH. Thirteen fractions were collected and their compositions were monitored by TLC (solvent: CHCl₃:MeOH=1:1 or CHCl₃ only) or HPLC. The purity was confirmed by HPLC, and solutions of 90% purity or greater were used in the experiments.

Animals and Measurement of Blood Pressure

Male SHR and male Wistar Kyoto (WKY) rats (11—12 weeks old, body weight 260—310 g) were purchased from Hoshino Laboratory Animals (Saitama, Japan). SHR and WKY rats were housed individually in steel cages in a room kept at 23 °C with a 12 h light–dark cycle (lights on 8:00—20:00), and fed a laboratory diet (CE-II, CLEA Japan Inc., Tokyo, Japan). Water was freely available. Systolic blood pressure and heart rate were measured by the tail-cuff method with a Softron BP system (BP-98A; Softron, Tokyo, Japan) after warming the animals in a chamber maintained at 39 °C for 5 min. At least six determinations were made in every session of systolic blood pressure measurements and the mean of six values was taken as the systolic blood pressure level.

Repeated Oral Administration of Propolis Fractions in SHR

After acclimation of SHR in the environment described above, animals (16 weeks old) with systolic blood pressure over 200 mmHg were used in the experiments. Using the starting blood pressure and body weight, animals were divided into 9 groups (control group, and Fr. 1 to Fr. 8). Each fraction was suspended in a 0.5% solution of gum Arabic and administered orally at 10:00 a.m. each day for 14 d at a dose of 10 mg/kg/10 ml. The control group was administered the same volume of gum Arabic solution. Systolic blood pressure and heart rate were measured at 0, 7, and 14 d before the administration.

Repeated Oral Administration of Flavonoid in SHR

Using the starting blood pressure and body weight, animals (13 weeks old) were divided into 5 groups (control group, and dihydrokaempferide-, isosakuranetin-, kaempferide-, and betuletol-treated groups) and 2 groups (control group, and amlodipine-treated groups). Each flavonoid was suspended in a 1% solution of gum Arabic (vehicle) and administered orally at 10:00 a.m. each day for 28 d at a dose of 10 mg/kg/10 ml. Amlodipine, calcium channel antagonist used as a positive control at a dose of 3 mg/kg/10 ml, was administered orally in the same method. The control group was administered the same volume of gum Arabic solution. Systolic blood pressure and heart rate were measured at 0, 7, 14, 21, 28 d before the administration and measured at 7, 14, 21, 28 d 2 h after the administration.

Repeated Oral Administration of Isosakuranetin and Amlodipine in WKY

Using the starting blood pressure and body weight, animals (13 weeks old) were divided into 3 groups (control group, isosakuranetin-, and amlodipine-treated groups). Isosakuranetin (10 mg/kg/10 ml) and amlodipine (3 mg/kg/10 ml) were administered orally at 10:00 a.m. each day for 28 d and the measurement of systolic blood pressure and heart rate was performed as described above.

Relaxation in the Aorta Isolated from SHR

Male SHR weighting 250—300 g were killed by cervical dislocation and exsanguination. The main branch of the superior mesenteric artery was excised and cut into helical strips (2—3 mm in width and 20—30 mm in length) as described previously. Arterial strips were then suspended between two stainless wire hooks in a 10 ml organ bath. The upper wire was connected to a force-displacement transducer and the lower wire was fixed to the bottom of the organ bath. The organ bath was filled with Krebs solution (m M: NaCl, 119; KCl, 4.7; NaHCO₃, 25; CaCl₂, 2.5; MgCl₂, 1; KH₂PO₄, 1.2; glucose, 11; and ascorbic acid, 0.3). The bath was continuously oxygenated with a mixture of 95% O₂ and 5% CO₂, and maintained at 37 °C to give a pH of approximately 7.4. The strips were placed under an optimal resting tension of 0.5 g, which had been determined by length–tension relationship experiments. The tissues were allowed to equilibrate for 90 min, during which time the bath solution was replaced with pre-
warmed and oxygenated Krebs solution every 20 min. The resting tension was readjusted to 1.0 g when necessary. The endothelial layer was mechanically removed by gently rubbing the intimal surface of the vessel. The absence of endothelium was confirmed by the absence of relaxing effects of acetylcholine (1 μM). The relaxing effects of propolis, flavonoid fraction mixed Fr. 6 with Fr. 7, and four flavonoids on the contraction induced by norepinephrine (10^{-6} M) were studied in the aorta without endothelium. Values for 50% inhibitory concentration (IC_{50}) were obtained by linear regression analysis.

**Data Analysis** The results are expressed as means and standard deviations (S.D.). The significance of the differences in systolic blood pressure and heart rate at each time point after repeated administration was analyzed by Dunnett's multiple-range test. The comparison of the control and amlodipine-treated group in SHR was evaluated by Student's t-test.

**RESULTS**

**Effects of Repeated Oral Administration of Fractions 6 and 7 in SHR** Repeated oral administration was performed using fractions of the ethanol extract. Only Fr. 6 and Fr. 7 produced significant decreases in blood pressure (Fig. 2). Measurements before the administration indicated significantly lower values in the groups that received Fr. 6 and Fr. 7 compared with the control group (p<0.01 on day 14). Other fractions were not decreased significantly, and throughout the period of administration, there were no differences between groups as regards heart rate and body weight of animals (data not shown).

**Isolation and Identification of Flavonoids in the Active Fractions** The results of the present study indicated that Fr. 6 and Fr. 7 obtained by LH-20 column chromatography exhibited antihypertensive effects in SHR. We isolated the active components in the ethanol extract of propolis (EEP-B20) fractionated by LH-20 column chromatography eluted in ethanol four times, and then obtained the corresponding fraction (10 g). These active fractions including Fr. 6 and Fr. 7 were subjected to silica gel column chromatography eluted gradually with CHCl3 and methanol. Thirteen fractions were collected and their compositions were monitored by TLC (solvent: CHCl3 : MeOH = 1:1 or CHCl3 only) or HPLC. For final purification, each was recrystallized in methanol, yielding compound 1 (0.92 g), compound 2 (0.1 g), compound 3 (1.09 g), and compound 4 (0.77 g), respectively. The purity of these flavonoids was confirmed to be 90% or more by HPLC analysis.

The NMR data of compounds 1 to 4 are as follows:

**Compound 1:** Dihydrokaempferide: \(^{1}H-NMR\) (DMSO-\(d_{6}\)) \(\delta\): 3.78 (3H, s, –OMe), 4.60 (1H, d, \(J=11.0\) Hz), 6.24 (1H, d, \(J=2.0\) Hz, H-6), 5.11 (1H, d, \(J=11.4\) Hz, H-2), 5.86 (1H, brs, H-6), 5.91 (1H, brs, H-8), 6.97 (1H, d, \(J=8.8\) Hz, H-3', 5'), 7.44 (2H, d, \(J=8.8\) Hz, H-2', 6'), 11.80 (1H, brs, –OH).

**Compound 2:** Isosakuranetin: \(^{1}H-NMR\) (DMSO-\(d_{6}\)) \(\delta\): 2.72 (1H, dd, \(J=17.0, 3.0\) Hz, Ha-3), 3.27 (1H, dd, \(J=17.0,13.0\) Hz, Hb-3), 3.77 (3H, s, –OMe), 5.50 (1H, dd, \(J=13.0, 3.0\) Hz, H-2), 5.88 (1H, d, \(J=2.0\) Hz, H-6), 5.89 (1H, d, \(J=8.5\) Hz, H-3', 5'), 7.44 (2H, d, \(J=8.5\) Hz, H-2', 6'), 12.14 (1H, s, –OH).

**Compound 3:** Kaempferide: \(^{1}H-NMR\) (DMSO-\(d_{6}\)) \(\delta\): 3.85 (3H, s, –OMe), 6.20 (1H brs, H-6), 6.45 (1H, brs, H-8), 7.11 (2H, d, \(J=8.9\) Hz, H-3', 5'), 8.14 (2H, d, \(J=8.9\) Hz, H-2', 6'), 12.44 (1H, brs, –OH).

**Compound 4:** Betuletol: \(^{1}H-NMR\) (DMSO-\(d_{6}\)) \(\delta\): 3.77 (3H, s, 6-OMe), 3.85 (3H, s, 4'-OMe), 6.57 (1H, s, H-8), 7.11 (2H, d, \(J=8.9\) Hz, H-3', 5'), 8.14 (2H, d, \(J=8.9\) Hz, H-2', 6'), 12.52 (1H, brs, –OH).

These compounds were identified as dihydrokaempferide and isosakuranetin in Fr. 6 and kaempferide and betuletol in Fr. 7 by HPLC (Fig. 3) and NMR, which showed that the
spectra were consistent with those reported previously.\textsuperscript{14–17} The structures of these four compounds were shown in Fig. 4. Contents of dihydrokaempferide and isosakuranetin in Fr. 6 and of kaempferide and betuletol in Fr. 7 were 10\%, 3.1\%, 36\%, and 40\%, respectively. The contents of these flavonoids in propolis extract were about 1.3\%, 0.2\%, 2.2\%, and 1.0\%, respectively (Table 1). The chemical structures of dihydrokaempferide, kaempferide, and isosakuranetin were A-ring 5,7-dihydroxyflavones (or flavonol) with B-ring 4'-methoxy groups. Betuletol had a 6,4'-methoxy group.

**Effects of Repeated Oral Administration of Flavonoids in SHR** Measurements before the administration revealed that lower values in the groups that received flavonoid compared with the control group (Fig. 5A). The systolic blood pressure in SHR administered dihydrokaempferide at a dose of 10 mg/kg/d was significantly lower than that in the control group on days 21 ($p<0.05$) and 28 ($p<0.01$) of administration. Isosakuranetin significantly reduced systolic blood pressure in SHR as compared with the control group on days 14 ($p<0.05$), 21 and 28 ($p<0.01$). Systolic blood pressure was significantly lower in SHR treated with betuletol than in the controls on days 28 ($p<0.05$) of administration. No significant effect on the systolic blood pressure was observed with kaempferide. Measurements conducted 2 h after administration showed that systolic blood pressure was significantly lower in the group that received isosakuranetin only ($p<0.05$ on day 14, 21 and 28, Fig. 5B). In contrast, amlodipine, calcium channel antagonist, significantly reduced systolic blood pressure before and 2 h after administration as compared with the control group on days 7 ($p<0.05$; before, $p<0.01$; 2 h after), 14, 21 and 28 (all $p<0.01$, Figs. 6A, B).

As regards the heart rate, no differences were observed between the control group and the groups before administration of flavonoid (Fig. 7A), however, in the group which received betuletol and dihydrokaempferide, a significant increase of heart rate was observed at 2 h after administration on days 21 ($p<0.05$ and $p<0.01$, respectively, Fig. 7B). In the group given amlodipine, no differences in the change of heart rate was observed before administration, however, the heart rate showed significant increase 2 h after administration as compared with the control group on days 7 ($p<0.01$), 14 ($p<0.05$), 21 and 28 ($p<0.01$, Figs. 8A, B).

**Repeated Oral Administration of Isosakuranetin and Amlodipine in WKY** In contrast to the SHR, no differences in the change of systolic blood pressure and heart rate of WKY were observed between the control group and the isosakuranetin-treated group before and 2 h after administration (Figs. 9A, B and Figs. 10A, B). Amlodipine significantly reduced systolic blood pressure 2 h after administration as compared with the control group on days 7 ($p<0.01$), 14 ($p<0.05$), 21 and 28 ($p<0.01$, Figs. 8A, B).

### Table 1. Flavonoid Contents of Ethanol Extract of Propolis

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Flavonoid contents from ethanol extract of propolis (solid) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydrokaempferide</td>
<td>1.3</td>
</tr>
<tr>
<td>Isosakuranetin</td>
<td>0.2</td>
</tr>
<tr>
<td>Kaempferide</td>
<td>2.2</td>
</tr>
<tr>
<td>Betuletol</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Fig. 4. Chemical Structures of 4 Compounds in Fractions 6 and 7 from the Ethanol Extract of Propolis

**Compound 1:** dihydrokaempferide. **Compound 2:** isosakuranetin. **Compound 3:** kaempferide. **Compound 4:** betuletol.

![Chemical Structures of 4 Compounds](https://example.com/chemical_structures.png)

Fig. 5. Effects of Repeated Oral Administration of Flavonoids from Propolis on Systolic Blood Pressure in SHR

Dihydrokaempferide, isosakuranetin, kaempferide, and betuletol were administered to SHR for 28 d (10 mg/kg). (A) Systolic blood pressure was measured before daily administration. (B) Systolic blood pressure was measured 2 h after daily administration. Differences in systolic blood pressure compared with the control group were examined for statistical significance. *$p<0.05$, **$p<0.01$* vs. control (Dunnett’s multiple-range test). Each point with a vertical bar represents the mean±S.D., $n=6–7$.

![Graph of Blood Pressure](https://example.com/blood_pressure_graph.png)
WKY. There were also no differences between groups as regards the body weight of animals (data not shown).

Relaxation Effect in the Aorta Isolated from SHR

Propolis and the flavonoids isolated from it markedly relaxed contractions induced by norepinephrine (10^{-7} M) in the aorta without endothelium. The IC_{50} values for propolis and some flavonoids were 0.9—16.6 mg/ml (Table 2). The inhibitory effects of flavonoids on relaxed contraction were observed in the order of kaempferide > isosakuranetin > flavonoid fraction > betuletol > propolis > dihydrokaempferide.

**DISCUSSION**

Propolis is usually extracted with ethanol or water, and these extracts have been used in folk medicine. The composition of propolis depends on the solvent used for its extraction. In addition, biological activities could be related to its chemical composition. Although 90—100% ethanol extract of Brazilian green propolis is commonly used as a health food, but little information is available regarding its biological activities and components. A recent study indicated that caffeoylquinic acid and its derivatives, which are among the components of the Brazilian green propolis extract, showed antihypertensive activities. However, the solvent used for extraction was 25% ethanol.

In this study, the ethanol extract of propolis was fractionated, and repeated oral administration of these fractions was performed in SHR. Only Fr. 6 and Fr. 7 produced significant decreases in blood pressure. The active constituents were purified and identified to be four flavonoids such as dihydrokaempferide, kaempferide, isosakuranetin, and betuletol.

Flavonoids are considered as an important part of the antioxidants in food. Dihydrokaempferide and betuletol isolated from Brazilian green propolis show anticancer activity *in vitro*. Kaempferide is known to have an antioxidative effect, and isosakuranetin is known to have antimicrobial activity. However, the antihypertensive effects of these flavonoids have not been clarified. Several previous studies have indicated various biological activities of flavonoids, including vasodilator effects in isolated aorta stimulated with noradrenaline or KCl. However, no antihypertensive activity was observed in SHR. Only a few reports have suggested that flavonoid shows an antihypertensive effect in SHR.

In this study, effects of four active flavonoids (dihydrokaempferide, kaempferide, isosakuranetin, and betuletol) from Brazilian green propolis on blood pressure and heart rate were examined using SHR. As a result, isosakuranetin, dihydrokaempferide and betuletol produced significant decrease in blood pressure before daily administration, especially marked were the effects observed in the group that received isosakuranetin; the values obtained 2 h after administration were significantly lower on days 14, 21 and 28.

In the present study, though dihydrokaempferide, kaempferide and betuletol were purified and administered at
10 mg/kg, the effect on lowering blood pressure was not more potent than those of Fr. 6 and Fr. 7 tested on days 14. It might be speculated that the response on lowering blood pressure is different by SHR used different age, in Fr. 6 and Fr. 7 experiment, with systolic blood pressure over 200 mmHg established, and there may be some unknown materials decreasing blood pressure in Fr. 6 and Fr. 7, although further studies are needed to elucidate the active constituents which purified and identified.

Four types of flavonoid have the flavonol, flavononol, and flavanon frame with a 4'-methoxyl group in the B ring. It was found that the chemical structures of flavonoids having the B-ring 3',4'-methoxy/hydroxyl groups were elevated for their inhibitory activities of NO production and the flavonols having methylation of the 3-, 5-, or 4'-hydroxyl group enhanced the inhibitory activity of NO production. Jeong et al. have suggested that flavonoids with 3-OH group play a positive role in antioxidant activities. Thus, it is well known that there are differences in the anti-oxidative revitalization and anti-inflammatory actions according to the structure of the B ring of flavonoids. The observation that these four flavonoids from Brazilian green propolis have a 4'-methoxyl group in the B ring is probably related to the antihypertensive activity.

Brazilian green propolis, Fr. 6 and Fr. 7, and the 4 active constituents relaxed the aorta isolated from SHR in a concentration-dependent manner. Its potency of four flavonoids was not necessarily corresponding to the antihypertensive effects in SHR. However, the vasodilating action may be partly involved in the mechanism of antihypertensive effect, though it is possible that the dissolubility and/or absorption of flavonoids in vivo might be different.

In conclusion, the findings presented here indicate that
ethanol extract of Brazilian green propolis and its main constituents would be beneficial for improving blood pressure as a functional food.

REFERENCES


Table 2. Relaxation Effect in the Aorta Isolated from SHR

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC₅₀ (95% confidential interval) (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydrokaempferide</td>
<td>16.6 (10.4—22.7)</td>
</tr>
<tr>
<td>Isosakuranetin</td>
<td>1.7 (1.2—2.5)</td>
</tr>
<tr>
<td>Kaempferide</td>
<td>0.9 (0.2—1.6)</td>
</tr>
<tr>
<td>Betuletol</td>
<td>8.2 (5.8—10.6)</td>
</tr>
<tr>
<td>Ethanol extract of propolis</td>
<td>15.0 (10.5—22.0)</td>
</tr>
<tr>
<td>Flavonoid fraction</td>
<td>4.4 (3.4—5.4)</td>
</tr>
</tbody>
</table>

IC₅₀ values (µg/ml) of different flavonoids to inhibit the contraction induced by 10⁻⁷ M norepinephrine. Data are means, n=3.