

Antihypertensive Effects of Brazilian Propolis: Identification of Caffeoylquinic Acids as Constituents Involved in the Hypotension in Spontaneously Hypertensive Rats

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Brazilian propolis was extracted with water or various concentrations of ethanol and were administered orally to spontaneously hypertensive rats (SHR) and the effects on blood pressure and heart rate were determined. Single oral administration of 100 mg/kg of propolis extracts decreased the blood pressure in SHR. Significant decrease in blood pressure was observed with propolis extracted with 25 and 70% ethanol. SHR were given orally 5 mg/kg of propolis extracted with 25 or 70% ethanol, twice a day for 28 d and the effects on blood pressure and heart rate were compared with control rats. While the blood pressure in the control group increased day by day, the increase was slower in rats treated with 25 and 70% ethanol extracts of propolis. The hypotensive activity of propolis extracted with 25% ethanol was more significant compared with control group than with 70% ethanol. Di- and tri-caffeoylquinic acids (CQAs) were found to be characteristic components of propolis extracted with 25% ethanol. A single oral administration of 3,4-diCQA, 3,5-diCQA, and 3,4,5-triCQA each at a dose of 10 mg/kg were conducted in SHR. These three components were found to have antihypertensive effects and therefore contribute to the antihypertensive effects of propolis extract. These results suggest that 25% ethanol extract of propolis is useful for prevention and treatment of hypertension.

Key words propolis; caffeoylquinic acid; antihypertension

Recently increases in life-style related diseases like hypertension, arteriosclerosis, heart disease, obesity, diabetes mellitus, and cancers have become a great social problem.¹⁾ As regards diseases of circulatory system such as hypertension, arteriosclerosis and heart disease, increasing numbers of people are showing symptoms of hypertension or pre-hypertension stage. Causes leading to hypertension include increase in the volume of body fluid, increase of the resistivity of the blood vessels, increases in factors leading to elevation of the blood pressure or decreases in factors bringing about lowering of the blood pressure.²⁾ These factors induce abnormal increase of blood pressure, either singly or in combination. Clinically, various drugs such as inhibitors of angiotensin converting enzymes, calcium antagonists, β -blockers, angiotensin II receptor antagonists, and hypotensive diuretics have been used for management of hypertension to improve the symptoms.³⁾ In addition, antihypertensive effects have been noted with various food and natural products.⁴⁾ Recently, various foods with differing hypotensive mechanisms have been specified for use as health foods. These foods have various activities such as the inhibitory activity of peptides derived from milk,⁵⁾ fish,⁶⁾ or plants,⁷⁾ on angiotensin converting enzymes, vasodilatory action by parasympathetic stimulation,⁸⁾ or inhibitory effects on release of noradrenaline from sympathetic nerves,⁹⁾ respectively. Furthermore, some flavonoids and polyphenol derivatives from natural products have been reported to have vasodilating and hypotensive effects.^{10–12)} In these points, we focused on propolis which has been shown to comprise more than 150 constituents, including cinnamic acid derivatives, flavonoids, and benzoic acids.¹³⁾

Propolis is called “bee resin”, which is a yellow to dark brown-colored adhesive substance collected from sprouts of plants by honeybees and thought to serve a protective role against potential predators. Propolis has been reported to possess some pharmacological functions in humans such as

antibacterial, antioxidative, anti-inflammatory, antitumor, and immunostimulatory activities and has been used in folk medicine.^{14–24)} The composition of propolis primarily depends upon the vegetation of the area from where it was collected and secondarily upon the solvents used for its extraction.²⁵⁾ Although pharmacological activities of propolis could be related to its chemical composition,²⁶⁾ water or 90–99.5% ethanol extract of propolis is commonly used for health food and beverages in recent years. Since *Baccharis dracunculifolia* is an important source of propolis collected from Minas Gerais state, Brazil,²⁷⁾ which is of good quality to provide experimental reproducibility, we have used this Brazilian propolis in the current study.

In the course of studies on diverse pharmaceutical activities of propolis, we examined hypotensive effects of Brazilian propolis extracts. Recent study reported that caffeic acid phenethyl ester (CAPE), which is one of the major components of propolis from Europe and Asia but not be contained in that from Brazil, has vasodilating activity on isolated rat aorta.¹¹⁾ Furthermore, the hypotensive effect of propolis extract in spontaneously hypertensive rats (SHR) was reported.²⁸⁾ However, information concerning effective extraction and ingredients underlying the hypotensive effect of Brazilian propolis have not been available.

In this study, at an initial effort insight into hypotensive effects of Brazilian propolis, effects of various propolis extracts on blood pressure and heart rate were examined using SHR. Furthermore, the potency of caffeoylquinic acid derivatives from propolis to induce hypotensive effects were elucidated.

MATERIALS AND METHODS

Materials Brazilian propolis was collected in Minas Gerais state, where *Baccharis dracunculifolia* DC. is the main botanical source of the propolis.²⁷⁾ Chlorogenic acid

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and *p*-coumaric acid were purchased from Nacalai Tesque, Ltd. (Kyoto, Japan); isosakuranetin was from Funakoshi, Ltd. (Kyoto, Japan); artepillin C was from Wako Pure Chemicals, Ltd. (Osaka, Japan).

Propolis Extracts Coarsely powdered Brazilian propolis was extracted with water or 10–95% ethanol to yield water extract of propolis (WEP) or ethanol extract of propolis (EEP), respectively. Propolis extracted with 10, 25, 50, 70, or 95% ethanol was designated as 10%-EEP, 25%-EEP, 50%-EEP, 70%-EEP, or 95%-EEP, respectively.

75 ml of water, 10, 25, or 50% ethanol was added to 15 g of propolis and stirred for 16 h at 40 °C. The insoluble part was removed by centrifugation at 2500 rpm for 10 min, and the supernatant was kept overnight at 4 °C. Following removal of insoluble by centrifugation, the supernatant was freeze-dried to yield WEP (1.9 g), 10%-EEP (2.0 g), 25%-EEP (2.4 g), or 50%-EEP (2.8 g). 75 ml of 70 or 95% ethanol was added to 15 g of propolis and stirred for 16 h at 40 °C. The insoluble part was removed by centrifugation at 2500 rpm for 10 min, and the supernatant was kept overnight at –20 °C, and filtered to remove waxes. The filtrate was taken to dryness under decompression heating to yield 70%-EEP (5.9 g) or 95%-EEP (4.3 g).

The main constituents compositions of WEP and EEP were analyzed using HPLC as described previously.²⁵⁾

Isolation of Caffeoylquinic Acids for Animal Experiments 25%-EEP (8 g) was dissolved in 25 ml of 10% ethanol, applied to a column ($\phi 62 \times 270$ mm) of AMBER-LITE XAD-2 gel (Organo, Ltd., Tokyo, Japan), and successively eluted with 10, 20, 40, or 99.5% ethanol. The amount of eluant used was 2.4 l of each. Each fraction denoted as Fr. 1, Fr. 2, Fr. 3, and Fr. 4 for eluate with 10% ethanol, 20% ethanol, 40% ethanol, and 99.5% ethanol, respectively, was taken to dryness. For isolation of 3,4-dicaffeoylquinic acid (3,4-diCQA) and 3,5-dicaffeoylquinic acid (3,5-diCQA), Fr. 2 was dissolved in a solvent containing 17.5% acetonitrile containing 2% acetic acid and applied to an HPLC system (Model PU-980 and UV-970, JASCO Co., Japan) with a Capcell Pak ACR (Shiseido, Tokyo, Japan) C18 column ($\phi 20 \times 250$ mm), and the column was eluted with the same solvent. As a result of detection at a wavelength of 325 nm, two major peaks were individually collected, and finally confirmed to be 3,4-diCQA and 3,5-diCQA by NMR (MERCURYplus 300NB; Varian, Inc., Palo Alto, CA, U.S.A.) analysis as described previously.²⁹⁾ For isolation of 3,4,5-tricaffeoylquinic acid (3,4,5-triCQA), Fr. 3 was dissolved in a solvent containing 30% acetonitrile containing 2% acetic acid and applied to an HPLC system with a Capcell Pak ACR C18 column, and the column was eluted with the same solvent. As a result of detection at a wavelength of 325 nm, three major peaks were individually collected. One of three peaks was identified as 3,4,5-triCQA by NMR analysis.

Animals and Measurements Male SHR (9–11 weeks old) and male Wistar Kyoto (WKY) rats (12 weeks old) were obtained from Hoshino Experimental Animal Breeding Farm, Inc. Animals were fed with a rat chow (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) at a room temperature of 23 ± 1 °C and at a relative humidity of $55 \pm 10\%$, and under conditions of lighting of 12 h/day (8:00–20:00). Tap water was given *ad libitum* as drinking water. After arrival the animals were acclimatized for 1–2 weeks. Animals showing no

abnormalities of health were used for the experiments. Body weight of the animals at the start of the experiments ranged between 230–275 g both for SHR and WKY. Experiments were conducted in accordance with the institutional Guideline for Feeding and Keeping of Experimental Animals. The blood pressure and heart rate were measured non-invasively with a tail-cuff method using Softron BP system (BP-98A, Softron, Tokyo, Japan), after warming the animals at 37 °C for 10 min. At least six determinations were made in every session of systolic blood pressure (SBP) measurements and the mean of six values within 5 mmHg was taken as the SBP level. In each experiment of single or repeated administration, we always confirmed when 30 mg/kg captopril³⁰⁾ or 10 mg/kg Val-Tyr³¹⁾ was administered in SHR as a positive control, the SBP decreased compared with vehicle control, which is consistent with previous reports.

Single Oral Administration of Propolis Extracted with Various Concentrations of Ethanol in SHR After preliminary acclimatization of SHR, in an environment as described above, animals showing an SBP over 185 mmHg (11–13 weeks old) were used.

Using the baseline (starting) blood pressure and body weight, animals were divided into 7 groups. These were: a control group and 6 groups to which propolis extracts (WEP, 10%-EEP, 25%-EEP, 50%-EEP, 70%-EEP, and 95%-EEP) were administered. Each group consisted of 6 animals. For animals that received propolis, each powder was either dissolved or suspended in 5% gum arabic solution and administered by gavage (100 mg/kg/10 ml). The same amount of 5% gum arabic solution was administered to the control group. In our preliminary experiments, we confirmed that the dose 100 mg/kg was enough to observe hypotensive effects of propolis extract. SBP and heart rate were measured before and 2, 4, and 6 h after administration.

Repeated Oral Administration of 25%-EEP and 70%-EEP in SHR After acclimatization of SHR, in an environment as described above, animals (11 weeks old) with SBP over 175 mmHg were used. The baseline blood pressure and body weight were recorded for reference. Animals were then divided into 3 groups (the control group and the groups that received 25%-EEP and 70%-EEP). EEP was suspended in 5% solution of gum arabic and administered orally twice a day (10:00 a.m. and 2:00 p.m.) for 28 d. Each dose was 5 mg/kg/10 ml. The same volume of 5% gum arabic solution was administered to the control group. SBP and heart rate were measured once a week before the first administration and 2 h after the second administration of test substances.

Single Oral Administration of 25%-EEP in SHR and WKY After acclimatization of SHR, in an environment as described above, animals (12 weeks old) with SBP over 185 mmHg were used. SHR and WKY rats (13 weeks old) were given single oral doses of 25%-EEP at 25, 50, and 100 mg/kg/10 ml. SBP and heart rate were measured before and 1, 2, 4, and 6 h after administration of extract and results were compared with those of the control group.

Single Oral Administration of Caffeoylquinic Acids in SHR After acclimatization of SHR in an environment as described above, animals (15 weeks old) with SBP over 185 mmHg were used. Single oral administration of caffeoylquinic acids (3,4-diCQA, 3,5-diCQA, and 3,4,5-triCQA) was conducted. The dose of each compound was

Table 1. Main Constituent's Contents in Water or Ethanol Extract of Propolis

| | Content (%) | | | | | |
|------------------------------|--------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| | WEP ^{b)} | 10%-EEP ^{b)} | 25%-EEP ^{b)} | 50%-EEP ^{b)} | 70%-EEP ^{b)} | 95%-EEP ^{b)} |
| Chlorogenic acid | 2.67 | 2.61 | 2.85 | 2.11 | 0.79 | 0.57 |
| Caffeic acid | 0.24 | 0.21 | 0.19 | 0.17 | 0.12 | 0.10 |
| <i>p</i> -Coumaric acid | 3.46 | 3.56 | 4.18 | 4.31 | 2.34 | 2.28 |
| Ferulic acid | 0.11 | 0.15 | 0.16 | 0.13 | 0.06 | 0.06 |
| 3,5-Dicaffeoylquinic acid | 4.30 | 4.69 | 5.69 | 5.16 | 3.06 | 2.40 |
| 3,4-Dicaffeoylquinic acid | 3.29 | 3.51 | 5.41 | 5.25 | 2.51 | 1.88 |
| 3,4,5-Tricaffeoylquinic acid | 0.20 | 0.26 | 0.86 | 1.17 | 0.72 | 0.60 |
| Isosakuranetin | N.D. ^{a)} | N.D. ^{a)} | N.D. ^{a)} | 0.79 | 1.29 | 1.19 |
| Artepillin C | 0.19 | 0.23 | 0.27 | 2.00 | 10.45 | 11.43 |

a) Not detected. b) Water extract of propolis (WEP) and ethanol extract of propolis (EEP).

10 mg/kg/10 ml, and SBP and heart rate were measured before and 1, 2, 4, and 6 h after administration of the compounds and results were compared with those of the control group.

Statistical Analysis Statistical analyses of differences of SBP values between the control group and the groups which received propolis was performed using the multiple comparison test of Dunnet for both the single and the repeated administration tests. In each group that received daily dosing of 25%-EEP or 70%-EEP, Student's paired *t*-test was performed to compare the values of SBP before repeated dosing (day 0) and those obtained during the period of administration. Bonferroni's test was performed to compare the values of SBP between the groups that received daily dosing of either 25%-EEP or 70%-EEP. The values obtained were expressed as mean \pm S.D. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Effects of Single Oral Administration of Propolis Extracted with Various Concentrations of Ethanol in SHR Brazilian propolis was extracted with water or various concentrations of ethanol, respectively, and their main constituents were analyzed by HPLC (Table 1). Each propolis extract was characterized by its phenolic and flavonoid contents.

Then single oral administration was performed using propolis (100 mg/kg) extracted with water or various concentrations of ethanol (10–95%). Comparisons of SBP were performed by Dunnet test between the control group and the groups to which propolis was administered. There was no significant difference in SBP in the control group before and after administration of vehicle, while hypotensive effects were observed in the groups that propolis extracts were administered (Fig. 1). 25%-EEP and 70%-EEP produced significant decrease in blood pressure. Especially marked were the effects observed in the group that received 25%-EEP; the values obtained 2 h after administration were significantly lower. The SBP decreased by 19.4 mmHg in the test group ($p < 0.01$). Thereafter, the decrease in the blood pressure started to rebound. However, even 6 h after administration, the SBP was 14.3 mmHg lower compared with the starting value. There were no changes in heart rates either in the control group or in the groups that received propolis (data not shown).

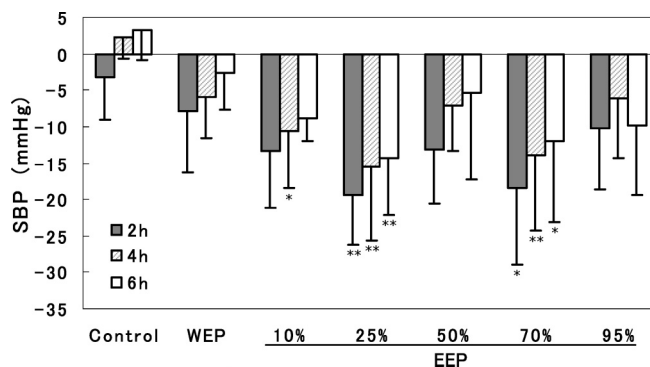


Fig. 1. Effects of Single Oral Administration of Propolis Extracted with Various Concentrations of Ethanol in SHR

Propolis was extracted with water or various concentrations (10, 25, 50, 70, 95%) of ethanol. 100 mg/kg of each extract was administered to SHR and systolic blood pressure (SBP) was measured 2, 4, and 6 h after administration. An SBP decrease in each time point compared with starting SBP was shown. Statistical significance between the control group and each group which received propolis was evaluated by Dunnet test ($n = 6$, $*p < 0.05$; $**p < 0.01$). Values are expressed as means \pm S.D.

Effects of Repeated Oral Administration of 25%-EEP and 70%-EEP in SHR Because 25%-EEP and 70%-EEP produced significant effects by single administration, we next examined effects of repeated oral administration of 25%-EEP and 70%-EEP. Comparisons of SBP were performed by Dunnet test between the control group and the groups to which propolis was administered. Measurements before the first administration revealed that significantly lower values in the groups that received 25%-EEP compared with the control group on any day of measurement ($p < 0.05$ on day 7, $p < 0.01$ on day 14, 21, and 28) (Fig. 2A). In the group that was given 70%-EEP, the SBP was significantly lower than that of the control group ($p < 0.05$), except for the 21st day (Fig. 2A). Measurements conducted 2 h after administration showed that SBP was significantly lower in the group that received 25%-EEP on the 14th day and thereafter compared with the control group ($p < 0.05$ on day 14, $p < 0.01$ on day 21 and 28) (Fig. 2B). The group that was given 70%-EEP showed a significantly lower SBP compared with the control group on the 14th and 28th days ($p < 0.05$) (Fig. 2B). In the control group, SBP increased by 20.0 mmHg on the 28th day compared with starting SBP before administration of vehicle and an increase of 19.2 mmHg 2 h after administration of vehicle. In contrast, in the group which received 25%-EEP, a decrease of 4.1 mmHg and an increase of 5.1 mmHg was observed at the same time points, respectively. For the group that received

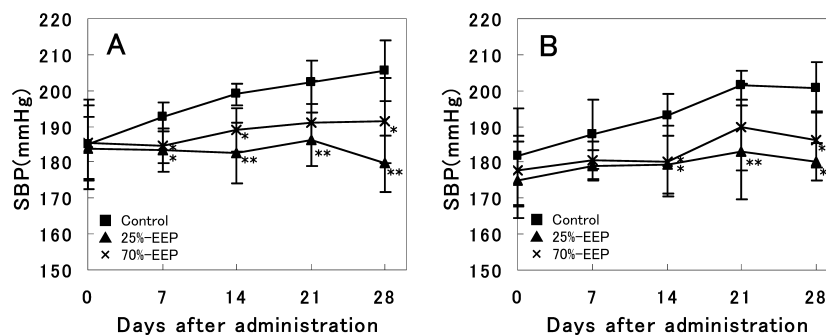


Fig. 2. Effects of Repeated Oral Administration of 25%-EEP and 70%-EEP on SBP in SHR

25%-EEP or 70%-EEP was administered to SHR for 28 d at a dose of 10 mg/kg. (A) SBP was measured before daily administration. (B) SBP was measured 2 h after daily administration. Statistical significance of difference of SBP values between the control group and each group which received propolis was evaluated by Dunnett test ($n=6$, $*p<0.05$; $**p<0.01$). Each point with a vertical bar represents the mean \pm S.D.

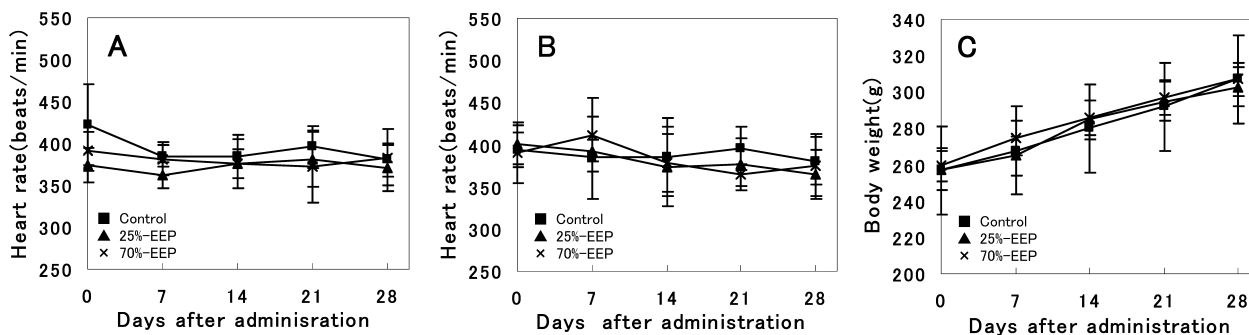


Fig. 3. Effects of Repeated Oral Administration of 25%-EEP and 70%-EEP on Heart Rate and Body Weight in SHR

25%-EEP or 70%-EEP was administered to SHR for 28 d at a dose of 10 mg/kg. Heart rate was measured before (A) and 2 h after (B) the daily administration. (C) Body weight was measured before daily administration. Each point with a vertical bar represents the mean \pm S.D.

70%-EEP, mean SBP increased by 6.0 and 8.7 mmHg, before and 2 h after administration, respectively, compared with starting SBP (day 0), indicating that the both extracts of propolis exerted inhibitory effects on the elevation of blood pressure.

In each group that received 25%-EEP or 70%-EEP, Student's paired *t*-test was performed to compare the values of SBP before repeated dosing (day 0) and those obtained during the period of administration. No significant differences were observed in the group that received 25%-EEP, while a significant increase was observed on the 28th day in the group that received 70%-EEP as compared with the values obtained before administration. Comparison made between the groups that received either 25%-EEP or 70%-EEP revealed that the elevation of the blood pressure was more potently suppressed in the group administered with 25%-EEP, although the differences between the groups were not significant (Bonferroni's test).

As regards the heart rate, no differences were observed within and between groups before and after administration of extracts (Figs. 3A, B). Throughout the period of administration, there were no abnormal findings as regards the general condition of the animals. There were also no differences within and between groups as regards the body weight of animals receiving propolis (Fig. 3C).

Single Oral Administration of 25%-EEP in SHR and WKY By single and repeated administration, 25%-EEP showed the most significant hypotensive activity, then we examined effects of dose responses of 25%-EEP on SBP in SHR and compared with WKY. SHR were administered with

a single oral dose of 25, 50, and 100 mg/kg of 25%-EEP. Comparisons of SBP were performed by Dunnett test between the control group and the groups to which propolis was administered. No significant effects on the blood pressure were observed with 25 mg/kg. In contrast, significant hypotensive effects were observed beginning 1 h after administration of 50 and 100 mg/kg (Fig. 4A). An SBP decrease of 9.7 mmHg was observed 1–2 h after administration of 50 mg/kg 25%-EEP compared with starting SBP and a maximum SBP decrease of 19.7 mmHg was observed 1 h after administration of 100 mg/kg. The effect of 100 mg/kg was durable; a significant decrease of 11.9 mmHg was observed even 6 h after administration. There were no differences as regards heart rate within and between groups before and after administration (data not shown).

In contrast to the SHR, no differences in the change of SBP (Fig. 4B) and heart rate (data not shown) of WKY were observed between the control group and the group administered with 25%-EEP. Figure 4C depicts the differences between the blood pressures before and 1 h after the administration of 25%-EEP observed in SHR and WKY rats. While administration of 25%-EEP resulted in a dose-dependent decrease of the blood pressure in SHR, the treatment produced almost no effect on the blood pressure in WKY.

Effects of Caffeoylquinic Acids on SBP in SHR As shown in Table 1, 25%-EEP was characterized by its contents of caffeoylquinic acids which were most abundant among WEP and EEP prepared in this study. At an initial effort toward identification of constituents involved in the hypotensive activity of propolis, effects of three kinds of caf-

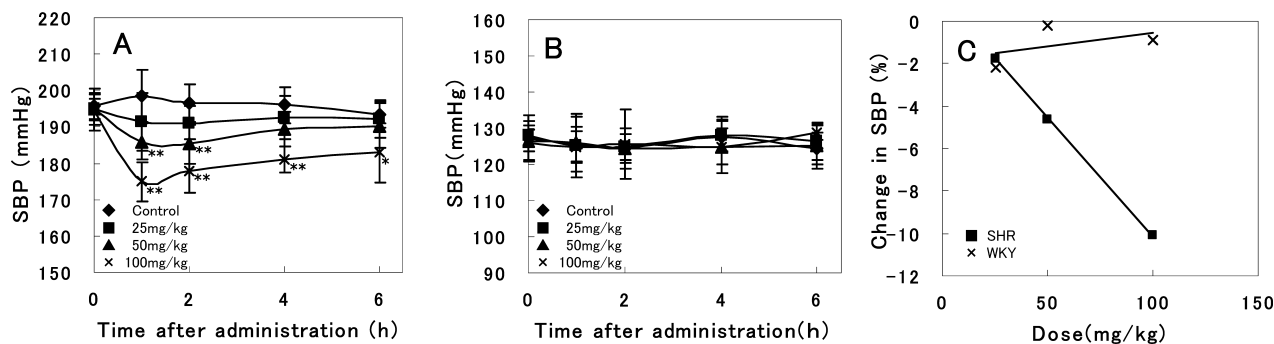


Fig. 4. Effects of Single Oral Administration of 25%-EEP on SBP in SHR and WKY

25%-EEP was administered to SHR (A) or WKY (B) at a dose of 25, 50, and 100 mg/kg, respectively, and SBP was measured 1, 2, 4, and 6 h after administration. Statistical significance of difference of SBP values between the control group and each group which received propolis was evaluated by Dunnet test ($n=6$, $*p<0.05$; $**p<0.01$). Each point with a vertical bar represents the mean \pm S.D. (C) The differences between SBP before and 1 h after the administration of 25%-EEP observed in SHR (A) and WKY (B).

caffeoylquinic acids on SBP in SHR were examined. Single oral administration of 10 mg/kg each of 3,4-diCQA, 3,5-diCQA, and 3,4,5-triCQA, was conducted in SHR, and SBP was measured. Each of the three components produced a significant decrease of blood pressure beginning approximately 1 h after administration (Fig. 5). When 3,4,5-triCQA was administered, the decrease was significant at all points of measurements compared with SBP of the control group. The maximum decrease of SBP was observed about 1 h after administration with all three components. The SBP decreased 9.3 mmHg with 3,4-diCQA, 8.4 mmHg with 3,5-diCQA, and 13.0 mmHg with 3,4,5-triCQA compared with starting SBP. As regards the heart rate, no differences were observed within or between groups with all three components before and after administration (data not shown).

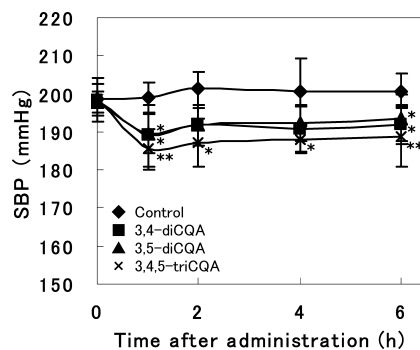


Fig. 5. Effects of Single Oral Administration of Caffeoylquinic Acids on SBP in SHR

3,4-DiCQA, 3,5-diCQA, or 3,4,5-triCQA was administered to SHR at a dose of 10 mg/kg and SBP was measured 1, 2, 4, and 6 h after the administration. Statistical significance of difference of SBP values between the control group and each group which received CQA was evaluated by Dunnet test ($n=6$, $*p<0.05$; $**p<0.01$). Each point with a vertical bar represents the mean \pm S.D.

DISCUSSION

Propolis is usually extracted with water or 90–99.5% ethanol, and they have been used in folk medicine. In recent years, they have become common additives in health foods and beverages. Although there have been a report on hypotensive activity of Brazilian propolis extract,²⁸ little information is available concerning effective extraction, including ingredients, underlying the hypotensive effect. In this study, at an initial effort insight into hypotensive effects of propolis, effects of various propolis extracts on blood pressure and heart rate were examined using SHR.

Considering that the composition of propolis extract and, thus, the hypotensive effects, differ depending on the water content of ethanol used for extraction of propolis, in this study we extracted propolis with water and 10, 25, 50, 70, and 95% ethanol and examined the hypotensive effects of these extracts in SHR. As a result, it became clear that significant hypotension was observed in animals that received 25%-EEP or 70%-EEP (Fig. 1).

Each propolis extract was characterized by its phenolic and flavonoid contents (Table 1). Noteworthy were the findings that caffeoylquinic acids such as 3,4-diCQA (5.41%), 3,5-diCQA (5.69%), and 3,4,5-triCQA (0.86%) comprised the 11.96% of 25%-EEP. In 70%-EEP, the amount of cinnamic acid derivatives including chlorogenic, *p*-coumaric, and caffeoylquinic acids decreased, and flavonoids increased. These results suggest that components involved in the production of hypotensive effects are different between 25%-

EEP and 70%-EEP, although there is some overlap.

As a result of repeated oral administration, the hypotensive activity of 25%-EEP was more significant compared with control group than that of 70%-EEP, although the differences between the groups were not significant (Bonferroni's test). So we firstly focused on 25%-EEP to support the idea that it is an effective extraction for the hypotensive effect. There was a tendency for blood pressure to decrease slightly after single oral administration of 25 mg/kg of 25%-EEP in SHR. While the maximum decrease of blood pressure was observed 2 h after administration of 50 mg/kg, it was observed 1 h after administration with 100 mg/kg, indicating the hypotensive effect was dose-dependent. With 100 mg/kg, the decrease of blood pressure was maintained up to 6 h after administration, suggesting that the hypotensive effects of 25%-EEP was long lasting, as was observed in experiments with repeated oral administration. In view of the findings that the blood pressure and heart rate of groups of WKY rats that received this compound did not differ significantly from those of the control group, it was inferred that 25%-EEP did not exert effects on the normal blood pressure and heart rate.

Since caffeoylquinic acids are the characteristic components of 25%-EEP, we assumed that the constituents involved in the hypotensive effects of 25%-EEP are 3,4-diCQA, 3,5-diCQA, and 3,4,5-triCQA. By single oral administration of these caffeoylquinic acids to SHR each at 10 mg/kg, significant hypotensive effects starting with 1 h after administration

were observed. Using the dose-response curve depicted with percent changes in SBP (%) observed after administration of 25%-EEP (Fig. 4C), comparison was made of the percent changes observed in SBP 1 h after single oral administration of 10 mg/kg of each of the caffeoylquinic acids. We found that administration of 10 mg/kg of 3,4-diCQA, 3,5-diCQA, and 3,4,5-triCQA was equivalent to that of 51.0 mg/kg, 46.5 mg/kg, and 67.2 mg/kg of 25%-EEP, respectively. As described previously,³¹⁾ the percentage contribution of the three caffeoylquinic acids to hypotensive effects was calculated using the following formula. Percentage contribution to hypotensive effects=(Amount of hypotensive component (mg) contained in propolis that showed hypotensive effects)/(amount of the component that showed hypotensive effects)×100. The contribution rate is found to be 27.6% for 3,4-diCQA, 26.5% for 3,5-diCQA, and 5.8% for 3,4,5-triCQA, respectively. The contribution rate of the total caffeoylquinic acids is over 50%. These results suggest that 3,4-diCQA, 3,5-diCQA, and 3,4,5-triCQA are the main constituents involved in the hypotensive effects of 25%-EEP. These caffeoylquinic acids are also comparatively abundant in 50%-EEP, while its hypotensive activity is not so potent. This might be speculated presence of some antagonists and/or competitive agonists in 50%-EEP, although further works are needed to elucidate the mechanisms for the hypotension by caffeoylquinic acids.

Yoshimoto *et al.*³²⁾ isolated 3,4-diCQA, 3,5-diCQA, and 3,4,5-triCQA from sweet potato leaf, and showed their antimutagenic activity. Matsui *et al.*³³⁾ isolated caffeoylquinic acid derivatives from propolis as constituents involved in α -glucosidase inhibitory activity, and demonstrated that 3,4,5-triCQA has most potent activity among them. It was also reported these caffeoylquinic acids have activities including antiinflammation, anti-HIV, and inhibition of melanin production.^{34–36)} In this context, the hypotensive activity of caffeoylquinic acid is one of the diverse physiological activities.

Recent study reported that caffeic acid phenethyl ester (CAPE), one of the major components of European and Asian propolis, has vasodilating activity on isolated rat aorta, which is only partially dependent on nitric oxide, and which is, at high concentrations, likely due to an inhibitory effect on calcium movements through cell membranes.¹¹⁾ These findings thus provide important clues for our further study of mechanisms for the hypotension by caffeoylquinic acids.

In summary, this study provides evidence that propolis extract (especially 25%-EEP) has hypotensive effects in SHR, which is at least in part mediated by caffeoylquinic acids. Further studies are in progress in our laboratory to support the idea that propolis is an effective dietary supplement for the improvement of hypertension.

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