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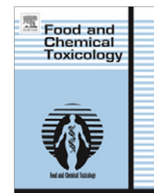

Antioxidant, anti-semicarbazide-sensitive amine oxidase, and anti-hypertensive activities of geraniin isolated from *Phyllanthus urinaria*

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ABSTRACT

The wrinkle-fruited leaf flower (*Phyllanthus urinaria* L.) (Euphorbiaceae) is widely used as a traditional folk medicine for inflammatory relief. Geraniin, the hydrolysable tannin, was purified by a series of chromatographic processes from the 70% aqueous acetone extracts of *P. urinaria* and identified by NMR [^{1}H (500 MHz) and ^{13}C NMR (126 MHz)] spectra and mass spectroscopy. The scavenging activities of geraniin against DPPH radicals (half-inhibition concentration, IC_{50}, were 0.92 and 1.27 μM, respectively, for pH 4.5 and pH 7.9), hydroxyl radicals (IC_{50} was 0.11 μM by deoxyribose method and 1.44 μM by electron spin resonance method), and superoxide radicals (IC_{50} were 2.65 μM) were determined in comparison with positive controls. The inhibitory activities against xanthine oxidase (IC_{50} were 30.49 μM) were measured. Geraniin also showed dose-dependent inhibitory activities against semicarbazide-sensitive amine oxidase (SSAO, IC_{50} were 6.58 μM) and against angiotensin converting enzyme (ACE, IC_{50} were 13.22 μM). For kinetic property determinations, geraniin showed competitive inhibitions against SSAO (the apparent inhibition constant, K_i, was 0.70 μM) and mixed noncompetitive inhibitions against ACE. Spontaneously hypertensive rats (SHR, 10-week age) were orally administered to once (5 mg geraniin/kg SHR), and changes of systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured over 24 h and compared with the positive control of captopril (2 mg/kg SHR). The geraniin showed antihypertensive activity in lowering SBP and DBP and showed a significant difference from the blank (distilled water) at 2, 4, 6, 8, and 24 h. Healthy food products could use geraniin for antioxidant protection and therapeutic effects in the future.

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1. Introduction

Active oxygen species and free radical-mediated reactions are involved in degenerative or pathological processes such as aging (Ames et al., 1993; Harman, 1995), cancer, coronary heart disease and Alzheimer's disease (Ames, 1983; Gey, 1990; Smith et al., 1996; Diaz et al., 1997). Meanwhile, many epidemiological results reveal an association between people who have a diet rich in fresh fruit and vegetables and a decrease in the risk of cardiovascular diseases and certain forms of cancer (Salah et al., 1995). Several reports have focused on the antioxidant activities of natural compounds in fruits and vegetables such as echinacoside in *Echinaceae* root (Hu and Kitts, 2000), anthocyanin (Espin et al., 2000), and various phenolic compounds (Rice-Evans et al., 1997).

Hypertension is considered to be the central factor in stroke with approximately 33% of deaths due to stroke attributed to untreated high blood pressure (Mark and Davis, 2000). Several classes of pharmacological agents have been used in the treatment of hypertension (Mark and Davis, 2000). One class of anti-hypertensive drugs, known as angiotensin I converting enzyme (ACE) inhibitors (i.e. peptidase inhibitors), has a low incidence of adverse side-effects and are the preferred class of anti-hypertensive agents when treating patients with concurrent secondary diseases (Fotherby and Panayiotou, 1999). ACE (peptidyldipeptide hydrolyase EC 3.4.15.1) is a dipeptide-liberating Zn-containing exopeptidase, which removes a dipeptide from the C-terminus of angiotensin I to form angiotensin II, a very hypertensive compound. Several antioxidant peptides (reduced glutathione and carnosine-related peptides) exhibit ACE inhibitory activities (Hou et al., 2003). Pomegranate juice (Aviram and Dornfeld, 2001), flavan-3-ols and procyanidins (Actis-Goretta et al., 2003), and tannins (Liu et al., 2003) have been reported to have ACE inhibitory activity. Sato et al. (2002) pointed out that three dipeptides, including AW

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(IC_{50} = 18.8 μM), VW (IC_{50} = 3.3 μM), and LW (IC_{50} = 23.6 μM), were potential ACE inhibitory peptides. However, none of them were able to effectively reduce the blood pressure of spontaneously hypertensive rats (SHRs) in animal models. Fujita et al. (2000) also found a similar phenomenon in SHRs.

Semicarbazide-sensitive amine oxidase (SSAO, EC 1.4.3.6) is the common name for a group of heterogeneous enzymes widely distributed in nature, in plants, microorganisms, and the organs of mammals (vasculature, dental pulp, eye and plasma) (Boomsma et al., 2000). SSAO converts primary amines into the corresponding aldehydes, generating hydrogen peroxide and ammonia. It was found that the endogenous compounds aminoacetone and methylamine are good substrates for most SSAOs (Lyles and Chalmers, 1992). Recent research has found that plasma SSAO was raised in diabetes mellitus and heart failure and is implicated in atherosclerosis (Yu and Zuo, 1996; Boomsma et al., 1997).

The *Phyllanthus urinaria* L., also called "pearls under the leaves" in Chinese, is widely used as a traditional folk medicine (Calixto et al., 1998). It was reported that boiling water extracts of *P. urinaria* exhibited cytotoxic activity against Lewis lung carcinoma cells (Huang et al., 2003) and human cancer cells such as HL-60, Molt-3, HT 1080, K-562, Hep G2, and NPC-BM1 (Huang et al., 2004). The boiling water extracts of *P. urinaria* were also reported to exhibit anti-tumor and anti-angiogenic effects against Lewis lung carcinoma in mice (Huang et al., 2006). The organic solvent (including acetone, ethanol, and methanol) extracts of *P. urinaria* were able to inhibit HSV-2 infection (Yang et al., 2005). The ethanolic extracts of *P. urinaria* were reported to have antioxidant and cardioprotective effects against doxorubicin-induced cardiotoxicity (Chularojmontri et al., 2005). The 50% methanolic extracts of *Phyllanthus niruri* were reported to have inhibitory activities against platelet aggregations (Iizuka et al., 2007). Several natural products were isolated from different *Phyllanthus* species, including flavonoids, lignans, alkaloids, triterpenes, and tannins (Calixto et al., 1998). The ellagic acid, a flavonoid isolated from *P. urinaria*, was reported to have anti-HBV infection activity (Shin et al., 2005). The gallic acid and geraniin isolated from *P. emblica* were the major compounds responsible for NO scavenging activities (Kumaran and Karunakaran, 2006). The geraniin and 1,3,4,6-tetra-*O*-galloyl-β-D-glucose isolated from *P. urinaria* exhibited anti-infection activities against HSV-1 and HSV-2 (Yang et al., 2007). The purpose of this study was to investigate the biological activities of purified geraniin from *P. urinaria*, including its antioxidant capacity, anti-SSAO activity and antihypertensive activity *in vitro* and *in vivo*. The results presented here will benefit the effort to develop healthy food products using geraniin for antioxidant protection and blood pressure regulation in the future.

2. Materials and methods

2.1. Materials

ACE (I unit, rabbit lung), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), benzylamine, bovine plasma (P-4639, reconstitute with 10 ml deionized water), butylated hydroxytoluene (BHT), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferrous sulfate, *N*-(3-[2-furyl]acryloyl)-Phe-Gly-Gly (FAPGG), horseradish peroxidase (148 units/mg solid), NADH, phenazine methosulfate (PMS), semicarbazide, xanthine, and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (33%) was from Wako Pure Chemical Industry (Osaka, Japan). Other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Geraniin isolation

The whole plants of fresh *P. urinaria* were collected from Taipei County and identified by Prof. Lih-Geeng Chen at the Graduate Institute of Biopharmaceutics, National Chiayi University, Chiayi. A voucher specimen (PU001) was deposited in the Graduate Institute of Pharmacognosy, Taipei Medical University. The whole plants of *P. urinaria* were washed and air-dried below 40 °C to yield 500 g of dried plants which were homogenized in 70% aqueous acetone (10 l) and filtered. The filtrate was concentrated under a vacuum using a rotary evaporator and then lyophilized for further use. Column chromatography was carried out on a Toyopearl HW-40 C (Tosoh Corp., Tokyo, Japan) and Diaion HP-20 (Mitsubishi Chemical Industry Co., Ltd.). The precipitates were dissolved in distilled water and chromatographed over a Diaion HP-20 column (50 cm × 7.0 cm i.d.) with aqueous MeOH (0% → 20% → 40% → 60% MeOH) and 70% acetone. The 40% MeOH eluate was rechromatographed over a TSK HW-40C column eluted with H_2O → 60% MeOH → 70% MeOH → 70% acetone. The crude geraniin was obtained from 70% MeOH elutant and recrystallized with cold aqueous MeOH. The yellow crystal of geraniin (Fig. 1) was obtained (200 mg) and identified by direct comparison of its NMR and mass spectroscopic data with authentic samples (Yoshida et al., 1988). Geraniin purity was shown by normal and reversed-phase high-performance liquid chromatography to exceed 95%. ^{1}H (500 MHz) and ^{13}C NMR (126 MHz) spectra were measured on a Bruker DRX 500 instrument. The ESI-MS were taken on a Waters ZQ-4000 mass spectrometer with a direct injection of geraniin solution (in MeOH). The geraniin was prepared as a stock solution (1 mM in distilled water) and stored at 4 °C for further use.

2.3. Scavenging activity of DPPH radical by spectrophotometry

Every 0.3 ml of geraniin (the final concentration was 0.08, 0.39, 0.78, 1.48, and 1.97 μM), BHT, and ascorbic acid (the final concentration was 2.4, 6.0, 12, 24, and 60 μM) was added to 0.1 ml of 1 M Tris–HCl buffer (pH 7.9) or acetate buffer (pH

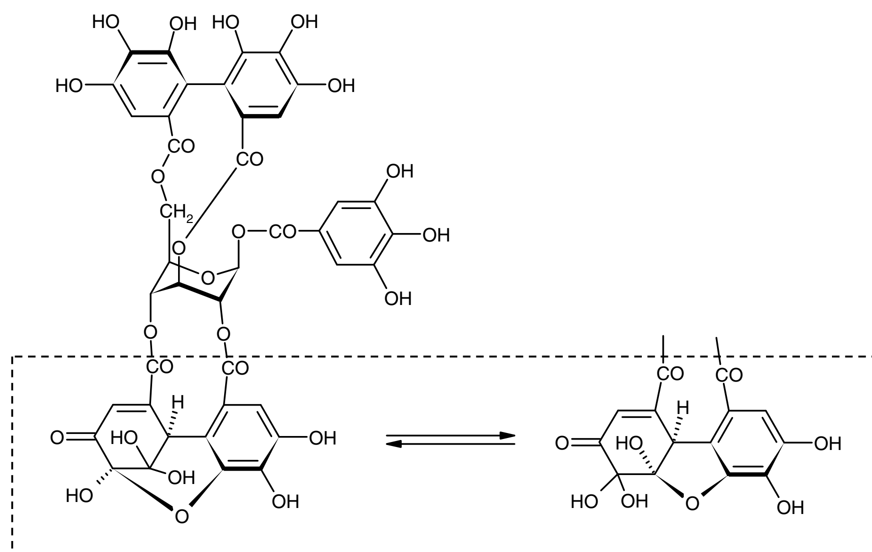

Fig. 1. The structure of geraniin.

4.5) and then mixed with 0.6 ml of 100 μM DPPH in methanol to a final concentration of 60 μM for 20 min under light protection at room temperature (Hou et al., 2002; Liu et al., 2003). The decrease of absorbance at 517 nm was measured and expressed as $\Delta A517$ nm. Deionized water or methanol (for BHT scavenging assay) was used instead of sample solution as a blank experiment. The scavenging activity of DPPH radical (%) was calculated with the equation: $\Delta A517_{blank}$ $\Delta A517_{sample}) \div \Delta A517_{blank} \times 100\%$. The IC_{50} stands for the concentration of half-inhibition. The KCl–HCl buffer (pH 2.0, 2.5, and 3.0), acetate buffer (pH 3.0, 3.5, 4.0, 4.5, 5.0, and 5.5), phosphate buffer (pH 6.0, 6.5, 7.0, 7.5, and 8.0), and Tris–HCl buffer (pH 7.0, 7.5, 7.9, 8.0, 8.5, and 9.0) were used to determine the optimal pH for the scavenging activity of the DPPH radicals of geraniin.

2.4. Scavenging activity of geraniin against metal ion-dependent hydroxyl radicals

The hydroxyl radical was generated by a metal ion-dependent reaction based on the method of Kohno et al. (1991). The scavenging activity of hydroxyl radical was determined by the deoxyribose method (Halliwell et al., 1987) or electron spin resonance (ESR) method. For deoxyribose method determination, every 0.5 ml sample containing a different amount (the final concentration was 0.0003, 0.03, and 0.3 μM) of geraniin was added to 1.0 ml solution of 20 mM potassium phosphate buffer (pH 7.4), 2.8 mM 2-deoxy-ribose, 104 μM EDTA, 100 μM FeCl3, 100 μM ascorbate, and 1 mM hydrogen peroxide. The mixtures were incubated for 1 h at 37 °C. After incubation, an equal volume of 0.5% thiobarbituric acid in 10% trichloroacetic acid was added, and the mixtures were boiled at 100 °C for 15 min. Deionized water was used instead of sample solution in a blank experiment. The absorbance at wavelength 532 nm was measured. The scavenging activity of hydroxyl radicals (%) was calculated with the equation: $(A532_{blank} - A532_{sample}) \div A532_{blank} \times 100\%$. The IC_{50} stands for the concentration of half-inhibition. For ESR determination, the mixture included different amounts of geraniin (the final concentration was 0.66, 1.64, 3.28, and 6.56 μM), 5 mM DMPO and 0.05 mM ferrous sulfate. After mixing, the solution was transferred to an ESR quartz cell, placed in the cavity of the ESR spectrometer, and then the hydrogen peroxide was added to a final concentration of 0.25 mM in a 500 l total volume. Deionized water was used instead of sample solution for control experiments. After 40 s, the relative intensity of the DMPO-OH spin adduct signal was measured. All ESR spectra were recorded at the ambient temperature (298 K) on a Bruker EMX-6/1 EPR spectrometer equipped with WIN-EPR SimFonia software, Version 1.2. The conditions of ESR spectrometry were as follows: center field 345.4 ± 5.0 mT; microwave power 8 mW (9.416 GHz); modulation amplitude 5 G; modulation frequency 100 kHz; time constant 0.6 s; scan time 1.5 min.

2.5. Scavenging activity of geraniin against superoxide radicals by spectrophotometry

The superoxide radical was generated by the PMS–NADH system (Liu et al., 2004). All 0.2 ml samples, containing different amounts of geraniin (the final concentration was 0.15, 1.54, 2.32, 3.86, and 7.72 μM), were added in sequence to 0.2 ml of 630 μM nitroblue tetrazolium, 0.2 ml of 33 μM PMS, and 0.2 ml of 156 μM NADH in 100 mM phosphate buffer (pH 7.4). Deionized water was used instead of geraniin solution as a blank experiment. Ascorbic acid (the final concentration was 6, 9, 12, and 24 μM) was used as a positive control. The changes of absorbance at 560 nm were recorded during 2 min and expressed as $\Delta A560$ nm/min. The scavenging activity of superoxide radicals was calculated as follows: $(\Delta A560\ \text{nm/min}_{blank} - \Delta A560\ \text{nm/min}_{sample}) \div \Delta A560\ \text{nm/min}_{blank} \times 100\%$. IC_{50} stands for the concentration of half-inhibition.

2.6. Inhibitory activity of geraniin against xanthine oxidase

The xanthine oxidase activity was measured by determining uric acid formation at 295 nm using xanthine as substrate (Kalckar, 1947). The different amounts of geraniin (the final concentration was 19.68, 26.24, 39.36, and 45.93 μM) were pre-mixed with 8 mU xanthine oxidase for 1 h at 4 °C, and then the 300 μl of 1 mM xanthine and 300 μl of 200 mM were added. The changes of absorbance at 295 nm were recorded over 3 min and expressed as $\Delta A295$ nm/min. The xanthine oxidase inhibitory activity was calculated as follows: $(\Delta A295\ \text{nm/min}_{blank} - \Delta A295\ \text{nm/min}_{sample}) \div \Delta A295\ \text{nm/min}_{blank} \times 100\%$. Deionized water was used instead of geraniin solution as a blank experiment. IC_{50} stands for the concentration of half-inhibition.

2.7. SSAO inhibitory activities of geraniin

SSAO inhibitory activity was determined by the spectrophotometric method of Szutowicz et al. (1984) with some modifications. The total 200-μl reaction solution [containing 50 μl of 200 mM phosphate buffer, pH7.4, 50 μl of 8 mM benzylamine, SSAO (2.53 units from bovine plasma) and different amounts of geraniin (the final concentration was 0.66, 1.64, 3.28, and 6.56 μM) and semicarbazide (the final concentration was 5, 10, 25, and 50 μM)] was placed at 37 °C for 1 h and then heated at 100 °C to stop the reaction. After cooling and a brief centrifugation, the 90-μl reaction solution was isolated and added to the 710-μl solution containing 200 μL of 200 mM phosphate buffer (pH 7.4), 100 μl of 2 mM ABTS solution, and 25 μl of horseradish peroxidase (10 μg/ml). The changes of absorbance at 420 nm were recorded during 1 min and expressed as $\Delta A420$ nm/min. Means of triplicates were recorded. Deionized water was used instead of geraniin solution as a blank experiment. The SSAO inhibition (%) was calculated with the equation: $(\Delta A420\ \text{nm/min}_{blank} - \Delta A420\ \text{nm/min}_{sample}) \div \Delta A420\ \text{nm/min}_{blank} \times 100\%$. IC_{50}stands for the concentration of 50% inhibition.

2.8. The kinetic properties of SSAO inhibition of geraniin

The kinetic properties of SSAO (2.53 units) without or with geraniin (1.64 μM) additions were determined from Lineweaver–Burk plots using different concentrations of benzylamine as substrates (0.67, 0.8, 1, 1.33 and 2 mM). The K_i was calculated using the equation of $K_i = [I]\ /(K'_m/K_m) - 1$, where $[I]$ was the concentration of 1.64 μM and K'_m was the Michaelis constant in the presence of geraniin at concentration $[I]$.

2.9. ACE inhibitory activity of geraniin

The ACE inhibitory activity was measured according to the method of Holmquist et al. (1979) with some modifications. Twenty microlitre (20 mU) commercial ACE (1 U/ml, rabbit lung) were mixed with 200 μl of different amounts of geraniin (the final concentration was 0.5, 1.0, 2.5, 5.0, 10, 15, and 20 μM) and then 1 ml of 0.5 mM FAPGG [dissolved in 50 mM Tris–HCl buffer (pH 7.5) containing 0.3 M NaCl] was added. The decreased absorbance at 345 nm ($\Delta A_{inhibitor}$) was recorded during 5 min at room temperature. Deionized water was used instead of geraniin solution as a blank experiment (ΔA_{blank}). The ACE activity was expressed as $\Delta A345$ nm and the ACE inhibition (%) was calculated as followed: $[1 - (\Delta A_{inhibitor} \div \Delta A_{blank})] \times 100\%$. Means of triplicates were recorded.

2.10. The kinetic properties of ACE inhibition of geraniin

The kinetic properties of ACE without or with geraniin (1.0 μM) were determined using different concentrations of FAPGG as substrates (0.1, 0.125, 0.25 and 0.5 mM). The K_m and K'_m were calculated from Lineweaver–Burk plots where the K'_m was the Michaelis constant in the presence of geraniin at concentration of 1.0 μM.

2.11. Antihypertensive effects of geraniin on SHR

The effects of orally administered geraniin or captopril by feeding tube (2.0 × 80 mm) on the reduced SBP and the reduced DBP were determined (Lin et al., 2006; Liu et al., 2007a). All animal experimental procedures followed published guidelines (National Science Council, 1994). The male SHRs (8 weeks of age, National Laboratory Animal Center, Taipei) were housed individually in steel cages kept at 24 °C with a 12-h light–dark cycle and had free acess to a standard laboratory diet (5001 Rodent Diet, St. Louis, MO) and water. SHRs were randomly divided into control and geraniin treatments for SBP and DBP determinations (six rats per group). For a short-term antihypertensive experiment, 0.5 ml of 5 mg geraniin/kg SHR or 2 mg captopril/kg SHR were orally administered once, and tail blood pressure was measured four times at each desired time over 24 h using an indirect blood pressure meter (BP-98A, Softron Co. Ltd. Tokyo, Japan) for each treatment. The 0.5 ml distilled water was used for a blank experiment. Before each blood pressure measurement, SHRs were warmed for 10 min in a 39 °C thermostated box. Means of triplicates were recorded. The one-way ANOVA followed by the post-hoc Tukey's test was performed at the same time. A value of $P < 0.05$ was considered to be statistically significant between geraniin and distilled water or captopril and distilled water or geraniin and captopril.

3. Results

3.1. Scavenging activity of DPPH radicals

In the beginning, 0.75 μM geraniin was used to screen the DPPH radical scavenging activity under different pH conditions (Fig. 2A). Different kinds of buffer and pH conditions were thought to influence DPPH scavenging activity. Under pH 7.0–8.0 conditions, the geraniin in Tris–HCl buffer had more DPPH scavenging activity than that in phosphate buffer (Fig. 2A). However, the acetate buffer at pH 4.5 resulted in the highest activity. Therefore, the acetate buffer (pH 4.5) and Tris–HCl buffer (pH 7.9) were selected for dose-dependent scavenging activities. Geraniin exhibited dose-dependent DPPH scavenging activities at either pH 4.5 or pH 7.9. At pH 4.5, there was 4.43%, 23.03%, 43.74%, 75.75% and 87.6% of scavenging activity, respectively, for 0.08, 0.39, 0.79, 1.48 and 1.97 μM of geraniin. At pH 7.9, there was 3.75%, 17.49%, 33.02%,

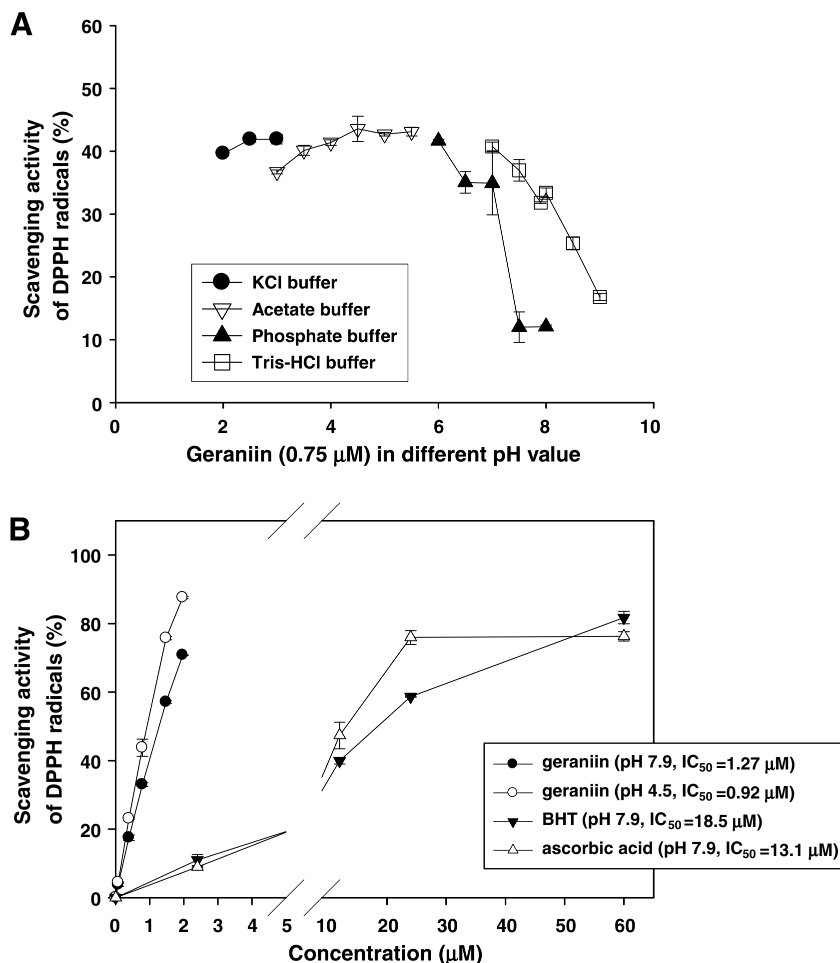

Fig. 2. (A) The DPPH scavenging activity of geraniin (0.75 μM) in different pH conditions, including the KCl–HCl buffer, pH 2.0, 2.5, and 3.0; the acetate buffer, pH 3.0, 3.5, 4.0, 4.5, 5.0, and 5.5; the phosphate buffer, 6.0, 6.5, 7.0, 7.5, and 8.0; and the Tris–HCl buffer, 7.0, 7.5, 7.9, 8.0, 8.5, and 9.0. (B) The effects of different amounts of geraniin on the scavenging activities of DPPH radicals in acetate buffer, pH 4.5 and Tris–HCl buffer, pH 7.9. The ascorbic acid and BHT were used as positive controls.

57.08% and 70.73% of scavenging activity, respectively, for 0.08, 0.39, 0.79, 1.48 and 1.97 μM of geraniin. The IC_{50} values were 0.92 μM and 1.27 μM, respectively, for pH 4.5 and pH 7.9 (Fig. 2B), much better than that of ascorbic acid (IC_{50} of 13.1 μM) and BHT (IC_{50} of 18.5 μM) at pH 7.9.

3.2. Scavenging activity of hydroxyl radicals

The hydroxyl radical was generated by a metal ion-dependent reaction according to the method of Kohno et al. (1991). The scavenging activity of hydroxyl radical was determined by the deoxyribose method (Fig. 3A) or ESR (Fig. 3B) method. Geraniin was found to exhibit dose-dependent OH**.** scavenging activities in the deoxyribose assay, and this activity was 27.48%, 45.64%, and 59.86%, respectively, for 0.0003, 0.03, and 0.3 μM of geraniin. The IC_{50} value was calculated to be 0.11 μM (Fig. 3A). In the ESR assay method OH**.** scavenging activities were 27.79%, 55.58%, 70.67%, and 81.92%, respectively, for 0.66, 1.64, 3.28, and 6.56 μM of geraniin. The IC_{50} value was calculated to be 1.44 μM (Fig. 3B).

3.3. Scavenging activity of superoxide radicals and inhibitory activity against xanthine oxidase

The PMS–NADH system was used to generate the superoxide radicals (Liu et al., 2004). Geraniin was found to exhibit dose-dependent superoxide radical scavenging activities of 6.01%, 15.44%, 44.20%, 70.65%, and 87.95%, respectively, for 0.15, 1.54, 2.32, 3.86, and 7.72 μM of geraniin. The IC_{50} value was calculated to be 2.65 μM (Fig. 4A), much better than that of ascorbic acid (IC_{50} 8.97 μM). For xanthine oxidase inhibition, geraniin was found to exhibit dose-dependent inhibitory activities of 30.81%, 41.08%, 68.65%, and 72.97%, respectively, for 19.68, 26.24, 39.36, and 45.93 μM of geraniin. The IC_{50} was calculated to be 30.49 μM (Fig. 4B).

3.4. SSAO inhibitory activities of geraniin and kinetic properties

The SSAO inhibitory activities of geraniin were compared with those of semicarbazide (5, 10, 25, and 50 μM), the positive control. Geraniin was found to exhibit dose-dependent SSAO inhibitory activities of 10.87%, 37.24%, 77.67%, and 95.77%, respectively, for 0.66, 1.64, 3.28, and 6.56 μM of geraniin. The IC_{50} was calculated to be 6.58 μM which was much lower than that semicarbazide (IC_{50} of 34.21 μM) (Fig. 5A). The 1.64 μM geraniin was used to determine the kinetic properties of SSAO inhibition. Geraniin showed competitive inhibitions against SSAO (Fig. 5B). The K_m was 2.18 mM, and the K'_m was 7.28 mM in the presence of geraniin. In our calculations, the K_i was 0.70 μM.

3.5. ACE inhibitory activities of geraniin and kinetic properties

Geraniin exhibited dose-dependent ACE inhibitory activities of 5.71%, 8.57%, 17.14%, 22.86%, 37.14%, 57.14%, and 65.71%, respectively, for 0.5, 1.0, 2.5, 5.0, 10, 15, and 20 μM of geraniin. The IC_{50}

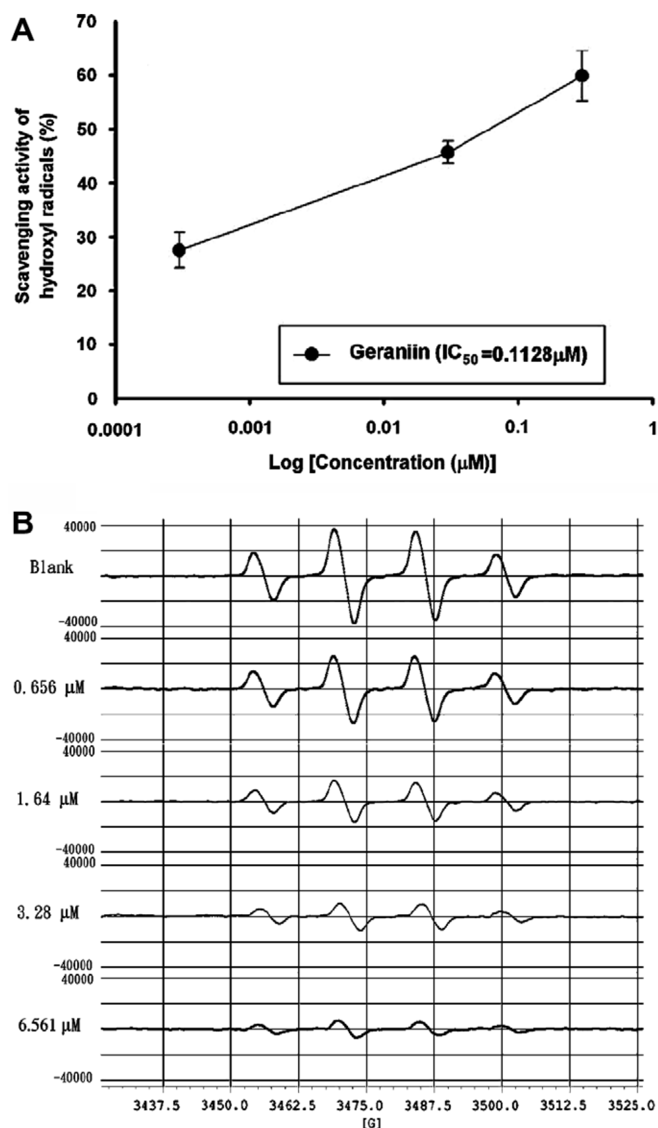

Fig. 3. Effects of different concentrations of geraniin on the scavenging activities of hydroxyl radical analyzed by (A) spectrophotometry of deoxyribose method and (B) by electron spin resonance spectrometry.

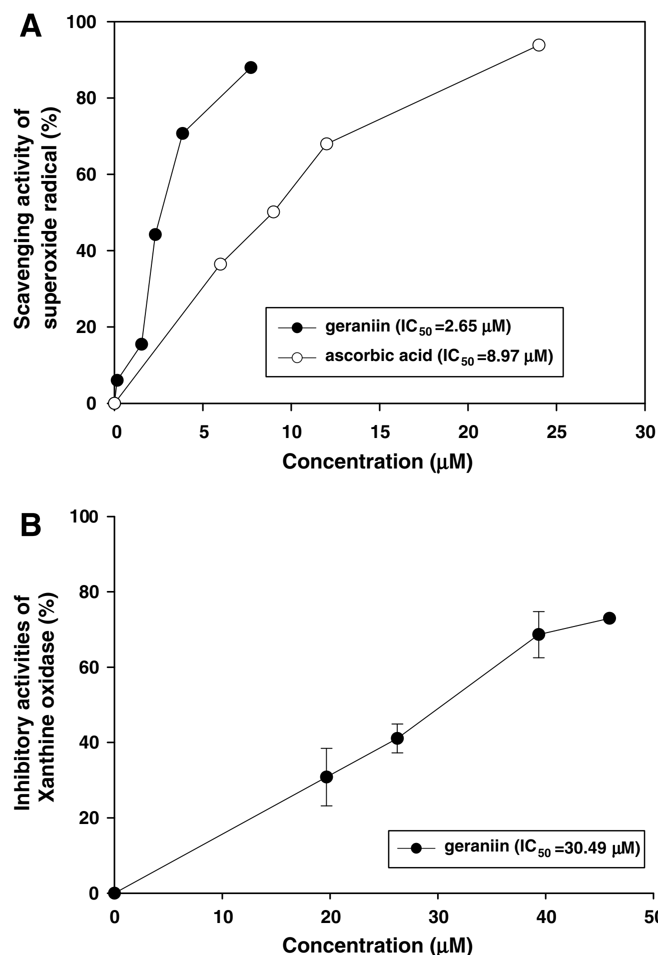

Fig. 4. (A) Effects of different concentrations of geraniin (0.15, 1.54, 2.32, 3.86, and 7.72 μM) and ascorbic acid (positive controls) on the scavenging activities of superoxide radical generating by the PMS–NADH generating system. The scavenging activity of superoxide radicals was calculated as follows: ($\Delta A560$ nm/min$_{blank}$ $\Delta\varpi$ $A560$ nm/min$_{sample}$) ÷ $\Delta A560$ nm/min$_{blank}$ × 100%. (B) Effects of different concentrations of geraniin (19.68, 26.24, 39.36, and 45.93 μM) on the inhibitory activities of xanthine oxidase. The inhibitory activity of xanthine oxidase was calculated as following: $\Delta A295$ nm/min$_{blank}$ $\Delta A295$ nm/min$_{sample}$) ÷ $\Delta A295$ nm/min$_{blank}$ × 100%.

was calculated to be 13.22 μM (Fig. 6A). The 1.0 μM geraniin was used to determine the kinetic properties of ACE inhibition, and it showed mixed noncompetitive inhibitions against it (Fig. 6B). The K_m was 0.21 mM, and the K'_m was 0.27 mM.

3.6. Antihypertensive effects of geraniin on SHR

SHRs received a single oral administration of geraniin (5 mg/kgSHR), and changes in SBP and DBP were recorded over 24 h. Geraniin was found able to reduce the SBP and showed significant differences ($P < 0.05$) at 2, 4, 6, 8 and 24 h (Fig. 7A). The reduced SBP was 18.3, 21.8, 15.5, 20.7, and 23.5 mmHg, respectively, for 2, 4, 6, 8, and 24-h after oral administration. DBP reductions were similar to those of SBP and showed significant differences ($P < 0.05$) at 2, 4, 6, 8 and 24 h (Fig. 7B). The reduced DBP was 21, 18, 15.5, 15.8, and 20.9 mmHg, respectively, for 2, 4, 6, 8, and 24 h after oral administration. It was noted that the reducing effects of geraniin on the blood pressure of the SHRs could last over 24-h before subsiding and showed significantly different to the positive control of captopril (Fig. 7A and B). The reduced SBP readings of captopril were 15.2, 17.9, 27.2, 34.5, and 8.1 mmHg, and the reduced DBP were 14.1, 12.3, 20.9, 32.6, and 8.8 mmHg, respectively, for 2, 4, 6, 8, and 24 h.

4. Discussions

Geraniin, the hydrolysable tannin, was decomposed to gallic acid, ellagic acid and corilagin after boiling water hydrolysis (Luger et al., 1998). Gallic acid and ellagic acid (Chen et al., 2007) and corilagin (Kinoshita et al., 2007) have all been reported to exhibit antioxidant activities, but few reports concerning the antioxidant activities of geraniin have appeared. Therefore, a series of antioxidant assay systems were used to determine the antioxidant effects of geraniin.

Geraniin proved to be a potent DPPH radical scavenger and its power was about 14.5- and 10.3-folds (for IC_{50} comparisons) that of BHT and ascorbic acid, respectively, under pH 7.9 conditions (Fig. 2B). It was also found that scavenging capacity of geraniin at pH 4.5 was higher than that at pH 7.9 for DPPH radical scavenging activity (for IC_{50} comparisons, 0.92 μM at pH 4.5 and 1.27 μM at pH 7.9). It was reported that DPPH scavenging activity might be affected by pH conditions (Hou et al., 2001; Yang et al., 2004; Liu et al., 2007b). DPPH radical assay belongs to the electron-transfer

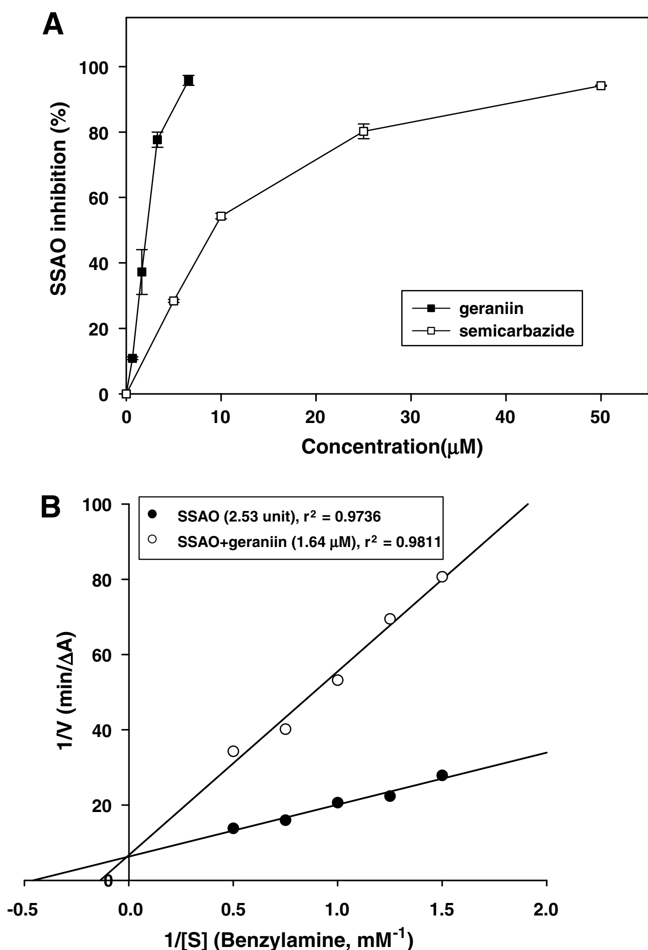

Fig. 5. (A) The inhibitory activities of geraniin (0.66, 1.64, 3.28, and 6.56 μM) and semicarbazide (5, 10, 25, and 50 μM; positive controls) on SSAO activities (2.53 units) from bovine plasma. (B) The kinetic properties of bovine SSAO (2.53 units) in the absence and presence of 1.64 μM geraniin in Lineweaver–Burk plots using different concentrations of benzylamine as substrates (0.67, 0.8, 1, 1.33, and 2 mM).

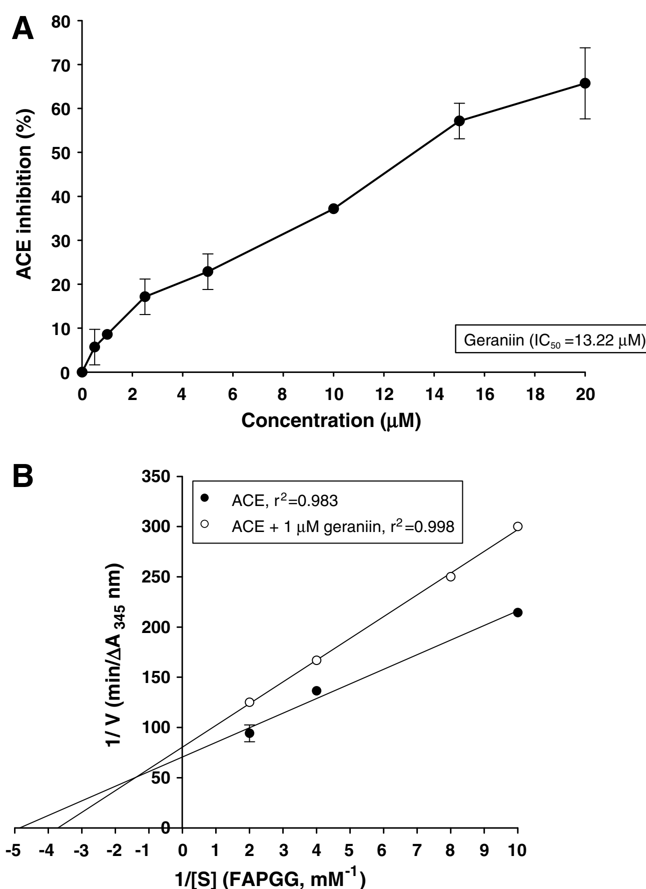

Fig. 6. (A) The inhibitory activities of geraniin on ACE activities (20 mU) from rabbit lung. The ACE activity was expressed as Δ*A*345 nm and the ACE inhibition (%) was calculated as followed: $[1 - (\Delta A_{\text{inhibitor}} \div \Delta A_{\text{blank}})] \times 100\%$. Means of triplicates were recorded. (B) The kinetic properties of ACE (15 mU) in the absence and presence of 1.0 μM geraniin in Lineweaver–Burk plots using different concentrations of FAPGG (0.1, 0.125, 0.25 and 0.5 mM) as substrates.

reaction (Huang et al., 2005), and pH conditions might affect the electron-transfer capacity of geraniin in the moiety of gallic acid, ellagic acid, and corilagin which contributes to its DPPH scavenging activity. The IC_{50} of OH**.** scavenging activity in the deoxyribose assay was 0.11 μM (Fig. 3A) and was 1.44 μM in the ESR method, which was lower than that of caffeic acid (4.4 μM), quercetin 3-*O*-rutinoside (7.5 μM) (Hou et al., 2005), myricetin galloylglycosides (Lee et al., 2006), and was about threefold that of Trolox (0.43 μM) in the ESR method (data not shown). Owing to the inhibitory activity of geraniin against xanthine oxidase (Fig. 4B), the PMS–NADH system was used to generate the superoxide radicals (Liu et al., 2004) instead of the xanthine–xanthine oxidase system. Geraniin exhibited dose-dependent superoxide radical scavenging activities, and the IC_{50} was about 1/3.38 that of ascorbic acid (Fig. 4A). Chen et al. (2001) reported that gallic acid and ellagic acid were effective scavengers against hydroxyl radical and superoxide radical. Kinoshita et al. (2007) showed that corilagin was a strong superoxide radical scavenger. In our present result, the strong antioxidant effects of geraniin might be from the component moiety of gallic acid, ellagic acid, and corilagin.

Geraniin was found to be a strong SSAO inhibitor, and its IC_{50} was about 1/5.2 that of semicarbazide (the positive control of SSAO). The geraniin showed the competitive inhibition against SSAO, which revealed that geraniin acted as a competitor with respect to the substrates (benzylamine) for substrate binding sites of SSAO. The calculated K_i was 0.7 μM, which was lower than that of hydroxyzine (1.5 μM), a histamine-1 receptor antagonist (O'Sullivan et al., 2006). SSAO played a key role in inflammation through its catalytic products, hydrogen peroxide, and reactive aldehydes. Therefore, the inhibition of SSAO activity might represent a target for anti-inflammation.

Geraniin isolated from *P. niruri* has been reported to have ACE inhibitory activity using hippuryl-L-His-His-Leu as a substrate and the IC_{50} was 0.4 mM and the geraniin was reported to exhibit a noncompetitive inhibition pattern (Ueno et al., 1988). In the present study geraniin was purified from *P. urinaria*, and FAPGG was used as an ACE substrate. The IC_{50} of geraniin against ACE was calculated to be 13.22 μM, which was lower than what Ueno et al. (1988) reported which the difference might be due to differing assay systems. Without the geraniin additions, the calculated K_m in this report was 0.21 mM FAPGG, which was close to the result (0.3 mM) of Holmquist et al. (1979) and exhibited a mixed noncompetitive inhibition pattern against FAPGG in the presence of geraniin, which revealed that geraniin acted as a competitor with respect to the substrates (FAPGG) or substrate (FAPGG)-enzyme (ACE) complex. Therefore, the antihypertensive effect of a single oral administration of geraniin on SHRs was investigated. Geraniin isolated from *Sapium sebiferum* has been reported to have antihypertensive effects (Cheng et al., 1994). The geraniin was intravenously injected into anaesthetized SHRs to rule out the adsorption factor. However, in our present study the geraniin was administrated orally into SHRs. Orally adminis-

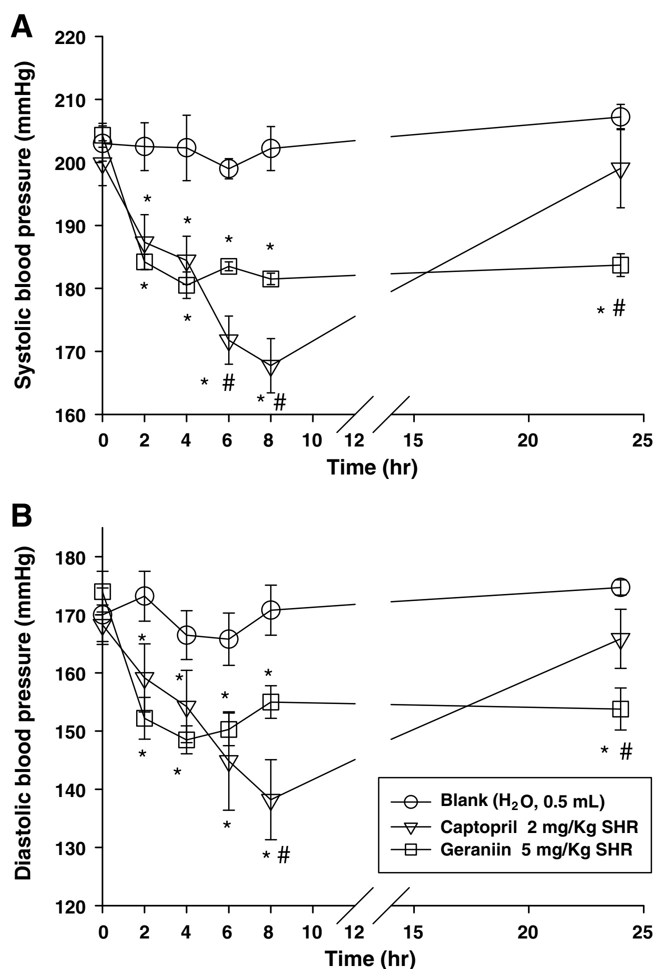

Fig. 7. Effects of geraniin (5 mg/kg SHR) or captopril (2 mg/kgSHR) on the systolic blood pressure (A) and diastolic blood pressure (B) of SHR after one oral administration during 24 h. The distilled water (0.5 ml) was used as a blank. The one-way ANOVA followed by the post-hoc Tukey's test was performed. A value of $P < 0.05$ was considered to be statistically significant at the same time. $^*P < 0.05$, geraniin vs. distilled water or captopril vs. distilled water; $^\#P < 0.05$, geraniin vs. captopril.

tered geraniin was found to exhibit antihypertensive effects that could last up to 24 h. Though the highest effect of reduced blood pressure was lower than that of captopril, the duration of the reduced blood pressure effect for geraniin was better than that of captopril in this report.

In conclusion, purified geraniin exhibited antioxidant activities, SSAO and ACE inhibitory activities, and antihypertensive effects on SHRs. The results presented here will benefit the effort to develop healthy food products using geraniin for antioxidant protection and therapeutic effects in the future.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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