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

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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 Echinacea 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 (peptidyl-dipeptidase hydrolyse 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 anti-oxidant 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 procy-anidins (Actis-Gorella 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...
(IC₅₀ = 18.8 µM), VW (IC₅₀ = 3.3 µM), and LW (IC₅₀ = 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 aminoaconite 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 cytoxic 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 (Chularojanmonti 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 (1 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]acyrlo]-The-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 (101) 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 recrystallized 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 recrystallized over a TSK HW-40C column eluted with H₂O → 60% MeOH → 70% MeOH → 70% acetone. The crude geraniin was obtained from 70% MeOH eluant 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%. 1H (500 MHz) and 13C 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
4.5) and then mixed with 0.6 ml of 100 mM 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 ΔA517 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: (ΔA517blank - ΔA517sample) / ΔA517blank × 100%. The IC50 stands for the concentration of half-inhibition. The KI was calculated using the equation: (ΔA440 nm/ΔA420 nm) / ΔA420 nm/ΔA400 nm × 100%. IC50 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–Bürk plots using different concentrations of benzylamine as substrates (0.67, 0.8, 1, 1.33 and 2 mM). The IC50 and KI were calculated using the equation of IC50 = [1 / (IC50 / KI)] - 1, where [I] is the concentration of 1.64 μM and KI 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 μl) 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 (ΔA345sample) was recorded during 5 min at room temperature. Deionized water was used instead of geraniin solution as a blank experiment. The ACE activity was expressed as ΔA345/min and the ACE inhibition (%) was calculated as follows: [1 – (ΔA345sample + ΔA345blank)] / ΔA345sample × 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 μM). The IC50 and KI were calculated from Lineweaver–Bürk plots where the KI 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 access 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/100 g SHR was administered orally once, and tail blood pressure was measured using a strain-gauge arterial cuff connected to a digital sphygmomanometer (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 15 min in a 39 °C thermostatted 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 or distilled water and 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%,
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 IC50 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 (IC50 of 13.1 μM) and BHT (IC50 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 assay (Fig. 3A) or ESR (Fig. 3B) method. Geraniin was found to exhibit dose-dependent OH•/C5 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 IC50 value was calculated to be 0.11 μM (Fig. 3A). In the ESR assay method OH•/C5 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 IC50 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 IC50 value was calculated to be 2.65 μM (Fig. 4A), much better than that of ascorbic acid (IC50 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 IC50 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 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 IC50 was calculated to be 6.58 μM which was much lower than that of semicarbazide (IC50 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_i 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 IC50
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 $K_0m$ was 0.27 mM.

3.6. Antihypertensive effects of geraniin on SHR

SHRs received a single oral administration of geraniin (5 mg/kg SHR), 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 18.3, 21.8, 15.5, 20.7, and 23.5 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, $0.92 \mu$M 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
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 IC50 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), myrcetin galloylglucosides (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 IC50 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 IC50 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 Ki 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 IC50 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 IC50 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 Km 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-

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**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 ΔA_{455} nm and the ACE inhibition (%) was calculated as followed: [1 – (ΔA_{substrate} – ΔA_inhibitor)] × 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.
tered geraniin was found to exhibit antihypertensive effects that could last up to 24 hr. 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 antithrombotic 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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References


