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INHIBITORY EFFECTS OF VARIOUS FLAVONOIDS ISOLATED FROM LEAVES OF PERSIMMON ON ANGIOTENSIN-CONVERTING ENZYME ACTIVITY

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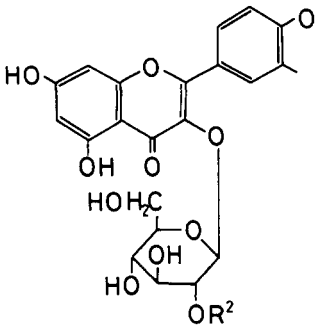
ABSTRACT.—The leaves of the persimmon *Diospyros kaki*, have been traditionally used for treatment of hypertensive diseases in Japan. We have studied the inhibitory effects of four flavonoids isolated from the leaves of the persimmon on angiotensin-converting enzyme activity. The four flavonoids astragalin [**1**], kaempferol-3-*O*-(2″-*O*-galloyl)-glucoside [**2**], isoquercitrin [**3**], and quercetin-3-*O*-(2″-*O*-galloyl)-glucoside [**4**] inhibited the angiotensin-converting enzyme activity in a dose-dependent fashion. Compounds **1-4** produced 67%, 53%, 33%, and 48% inhibition at a concentration of 300 μg/ml, respectively. The 50% inhibitory concentrations (IC_{50}) of **1** and **2** for the angiotensin-converting enzyme were 180 μg/ml and 280 μg/ml, respectively. On the other hand, **2** and **4** were shown to have tannin activities, but **1** and **3** had no tannin activities. These results suggest that there is no relationship between the inhibition for angiotensin converting enzyme activity and the tannin activity for the four flavonoids.

The fruits of a persimmon, *Diospyros kaki* Thunb. (Ebenaceae), have been used for treatment of apoplexy, hematemesis, chilblain, and burns. A persimmon tannin and leaf have been traditionally used for treatment of hypertensive diseases. Funayama (1) reported that two flavonol glucosides, astragalin [**1**] and isoquercitrin [**3**], isolated from the leaves of a persimmon have a hypotensive action in rats. Condensed tannins are contained in the fruits and leaves of persimmon (2). It seems likely that the tannin fractions of *D. kaki* may be related to the pharmacological actions.

It is well known that renin is released into the blood stream from the kidney and converted angiotensinogen into angiotensin I. Further, angiotensin I is converted into



1 $R^1=R^2=H$ astragalin

2 $R^1=H$, $R^2=CO$-(3,4,5-trihydroxybenzoyl) kaempferol 3-*O*-(2″-*O*-galloyl)-glucoside

3 $R^1=OH$, $R^2=H$ isoquercitrin

4 $R^1=OH$, $R^2=CO$-(3,4,5-trihydroxybenzoyl) quercetin 3-*O*-(2″-*O*-galloyl)-glucoside

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angiotensin II by angiotensin-converting enzyme (ACE) (3). Angiotensin II is well known to have a hypertensive activity . The inhibition of ACE is a useful in vitro model for developing hypotensive drugs (4,5). Most of the drugs used in the treatment of hypertension [for example, captopril (SQ 14,225) and nonapeptide (SQ 20,881)] are effective in vitro inhibitors of ACE activity (4,5).

In this study, we investigated the inhibitory effects of four flavonoids isolated from the leaves of a persimmon on ACE activity. In addition, we measured the tannin activity of the four flavonoids, and the relationship between the ACE inhibition and the tannin activity of the four flavonoids are discussed.

MATERIALS AND METHODS

MATERIALS.—Hippuryl-L-histidyl-L-leucine (Hip-His-Leu) was obtained from Sigma Co. and used as a substrate for ACE. We dissolved 2.5 mM Hip-His-Leu in phosphate buffered saline (PBS, 100 mM phosphate buffer containing 300 mM NaCl) (pH 8.3). Sephadex LH-20 (100 μm), silicic acid, MCI gel CHP-20P (75-150 μm), and YMC A324 ODS column were purchased from Pharmacia Fine Chemicals, Mallinckrodt, Mitsubishi Chemical Industries, and Yamamura Chemical Laboratories, respectively. Other chemicals were reagent grade.

ISOLATION OF FLAVONOIDS.—Dried leaves (100 g) of *D. kaki* cv. Saijo were homogenized in a mixture of Me_2CO-H_2O (7:3) and filtered. The filtrate was concentrated in vacuo and extracted with Et_2O, EtOAc, and *n*-BuOH, successively. The EtOAc extract (1.4 g) thus obtained was subjected to column chromatography over Sephadex LH-20 (100 μm, Pharmacia Fine Chemicals) with 60% EtOH as an eluent and then separated into four fractions. The second fraction was subjected to column chromatography over silicic acid with $CHCl_3$-EtOH (9:1) and then $CHCl_3$-EtOH (8:2) as eluents. The $CHCl_3$-EtOH (8:2) eluate afforded astragalin [**1**] (90 mg) and a mixture (0.38 g) containing three other flavonoids. The mixture was subjected to column chromatography over MCI gel CHP-20P with 20% MeOH, 40% MeOH, and 60% MeOH as eluents. The 40% MeOH eluate was further chromatographed over MCI gel CHP-20P, and the final purification was achieved by preparative (column: YMC A324 ODS, 10 mm i.d.×30 cm, solvent: MeOH-HOAc-H_2O, 35:5:60) to yield quercetin-3-(2″-*O*-galloyl)-glucoside [**4**] (8 mg) and isoquercitrin [**3**] (4 mg). Kaempferol-3-(2″-*O*-galloyl)-glucoside [**2**] (14 mg) was obtained from the 60% MeOH eluate of the MCI gel chromatography in an analogous way. The identification of these compounds was based on the comparison of the spectral data with the reported data (6-8) and direct comparison (for astragalin) with the authentic sample.

PREPARATIONS OF ACE.—ACE was isolated from rat lung by the methods of Takada *et al.* (9). ACE was dissolved in PBS (pH 8.3).

MEASUREMENT OF INHIBITORY EFFECTS ON ACE ACTIVITY.—A mixture of 2.5 mM Hip-His-Leu (0.15 ml) and ACE solution (0.1 ml; ACE activity: 2.64 units/mg protein, 1 unit: 1 μmoles/hippuric acid/min) was incubated with or without (control) the indicated amounts of test compounds at 37° for 30 min in a final volume of 0.35 ml. The reaction was stopped by adding 1 N HCl (0.25 ml), and the mixture was extracted with EtOAc (2.0 ml). The EtOAc phase was evaporated, and the residue was dissolved in H_2O (2.0 ml). The free hippuric acid was determined by uv absorption at 280 nm for detection. The amount of hippuric acid released was determined by the regression equation of the standard curve of an authentic hippuric acid. The activities of four flavonoids are expressed as percent inhibition as compared to each control value.

MEASUREMENT OF TANNIN ACTIVITY OF FOUR FLAVONOIDS.—The relative astringency (RA) and relative affinity for methylene blue (RMB) of the four flavonoids isolated from the leaves of the persimmon were determined by the methods previously reported (10-12). The RAG and RMBG values were expressed in units based on the use of the tannin geraniin, as the standard (10).

RESULTS

INHIBITORY EFFECTS OF FOUR FLAVONOIDS ON ACE ACTIVITY.—As shown in Table 1, astragalin [**1**], kaempferol-3-*O*-(2″-*O*-galloyl)-glucoside [**2**], isoquercitrin [**3**], and quercetin-3-*O*-(2″-*O*-galloyl)-glucoside [**4**] inhibited ACE activity in a dose-dependent fashion. Compounds **1**-**4**, at a concentration of 300 μg/ml, were shown to result in 67, 53, 33, and 48% inhibition, respectively. The IC_{50} for **1** and **2** were 180 μg and 280 μg/ml, respectively.

TABLE 1. Inhibitory Effects of Four Flavonoids in the Leaves of *Diospyros kaki* on Angiotensin-Converting Enzyme (ACE) Activity[a]

Concentrations of flavonoids (μg/ml)	Inhibition (%)			
	Astragalin [**1**]	Kaempferol-3-*O*-(2″-*O*-galloyl)-glucoside [**2**]	Isoquercitrin [**3**]	Quercetin-3-*O*-(2″-*O*-galloyl)-glucoside [**4**]
38	0±0	3±0.5	0±0	10±1.1
56	2±0.2	6±1.0	0±0	12±1.1
75	22±1.0	15±1.1	9±4.1	18±0.8
113	31±2.5	35±2.7	3±2.5	39±1.5
150	53±6.4	35±3.9	19±1.7	39±3.0
225	58±2.1	39±3.9	19±1.9	34±7.4
300	67±3.7	53±2.3	33±2.0	48±1.4

[a]The results are means ± standard errors for three experiments.

TANNIN ACTIVITY (RAG AND RMBG VALUES) OF FOUR FLAVONOIDS.—As shown in Table 2, astragalin [**1**] and isoquercitrin [**3**] had no tannin activities, while RAG values of their galloyl esters, kaempferol-3-*O*-(2″-*O*-galloyl)-glucoside [**2**] and quercetin-3-*O*-(2″-*O*-galloyl)-glucoside [**4**], were 0.25 and 0.64, respectively. On the other hand, RMBG values of compounds **1**-**4** were found to be 0.05, 0.52, 0.09, and 0.93, respectively.

TABLE 2. RAG and RMBG Values of Four Flavonoids in the Leaves of *Diospyros kaki*[a]

Flavonoids	RAG[b]	RMBG[c]
Astragalin [**1**]	0±0	0.05±0.003
Kaempferol-3-*O*-(2″-*O*-galloyl)-glucoside [**2**]	0.25±0.013	0.52±0.076
Isoquercitrin [**3**]	0±0	0.09±0.002
Quercetin-3-*O*-(2″-*O*-galloyl)-glucoside [**4**]	0.64±0.028	0.93±0.033

[a]The results are means ± standard errors for three experiments.

[b]RAG, relative astrigency (RA) value based on a tannin, geraniin isolated from the leaves of *Geranium thunbergii* as a standard.

[c]RMBG, relative affinity for methylene blue (RMB) based on a tannin, geraniin as a standard.

DISCUSSION

It is well known that tannins and related compounds are contained in the leaves of *D. kaki*, and it seems likely that the tannin fractions may be related to the pharmacological actions. In the present study we isolated two flavonoids having tannin activity from the leaves of a persimmon. It was found that tannin activities of two galloyl esters of flavonol glucosides, kaempferol-3-*O*-(2″-*O*-galloyl)-glucoside [**2**] and quercetin-3-*O*-(2″-*O*-galloyl)-glucoside [**4**], were stronger than those of the two flavonol glucosides astragalin [**1**] and isoquercitrin [**3**]. These findings suggest that a galloyl group at C-2″ position of the glucose moiety may be essential for the tannin activity (RAG and RMBG). On the other hand, all the four flavonoids inhibited the ACE activity. The degree of inhibition of ACE activity was in the order astragalin [**1**]>kaempferol-3-*O*-(2″-*O*-galloyl)-glucoside [**2**]>quercetin-3-*O*-(2″-*O*-galloyl)-glucoside [**4**]>isoquercitrin [**3**]. From the results of two experimental systems, it was shown that there is no relationship between the inhibition for ACE activity and tannin activity for the flavonoids used in this study. Therefore, these findings suggest that the

inhibitory effects of flavonol glucosides and their gallates on ACE activity might be due to the flavonol moiety. It was of great interest that flavonol glucosides and their gallates, as ACE inhibitors, were structurally different from the ACE inhibitors nonapeptide (SQ 20,881) and captopril (SQ 14,225).

Further work is needed to clarify the mechanism of ACE inhibition and the relevance of these flavonoids to the purported antihypertensive activity of the persimmon.

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