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Identification of the Strong Vasorelaxing Substance Scirpusin B, a Dimer of Piceatannol, from Passion Fruit (*Passiflora edulis*) Seeds

Shoko Sano,[*,†] Kenkichi Sugiyama,[‡] Tatsuhiko Ito,[†] Yumi Katano,[§] and Akira Ishihata[§]

[†]Health Care Division, Morinaga and Company, Limited, 2-1-1 Shimosueyoshi, Tsurumi-ku, Yokohama 230-8504, Japan

[‡]Research Institute, Morinaga and Company, Limited, 2-1-1 Shimosueyoshi, Tsurumi-ku, Yokohama 230-8504, Japan

[§]Division of Theoretical Nursing and Pathophysiology, Yamagata University School of Medicine, 2-2-2 Iida Nishi, Yamagata 990-9585, Japan

ABSTRACT: Piceatannol is present in passion fruit (*Passiflora edulis*) seeds in high amounts. In this study, we isolated the second major polyphenolic compound of passion fruit seeds and identified it as scirpusin B, which is a dimer of piceatannol. We investigated the antioxidant activities and vasorelaxing effects of these polyphenols. Their antioxidant effects were measured using an in vitro 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, and their vasorelaxant effects were determined ex vivo in rat thoracic aorta. Both polyphenolic compounds exhibited potent antioxidant activities and significant vasorelaxant effects in endothelium-intact aortas. More specifically, scirpusin B exerted a greater antioxidant activity and vasorelaxant effect compared with that of piceatannol. Additionally, the vasorelaxation effects of the compounds were induced via the NO derived from the endothelium. This study provides the possibility that polyphenols in passion fruit seeds are effective against cardiovascular diseases (CVDs).

KEYWORDS: *Passiflora edulis*, scirpusin B, piceatannol, vasorelaxation, passion fruit seed

INTRODUCTION

Cardiovascular diseases (CVDs) such as ischemic heart diseases and cerebrovascular diseases are the primary cause of death. Epidemiological studies report that the incidence of CVDs resulting from hyperlipidemia or atherosclerosis is related to the excess intake of animal fats, which are rich in saturated fatty acids. However, despite a much greater consumption of saturated fatty acids, the CVD-related mortality in France is lower than that observed in other European countries. This epidemiological fact is called the French paradox.[1] As the French usually take a great quantity of red wine with food, it is suggested that red wine polyphenolic compounds (RWPC) lower the mortality from CVDs in that country. Subsequent studies revealed that RWPC exhibit plasma-LDL-reducing,[2] antioxidant,[3] antiaggregating,[4] and endothelin-1 synthesis inhibitory effects.[5] It is speculated that these effects of RWPC play important roles in preventing CVDs.

Passiflora edulis is a plant that belongs to the family *Passifloraceae*. This is a native plant of South America. The fruit of this plant is called passion fruit, and the seeds are often eaten together with the yellow pulp. Previous studies reported that the leaves, vines, and flowers contain polyphenolic compounds and exhibit a variety of physiological effects, such as antianxiety,[6,7] anti-inflammatory,[8,9] and cough-suppressant effects.[10] Moreover, it was reported that the rind of passion fruit has a hypotensive effect.[11] However, the specific compound in passion fruit that contributes to these effects remains unknown.

Matsui et al. discovered that passion fruit seeds contain large amounts of piceatannol and that it exerts positive effects on cultured dermal cells: inhibition of melanogenesis and synthesis of collagen.[12] The aim of this study is to investigate the effect of polyphenol contained in passion fruit seed on preventing CVDs and to add new functionality to this material. Then, we isolated the major polyphenolic compound from passion fruit seed second to piceatannol and identified it as scirpusin B, which is a dimer of piceatannol. Subsequently, we investigated their antioxidant effects in vitro and their vasorelaxant effects in rat aortas.

MATERIALS AND METHODS

Animals. Male Fischer 344 rats (5−6 months of age) obtained from Charles River Japan (Atsugi, Japan) were used. Experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and under the regulations of the Animal Care Committee of the Yamagata University School of Medicine. Rats were euthanized by cervical dislocation under ether anesthesia.

Materials. The freeze-dried and milled seeds of *Passiflora edulis* (passion fruit) were purchased from Kobayashi Perfumery Co., Ltd. (Tokyo, Japan). The following reagents were used in this study. Acetylcholine chloride was obtained from Daiichi Pharmaceutical (Tokyo, Japan); 2-(*N*-morpholino)ethanesulfonic acid (MES) was obtained from Dojindo Laboratories (Kumamoto, Japan); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was obtained from Calbiochem-Novabiochem Corp. (San Diego, CA, USA); piceatannol was obtained from Cayman Chemical (Ann Arbor, MI, USA); and heparin sodium, N^G-nitro-L-arginine methylester (L-NAME) and phenylephrine (PE) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Extraction and Isolation of Plant Polyphenols. Optical rotation was determined using a Jasco P-2200 polarimeter (Jasco, Tokyo, Japan). IR spectra were measured on a Jasco FT/IR-6200 Fourier transform infrared spectrometer (Jasco, Tokyo, Japan). Liquid chromatography

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mass spectra (LC/MS) were acquired on an Agilent 1100 LC/MS system (Agilent Technologies, Tokyo, Japan). ^{1}H- and ^{13}C NMR spectra were recorded on a Varian Mercury 300 NMR spectrometer (300 and 75 MHz, respectively) (Varian, Tokyo, Japan) or a Bruker Avance 700 MHz NMR spectrometer (700 and 175 MHz, respectively) (Bruker, Yokohama, Japan). Chemical shifts are given in δ (ppm), with tetramethylsilane as an internal standard (s = singlet, d = doublet, dd = double doublet, and t = triplet). Ultraviolet (UV)−visible spectra were measured on a Jasco V-630 spectrophotometer (Jasco, Tokyo, Japan). Analytical and preparative HPLC were performed using a Jasco LC-2000 plus HPLC system (Jasco, Tokyo, Japan).

Fractionation of extracts was achieved using reverse-phase liquid chromatography. The collection of each fraction was performed on an Inertsil ODS-3 column (5 μm, 20 mm i.d. × 250 mm) purchased from GL Sciences Inc. (Tokyo, Japan). The conditions of this preparative HPLC were a (A) water and/or (B) acetonitrile mobile phase at a flow rate of 5 mL/min. A gradient elution of 0−80% (B) at 0−90 min was used for fractionation.

The analysis of each fraction was carried out on an Inertsil ODS-3 column (5 μm, 4.6 mm i.d. × 150 mm) purchased from GL Sciences Inc. (Tokyo, Japan). The conditions of this analytical HPLC were a (A) water and/or (B) acetonitrile mobile phase at a flow rate of 0.75 mL/min. A gradient elution of 0−45% (B) at 0−25 min was used for this analysis. All chromatographic processes were performed at ambient temperature and detected at a wavelength of 280 nm.

The freeze-dried and milled seeds of passion fruit (100 g) were refluxed at 90 °C with 90% ethanol (1,000 mL) for 90 min. After centrifugation (11,000*g* for 20 min), the supernatant was collected. For the purpose of defatting, the supernatant was extracted with *n*-hexane, and the hexane layer was removed. The remaining solution was concentrated in vacuo (10 g) and dissolved in 25% acetonitrile (40 mL). The dissolved solution was centrifuged (11,000*g* for 20 min), and the supernatant was filtered (0.22 μm). This solution was separated using preparative HPLC. The detected target peaks were purified by the same preparative HPLC.

Measurement of DPPH Radical Scavenging Activity. The radical scavenging activities of plant polyphenols were evaluated against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical in accordance with the conventional method. Two different solutions were prepared, as follows. Solution A was a mixture of samples in DMSO and 80% ethanol (1:4). Solution B was a mixture of 0.4 mM DPPH in ethanol, 200 mM MES buffer (pH 6.0), and 20% ethanol (1:1:1). Solution A (0.8 mL) was mixed well with solution B (2.4 mL) in a test tube and placed at room temperature for 60 min. The absorbance at 520 nm was then measured using a UV spectrophotometer. All samples were analyzed in triplicate, and the mean values of free radical scavenging activity were calculated. The DPPH radical scavenging activities of samples were compared with that of trolox, which was used as a control.

Measurement of Vascular Tension in Isolated Aortas. Thoracic aortas were isolated, and adherent fat and connective tissues were carefully removed. Aortas were cut into rings (approximately 2 mm long). In some preparations, the endothelium was removed by gently rubbing the internal surface with forceps. Removal of the endothelium was verified by the absence of relaxation in the presence of acetylcholine (ACh, 1 μM) in aortic rings precontracted with 1 μM phenylephrine.

Isolated rat aortic ring preparations (with or without endothelium) were suspended in organ baths containing 10 mL of Krebs−Henseleit solution. The solution contained 118 mM NaCl, 4.7 mM KCl, 24.9 mM $NaHCO_3$, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11.1 mM glucose, 1.8 mM $CaCl_2$, and 0.057 mM ascorbic acid. A solution containing a high concentration of K^+ was prepared by substituting NaCl with equimolar KCl. These solutions were saturated with a mixture of 95% O_2 and 5% CO_2 at 37 °C, pH 7.4. The developed tension was recorded using an isometric force transducer (7T−15−240, Orientec, Tokyo, Japan), for the measurement of changes in contractile force. The preparation was stretched to a resting tension of 0.8 g, and the solution was changed every 15 min. After an equilibration period of 1 h, each preparation was contracted with 66.7 mM KCl (high K^+) repeatedly, until reproducible contraction was obtained.

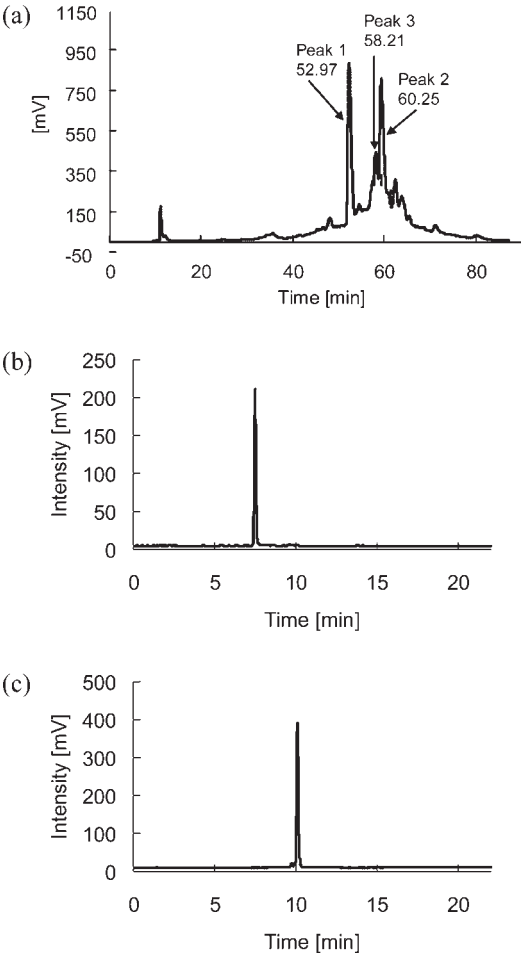

Figure 1. (a) Fractionation of the extract of passion fruit seeds using reverse-phase chromatography with an ODS column. The flow rate was 5 mL/min. Peak **1** (with a retention time of 52.97 min) was piceatannol. Peak **2** (with a retention time of 60.25 min) was scirpusin B. Peak **3** (with a retention time of 58.21 min) was resveratrol. (b and c) Analysis of each fraction using reverse-phase chromatography with an ODS column. The flow rate was 0.75 mL/min. (b) Piceatannol fraction. (c) Scirpusin B fraction. All chromatograms were detected at a wavelength of 280 nm.

After the aortic rings were precontracted using PE (1 μM), each drug was added cumulatively. In some experiments, the inhibitor L-NAME (100 μM) was added 15 min before and during stimulation with compounds.

Statistical Analyses. Data were expressed as the means ± SEM. Statistical analyses were performed with a Student's *t* test for comparisons between two groups and a Tukey−Kramer multiple comparison test for multiple comparisons, using SPSS software, version 13 (SPSS Inc.). Differences were considered significant at $p < 0.05$.

RESULTS

Identification of Stilbene Derivatives in Passion Fruit Seeds. To isolate and identify the compounds present in passion fruit seeds, freeze-dried and milled seeds were extracted with an organic solvent. The extract of seed was fractionated using

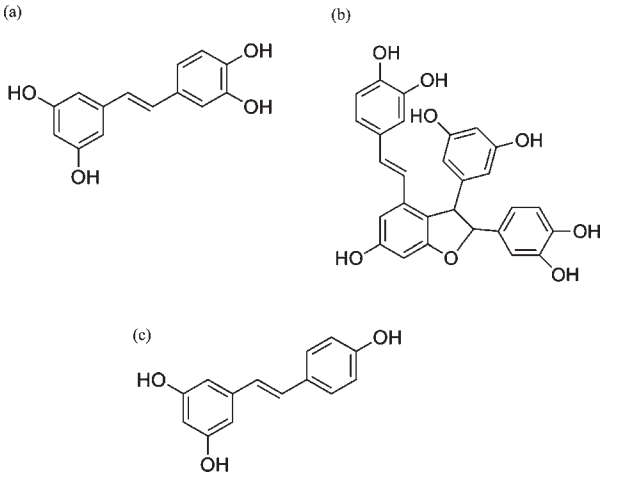

Figure 2. Chemical structures of the stilbene derivatives. (a) Piceatannol; (b) scirpusin B, a dimer of piceatannol; and (c) resveratrol.

reversed-phase chromatography with an ODS column, and two major peaks were detected (Figure 1a). Matsui et al. have shown previously that passion fruit seeds contain piceatannol (peak **1**) and resveratrol (peak **3**), which are stilbene derivatives, and piceatannol was present in large amounts.[12] However, peak **2**, the second major peak after piceatannol, was not identified. To investigate the biological activities of the two main substances in passion fruit seeds, we collected, purified, and analyzed peaks **1** and **2**. On the basis of the comparison of these data with those of the piceatannol reagent, peak 1 was confirmed as piceatannol. Finally, piceatannol was isolated as a pale pink powder (Figure 1b). LC/MS revealed that the molecular weight of peak **1** (piceatannol) was 244 g/mol and that the molecular weight of another major peak (peak **2**) was 486 g/mol. From this data, peak **2** was hypothesized as a piceatannol dimer. Peak **2** was isolated as a yellow amorphous powder (Figure 1c). The spectral data indicated that peak 2 was scirpusin B.[13]

Passion fruit seeds contained large amounts of piceatannol and scirpusin B (100 g of dried seeds contained 570 mg and 360 mg, respectively). We succeeded in obtaining piceatannol and scirpusin B as highly purified powders (99%, 210 mg and 96%, 190 mg from 100 g of dried seeds, respectively). The structures of piceatannol (peak **1**) and scirpusin B (peak **2**) are shown in Figure 2.

Piceatannol (Peak 1). MS (ESI) m/z: 243 (M^-). IR (KBr): 965, 1142, 1294, 1347, 1444, 1520, 1601 cm^{-1}. UV (25% acetonitrile): λ_{max} = 220, 236, 303, 324 nm. ^1H NMR (300 MHz, CD_3OD) δ: 6.15 (1H, t, J = 2.2 Hz), 6.43 (2H, d, J = 2.3 Hz), 6.71 (1H, d, J = 2.5 Hz), 6.74 (1H, s), 6.76 (1H, s), 6.81 (1H, d, J = 1.9 Hz), 6.84 (1H, d, J = 2.2 Hz), 6.86 (1H, s), 6.91 (1H, s), 6.97 (2H, d, J = 2.2 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ: 101.4, 104.5, 112.6, 115.2, 119.0, 125.8, 128.5, 129.9, 140.1, 145.3 × 2, 158.5.

Scirpusin B (Peak 2). $[\alpha]_D^{23}$ 0.0° (c = 1.01, MeOH). MS (ESI) m/z: 485 (M^-). IR (KBr): 1002, 1116, 1156, 1197, 1282, 1339, 1445, 1520, 1605 cm^{-1}. UV (25% acetonitrile): λ_{max} = 286, 303, 332, 392 nm. ^1H NMR (700 MHz, CD_3OD) δ: 4.38 (1H, d, J = 5.7 Hz), 5.32 (1H, d, J = 5.7 Hz), 6.19 (2H, d, J = 2.0 Hz), 6.21 (1H, t, J = 2.0 Hz), 6.29 (1H, d, J = 2.0 Hz), 6.58 (1H, d, J = 16.2 Hz), 6.62 (1H, dd, J = 8.1, 2.0 Hz), 6.66 (1H, d, J = 2.0 Hz), 6.68 (1H, dd, J = 8.1, 2.0 Hz), 6.74 (1H, d, J = 2.0 Hz), 6.78 (1H, d, J = 8.1 Hz), 6.80 (1H, d, J = 2.0 Hz), 6.81 (1H, d, J = 16.2 Hz). ^{13}C NMR (175 MHz, CD_3OD) δ: 58.0, 94.8, 96.8, 102.3, 104.5, 107.4 × 2, 113.7, 114.0, 116.3 × 2, 118.5, 119.8, 120.0, 123.7, 130.9, 131.0, 134.9, 137.0, 146.2, 146.3, 146.4, 146.5, 147.6, 159.8, 159.8 × 2, 162.7.

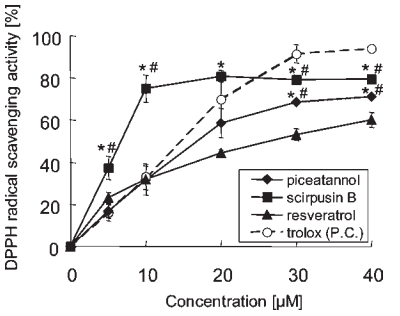

Figure 3. DPPH radical scavenging activities of stilbene derivatives in vitro. Piceatannol and scirpusin B were derived from passion fruit seeds. Trolox was used as the positive control. Data are the means ± SEM (n = 3−4). Significance was set at $p < 0.05$ using a Tukey−Kramer multiple comparison. $^*p < 0.05$ vs resveratrol, $^\#p < 0.05$ vs trolox (positive control).

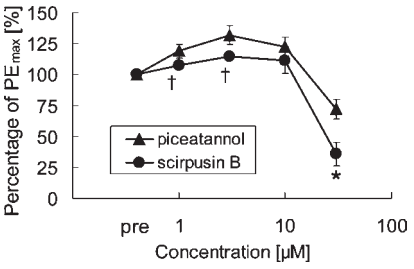

Figure 4. Vasorelaxant effect of piceatannol and scirpusin B in endothelium-intact rat aorta. Data are the means ± SEM (n = 6−7). Significance was set at $p < 0.1$ using a t test. $^\dagger p < 0.1$, $^*p < 0.05$ vs piceatannol.

Evaluation of the Antioxidant Activities of the Stilbene Derivatives of Passion Fruit Seeds. To investigate the antioxidant activities of piceatannol and scirpusin B in passion fruit seeds, we measured DPPH radical scavenging activities. Trolox and resveratrol reagents were used as positive controls. Trolox is a water-soluble derivative of vitamin E. Resveratrol is a stilbene derivative with a chemical structure similar to that of piceatannol (Figure 2). Additionally, resveratrol is reportedly a major polyphenolic compound in red wine and has a high antioxidant activity.

As shown in Figure 3, scirpusin B was a very strong antioxidant material. At low concentrations (5−10 μM), the antioxidant activity of scirpusin B was the highest and was more than twice that of other compounds. In contrast, at high concentrations (30−40 μM), trolox had the highest antioxidant activity, followed by scirpusin B, piceatannol, and resveratrol.

Stilbene-Derivative-Induced Vasorelaxation in Rat Aortas. We investigated whether piceatannol and scirpusin B induced endothelium-dependent vasorelaxation of rat aortas. Figure 4 represents the concentration−response curves (1−30 μM) of the vasorelaxation induced by piceatannol and scirpusin B. Although both compounds induced weak vasocontraction at low concentrations (1−10 μM), they showed strong vasorelaxation at 30 μM. Scirpusin B exerted greater vasorelaxation and smaller vasoconstriction effects compared with those of piceatannol.

Mechanism of Stilbene-Derivative-Induced Vasorelaxation. To investigate the mechanisms underlying the vasorelaxation

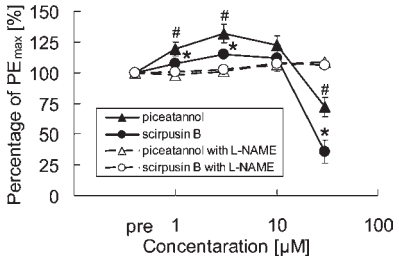

Figure 5. Vasorelaxant effect of piceatannol and scirpusin B in L-NAME-pretreated endothelium-intact rat aorta. Data are the means ± SEM ($n = 5-7$). Significance was set at $p < 0.05$ using a Tukey−Kramer multiple comparison. $^{*}p < 0.05$ piceatannol compared with piceatannol with L-NAME. $^{\#}p < 0.05$ scirpusin B compared with scirpusin B with L-NAME.

induced by piceatannol and scirpusin B, we evaluated the possible involvement of the endothelium and of NO derived from the endothelium. Vascular effects were not observed in endothelium-denuded aortas (data not shown). Additionally, the reactions induced by both piceatannol and scirpusin B in endothelium-intact aortas were significantly inhibited by pretreatment with L-NAME, which is a NO synthase inhibitor (Figure 5).

DISCUSSION

In this study, we isolated scirpusin B, a dimer of piceatannol, from passion fruit seeds. This is the first report that passion fruit seeds contain scirpusin B in large amounts. Passion fruit contains piceatannol, which is a stilbene derivative with a structure that is closely related to that of resveratrol. Piceatannol has an additional phenolic hydroxyl group at the 3′ position (Figure 2). Because piceatannol can be converted to resveratrol by CYP1B1 and exhibits strong anticancer activity in tumor cells,[14] considerable attention has been focused on piceatannol as a possible anticancer agent. Scirpusin B was first extracted from *Scirpus fluviatilis* (Torr.) A. Gray in 1978 by Nakajima et al.;[15] however, there are few reports concerning its physiological role. Anti-HIV activity,[16] superoxide anion scavenging activity,[17] and α-amylase inhibitory activity[13] have been reported, among others.

In this study, we examined the vasorelaxant effects of piceatannol and scirpusin B derived from passion fruit seeds in rat thoracic aortas. In endothelium-intact aortas, both piceatannol and scirpusin B yielded significant vasorelaxation. Importantly, we found that the vasorelaxation associated with scirpusin B was greater than that induced by piceatannol, although a previous study reported that piceatannol caused strong vasorelaxation in isolated aortas.[18]

To investigate the mechanisms of piceatannol- and scirpusin B-induced vasorelaxant effects, we compared their effects in endothelium-denuded versus endothelium-intact aortas. In endothelium-denuded aortas, the vasorelaxing effects induced by both piceatannol and scirpusin B were abolished. These results suggest that both piceatannol and scirpusin B induce vasorelaxation via endothelium-derived factors. In addition, pretreatment of endothelium-intact aortas with L-NAME abolished the vasorelaxation induced by both piceatannol and scirpusin B. L-NAME is an inhibitor of NO synthesis from L-arginine. These results suggest that endothelium-derived NO is responsible for piceatannol- and scirpusin B-induced vasorelaxation. Then, regarding the relative contribution of NO and endothelium-derived hyperpolarizing factor (EDHF) within the vascular tree, it appears that EDHF becomes more important when vessel diameter decreases, whereas NO seems to be predominant in large arteries.[19] Therefore, further studies on other vasodilating factors using various vessels and on more detailed mechanisms of producing the factors will be needed.

Although piceatannol and scirpusin B caused vasorelaxation, both compounds induced slight vasoconstriction at lower concentrations (1−10 μM). Consistent with our present results, it was reported that low concentrations (1−30 μM) of catechins induce vasoconstriction, whereas they caused vasorelaxation at a higher concentration (100 μM) in rat aortas.[20] As NO is an unstable radical, catechins with the antioxidant properties and free radical scavenging activity would influence the contractile state of vascular tissue. Additionally, it was reported that quercetin exerts dual effects on the O_2^- radical and NO.[21] At physiological conditions of pH, O_2^- concentration, and NO concentration, lower concentrations of quercetin scavenge NO effectively. Quercetin may contribute to limiting NO diffusion in vivo, exclusively at low NO concentrations. The prevention of the extracellular NO scavenging effect of quercetin by SOD led to a quercetin-mediated increase in O_2^- radical scavenging properties, inhibition of tissue O_2^- radical generation, and increase in the biological activity of NO. This mechanism of the dual action of quercetin could help explain the concentration-dependent biphasic vascular response to stilbene derivatives (piceatannol and scirpusin B). It is possible that low concentrations of piceatannol and scirpusin B induce vasoconstriction via NO scavenging properties, whereas at high concentrations, they cause vasorelaxation via endothelial NO-producing and O_2^- radical scavenging activities.

The pharmacokinetics of piceatannol and scirpusin B has not been elucidated. However, the initial serum piceatannol level after intravenous administration of piceatannol at 10 mg/kg was 8.5 μg/mL (35 μM).[22] On the basis of the present results, this concentration of piceatannol is sufficient to exert vasorelaxant effects. In contrast, the pharmacokinetics of scirpusin B is not known at present. In this study, we developed a method to obtain large amounts of highly purified piceatannol and scirpusin B from passion fruit seeds at a reasonably low cost. Further studies are necessary to identify their pharmacokinetics, such as the absorption and metabolism of these polyphenols. Furthermore, in vivo studies using animal models (SHR, KKAy, ZDF etc.) will elucidate the effects of piceatannol and scirpusin B on blood pressure, glucose, oxidized LDL, etc. in the future.

Here, we compared the DPPH radical scavenging activities of the two stilbene derivatives. Scirpusin B had the highest antioxidant activity, followed by piceatannol and resveratrol. On the basis of their chemical structures, it was suggested that the catechol and/or phenolic hydroxyl moieties in their structures were involved in the radical scavenging activities. In general, it is known that the catechol moiety is effectively involved in antioxidant activity. For example, resveratrol has no catechol moiety, whereas piceatannol has one catechol moiety. Scirpusin B (a dimer of piceatannol) has two catechol moieties. Consistent with our present data, several other studies indicate that dimers or oligomers have higher antioxidant activity compared with that of monomers, such as ε-viniferin, a dimer of resveratrol,[23] or procyanidin, an oligomer of catechin.[24]

In conclusion, we identified two stilbene derivatives in passion fruit seeds, piceatannol, and scirpusin B. These two compounds exhibited a vasorelaxant activity, and the activity of scirpusin B was greater than that of piceatannol. Moreover, scirpusin B exhibited a negligible vasoconstriction effect. The vasorelaxation activity of piceatannol and scirpusin B was induced via endothelium-derived NO. In addition, scirpusin B exhibited higher DPPH radical

scavenging activity than piceatannol. The potential effects of piceatannol and scirpusin B in pathophysiological conditions, such as CVDs, remain a matter of further discussion.

AUTHOR INFORMATION

Corresponding Author

*Tel: +81-45-571-2941. Fax: +81-45-571-6109. E-mail: s-sano-jh@morinaga.co.jp.

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Notes

We have no conflicts of interest.

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ABBREVIATIONS USED

ACh, acetylcholine; CVD, cardiovascular disease; DMSO, dimethyl sulfoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EDHF, endothelium-derived hyperpolarizing factor; FT/IR, Fourier transform infrared spectrometry; HIV, human immunodeficiency virus; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography mass spectrometry; LDL, low-density lipoprotein; L-NAME, N^G-nitro-L-arginine methylester; MES, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; NO, nitric oxide; ODS, octadecylsilyl; PE, phenylephrine; RWPC, red wine polyphenolic compound; SEM, standard error of the mean; SOD, superoxide dismutase; UV, ultraviolet.

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