Identification of a New Natural Vasorelaxatant Compound, (+)-Osbeckic Acid, from Rutin-free Tartary Buckwheat Extract

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The candidates responsible for vasorelaxation action of rutin-free tartary buckwheat extract (TBSP) were examined in this study. As a result of reversed-phase high-performance liquid chromatography (HPLC) separations, five prominent peaks in the acidic fraction of TBSP were obtained at 260 nm. Among the five collected peaks, we successfully identified four compounds by nuclear magnetic resonance (NMR) and mass spectrometry (MS) measurements: (+)-osbeckic acid as a dimer ([M − H]$^-$ *m/z*: 371.2 > 184.9 > 140.9), 5-hydroxymethyl-2-furoic acid, protocatechuic acid, and *p*-hydroxybenzoic acid. A vascular contractive measurement in 1.0 μM phenylephrine-contracted Sprague−Dawley rat thoracic aorta rings revealed that (+)-osbeckic acid dimer evoked a potent vasorelaxant effect with an EC$_{50}$ value of 887 μM compared to other isolates (EC$_{50}$: 5-hydroxymethyl-2-furoic acid, 3610 μM; protocatechuic acid, 2160 μM; *p*-hydroxybenzoic acid, no inhibition). Dimeric (+)-osbeckic acid was stable in solutions and at high temperatures, while its degraded peak on the HPLC chromatogram was observed when it was dissolved in dimethyl sulfoxide.

KEYWORDS: (+)-Osbeckic acid; tartary buckwheat; vasorelaxation; aorta

INTRODUCTION

It is said that buckwheat is effective for health promotion, in particular the prevention of hypertension (*1*). In our recent study on the potential physiological roles of buckwheat, we obtained useful information that a hot-water extract of tartary buckwheat (*Fagopyrum tataricum* Gaertn.) containing no rutin (TBSP) played a role in the regulation of vascular functions (*2*); in a contractile experiment using Sprague−Dawley (SD) rat thoracic aorta rings contracted by phenylephrine (PE), the acidic fraction of the extract elicited an endothelium-dependent vasorelaxation effect via NO/cGMP pathways (EC$_{50}$ value of 0.25 mg/mL).

As far as the authors know, no research has been conducted concerning the candidates responsible for vasorelaxation of tartary buckwheat, besides rutin (*3*) or its aglycon (i.e., quercetin) (*4*). Although some physiologically functional compounds in buckwheat, such as fagopyritols (*5*) and quercitrin (*6*), were also identified as antidiabetic compounds, the possible involvement of these buckwheat compounds in the vasorelaxation of TBSP would be excluded from the candidates, because they could not elute into a non-absorbed fraction (or TBSP fraction) of hot-water extract of tartary buckwheat on an SP-70 absorption chromatography (*2*). The aim of the present study was thus to identify vasoactive candidates from TBSP. High-performance liquid chromatography (HPLC), followed by nuclear magnetic resonance (NMR) and mass spectrometry (MS), allowed us to identify four natural compounds, including (+)-osbeckic acid [2*S*-hydroxy-2-(5-carboxy-2-furyl)acetic acid]. We also

examined their vasorelaxation powers on contracted SD thoracic aorta rings.

MATERIALS AND METHODS

Materials. Tartary buckwheat was supplied from ITO EN, Ltd. (Shizuoka, Japan). The acidic fraction of a non-absorbed fraction of hot-water extract of buckwheat (TBSP) on a SP70 open column chromatography (Φ 60 × 120 mm, Mitsubishi Chemical Co., Tokyo, Japan) was obtained according to our previous paper (*2*). PE was purchased from Wako Pure Chemical (Osaka, Japan). Synthetic osbeckic acid was provided from Shinsei Chemical Co. (Osaka, Japan). Apocynin was purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical reagent grade and were used without further purification.

Instruments. ^{1}H and ^{13}C NMR spectra were recorded on a JNM A400 NMR instrument (JEOL, Tokyo, Japan). CD$_3$OD and tetramethylsilane (TMS) (Wako Pure Chemical) were used as solvent and internal standard, respectively. MS^n analysis was taken on an Esquire6000 electrospray ionization (ESI)−ion trap (IT) mass spectrometer (Bruker Daltonics, Bremen, Germany). The conditions of ESI−ITMS measurements in negative ionization mode were as follows: nebulizer gas, 10 psi; dry gas, 7 L/min; dry temperature, 300 °C; capillary, 50.0 V; trap drive, 45.3 V; octapole 1 DC, 9.74 V; octapole 2 DC, 9.74 V; resolution, *m/z* 0.25; target IT, 20 000. ESI−time of flight (TOF) MS spectra were measured for (+)-osbeckic acid using a microTOF II mass spectrometer (Bruker Daltonics). The sample for MS measurements was dissolved in methanol containing 0.1% formic acid (FA).

HPLC Separation. The acidic fraction of TBSP was applied to a Shimadzu LC-10A HPLC system (Kyoto, Japan) on an ODS column (Cosmosil 5C$_{18}$-ARII, Φ 10 × 250 mm, Nacalai Tesque, Kyoto, Japan) with a linear gradient solvent system of CH$_3$CN (0−20%, 60 min) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 2.0 mL/min at 260 nm at 35 °C. Namely, 250 μL of acidic fraction (10 mg/mL) was applied to the

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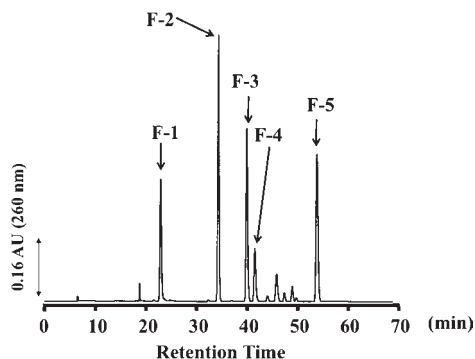
Figure 1. HPLC profiles of the acidic fraction of tartary buckwheat extract. The acidic fraction of the hot-water extract of tartary buckwheat containing no rutin was applied on a reversed-phase HPLC system. HPLC conditions were as follows: column, Cosmosil $5C_{18}$-ARII (Φ 10 × 250 mm); elution, CH_3CN (0−20%, 60 min) in 0.1% TFA; flow rate, 2.0 mL/min; absorbance, 260 nm; temperature, 35 °C. Arrowed peaks denoted as F-1−F-5 were collected to dryness.

reversed-phase HPLC, and the observed five major peaks, denoted as F-1−F-5, were individually collected to dryness: F-1, white powder, 22.0 min on the HPLC retention, 46.1 mg/g in yield from acidic fraction; F-2, light brown powder, 34.0 min, 72.5 mg/g; F-3, white powder, 40.0 min, 56.8 mg/g; F-4, yellow powder, 42.0 min, 31.2 mg/g; and F-5, white powder, 53.0 min, 37.3 mg/g.

Stability Experiments of F-1. An aliquot of the dried F-1 fraction was dissolved in water, 0.1% TFA solution, or dimethyl sulfoxide (DMSO) to examine its stability. After voltex, each solution was incubated at 4 °C for 2 weeks or 90 °C for 90 min. The stability was evaluated in the above-described HPLC conditions. F-1 dissolved in DMSO was immediately (or without incubation) injected to the above-described HPLC, and further separation was performed by HPLC (column: Cosmosil $5C_{18}$-ARII, Φ 10 × 250 mm) with an isocratic 0.1% TFA solution at a flow rate of 2.0 mL/min at 260 nm at 35 °C.

Contractive Study. Preparation of thoracic aorta rings from male 8−9-week-old SD rats (SPF/VAF Crj:SD, Charles River Japan, Kanagawa, Japan) was performed by our previous study (*2*). The prepared thoracic aorta ring was mounted between two stainless-steel hooks in a 5 mL organ bath filled with PSS buffer maintained at 37 °C. PSS buffer had the following composition (in mM): NaCl, 145; KCl, 5.0; Na_2HPO_4, 1.0; $CaCl_2$, 2.5; $MgSO_4$, 0.5; glucose, 10; and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 5. The rings were allowed to equilibrate for 45 min under a resting tension of 2 g before experiments were begun. During the equilibration period, the PSS buffer adequately bubbled with the gas mixture (95% O_2/5% CO_2) was exchanged every 15 min. Aorta rings were contracted by 1.0 μM PE, and isolates from the acidic fraction of TBSP were then added to the bath in a cumulative manner (0.5−2.5 mg/mL) after maximal contractile tension was achieved. Each isolate collected from the acidic fraction was used for this experiment. The changes in isometric tension (g) were measured with a force transducer, which was connected via an amplifier (Bridge 8, World Precision Instruments, Inc., Berlin, Germany) to a data acquisition system (Biopac System, model MP100, Santa Barbara, CA). In all experiments for endothelium-dependent vascular responses, special care was taken to avoid damaging the luminal surface of the endothelium. The results are expressed as the mean ± standard error of the mean (SEM) (*n* = 3). Rat experiments were carried out under the Guidance for Animal Experiments in Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105, 1973) and Notification (No. 6, 1980 of the Prime Minister's Office) of the Japanese Government.
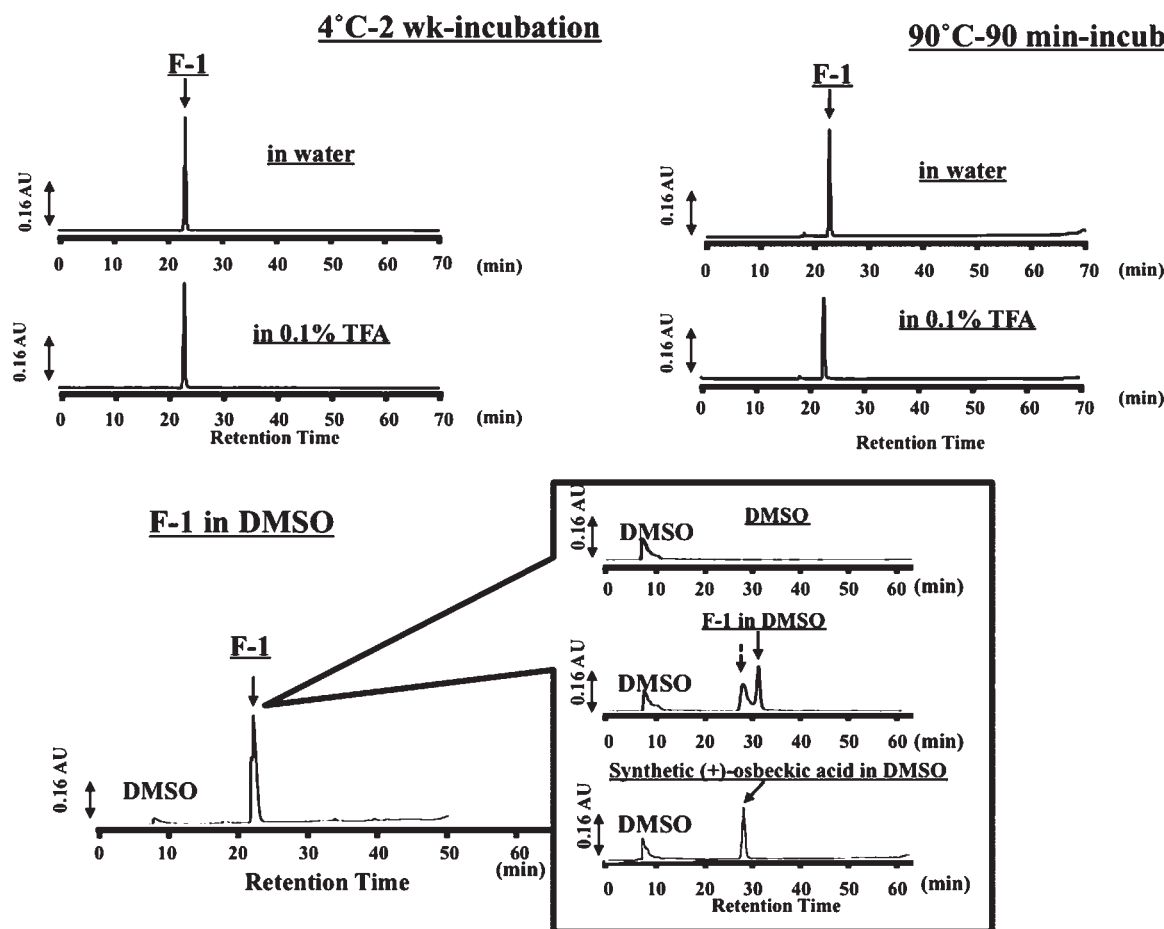
RESULTS AND DISCUSSION

Identification of Compounds in Rutin-free Tartary Buckwheat Extract. Figure 1 shows the typical HPLC chromatogram of the acidic fraction of TBSP extract, while monitoring at 260 nm. As depicted with arrows, five major peaks denoted as F-1−F-5 were
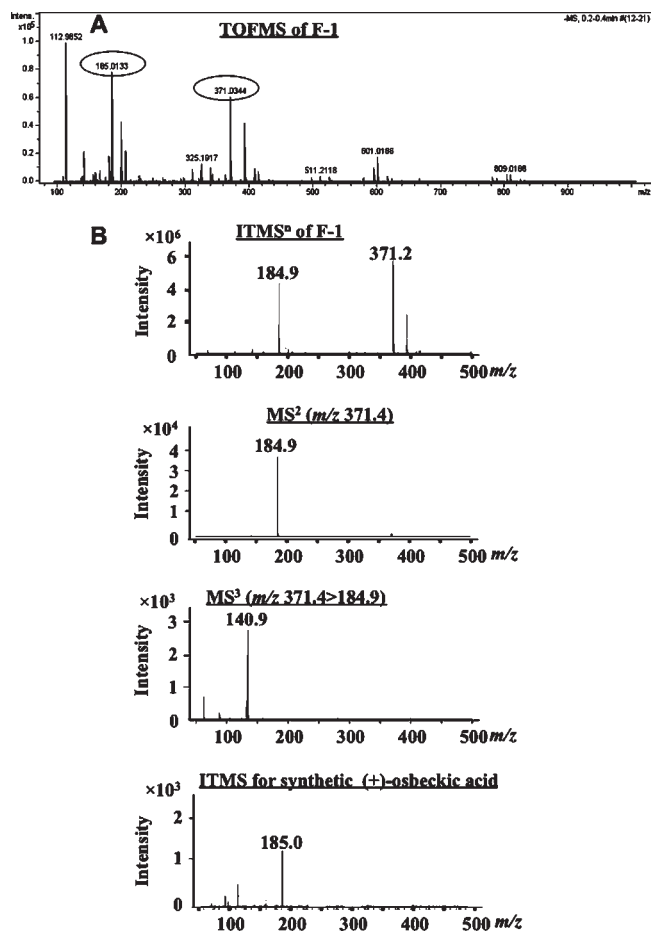
Figure 2. ESI−MS chromatograms of F-1 and synthetic osbeckic acid. (A) ESI−TOFMS and (B) ESI−ITMS^n analyses of F-1 were performed in negative ionization mode. ESI−ITMS was also performed for synthetic osbeckic acid.

observed under the linear gradient solvent condition of CH_3CN (0−20%, 60 min) in 0.1% TFA. Although data were not shown, the elution profile at 220 nm was similar to that at 260 nm and few peaks were observed over 20% CH_3CN elution, indicating that most of the compounds in the acidic fraction of the TBSP extract were eluted within the assayed HPLC conditions.

As a result of reversed-phase HPLC separations of each peak in combination with NMR and ESI−MS analyses, we successfully identified four compounds from five arrowed peaks (**Figure 1**). F-2 was identified as 5-hydroxymethyl-2-furoic acid from the following. ^{1}H NMR (400 MHz, CD$_3$OD) δ: 7.13, 1H, *d*, *J* = 3.4 Hz, H-3; 6.45, 1H, *d*, *J* = 3.4 Hz, H-4; 4.56, 2H, *s*, C$\underline{H_2}$OH). ^{13}C NMR (100 MHz, CD$_3$OD) δ: 160.5, C-5'; 119.7, C-4'; 110.2, C-3'; 57.6, C-1. ESI−ITMS ([M − H]$^-$) *m*/*z*: 141.2. Analyses of ^{1}H NMR and ESI−ITMS spectra of F-3 revealed protocatechuic acid. ^{1}H NMR (400 MHz, CD$_3$OD) δ: 7.43, 1H, *s*, H-2'; 7.40, 1H, *d*, *J* = 2.0 Hz, H-6'; 6.78, 1H, *d*, *J* = 7.8 Hz, H-5'). ESI−ITMS ([M − H]$^-$) *m*/*z*: 153.0. NMR and ESI−ITMS spectra of F-5 showed that F-5 was *p*-hydroxybenzoic acid. ^{1}H NMR (400 MHz, CD$_3$OD) δ: 7.87, 2H, *d*, *J* = 8.8 Hz, H-2' and H-6'; 6.81, 2H, *d*, *J* = 8.8 Hz, H-3' and H-5'. ^{13}C NMR (100 MHz, CD$_3$OD) δ: 163.3, C-1; 133.0, C-2'; 122.9, C-4'; 116.0, C-3'. ESI−ITMS ([M − H]$^-$) *m*/*z*: 137.0. F-4 could not be identified because of low ionization efficiency in the negative ESI mode. For the identification of F-1, NMR analyses revealed it as 2*S*-hydroxy-2-(5-carboxy-2-furyl)acetic acid [or (+)-osbeckic acid]. ^{1}H NMR (400 MHz, CD$_3$OD) δ: 7.25, 1H, *d*, *J* = 3.9 Hz, H-4'; 6.64, 1H, *d*, *J* = 3.9 Hz,

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Figure 3. Stability of F-1 in different solvent systems. F-1 was incubated in either water or 0.1% TFA at 4 °C for 2 weeks, followed by the reversed-phase HPLC analysis (see the caption of **Figure 1**). Another set of incubation of F-1 was performed in either water or 0.1% TFA at 90 °C for 90 min. F-1 dissolved in DMSO without incubation was analyzed in the above HPLC conditions. Insets show the second HPLC separation [column, Cosmosil $5C_{18}$-ARII (Φ 10 × 250 mm); an isocratic elution, 0.1% TFA solution; flow rate, 2.0 mL/min; absorbance, 260 nm; temperature, 35 °C]. Synthetic (+)-osbeckic acid in DMSO and DMSO alone were also analyzed in the second HPLC conditions.

H-3′; 5.36, 1H, *s*, H-2. ^{13}C NMR (100 MHz, CD$_3$OD) δ: 176.7, COOH; 165.2, C-1; 158.2, C-5′; 147.8, C-2′; 122.0, C-4′; 113.8, C-3′; 69.6, C-2. However, the NMR data obtained in this study were not completely matched with the reported ones (*7*). Thus, to make sure that the structure of F-1 was (+)-osbeckic acid, we tried to determine the precise molecular weight by an ESI−TOFMS spectrum. As a result, F-1 gave two [M − H]$^-$ of *m/z* 185.0132 and 371.0342, and their molecular formulas were found to be $C_7H_5O_6$ and $C_{14}H_{11}O_{12}$, respectively (**Figure 2A**). The results of TOFMS spectra, including the neutral loss of *m/z* 186 from F-1, strongly indicated that the formula of $C_{14}H_{11}O_{12}$ with *m/z* 371.0342 is a dimer of (+)-osbeckic acid ($C_7H_6O_6$). In addition, in ESI−ITMS^n analyses (**Figure 2B**), F-1 gave a fragmentation pattern of *m/z* 371.2 > 184.9 > 140.9. As shown in **Figure 2B**, the MS spectrum of synthetic osbeckic acid gave the same molecular ion ([M − H]$^-$) of *m/z* 185.0 as that of F-1, except for *m/z* 371.2. As far as the authors know, (+)-osbeckic acid has already been identified as an antioxidant compound from the shrub *Oskecia chinensis* L. (*8*), while no report was found that it forms a dimer.

Formation and Stability of (+)-Osbeckic Acid Dimer. To confirm whether (+)-osbeckic acid occurs as a dimer in the acidic fraction of TBSP extract, we challenged to examine the stability of F-1 at different incubation conditions. As shown in **Figure 3**, HPLC chromatograms of F-1 dissolved in either acidic solution of 0.1% TFA or deionized water gave a single peak even after

2 weeks of incubation at 4 °C or 90 min of incubation at 90 °C. This implies that F-1 isolated from the acidic fraction was stable against both acid and heat treatments. In contrast, a HPLC chromatogram of F-1 dissolved in DMSO gave two apparent peaks, with their retention times of 29.1 and 31.3 min (shown in the inset of **Figure 3**), upon which the former eluted peak corresponded to the retention time of synthetic (+)-osbeckic acid monomer treated with DMSO, while DMSO alone did not show any peaks. These findings indicate that F-1 formed as a dimer of (+)-osbeckic acid was degraded into a monomer (retention time of 29.1 min) by treating with DMSO, being matched with the results of MS analyses (**Figure 2**). The pK_a of (+)-osbeckic acid was estimated to be 3.01 by ChemAxon Marvin software, version 4.0.6, available on the website (*9*). Taken together, intermolecular hydrogen bonds between (+)-osbeckic acids may be responsible for the high stability of (+)-osbeckic acid dimer in water and acidic solutions (**Figure 4**). Lichtenthaler et al. (*7*) have reported that (+)-osbeckic acid (monomer) was endogenously generated from daucic acid in plants, suggesting that a dimeric (+)-osbeckic acid may be formed during the hot-water extraction and/or subsequent fractionation processes of TBSP preparations.

Vasorelaxation Effect of Identified Compounds. We evaluated the vasorelaxation effects of isolates from TBSP in a contractile force measurement, all of which were prepared from the acidic fraction for this study. In endothelium-intact PE-contracted thoracic aorta rings, (+)-osbeckic acid dimer, 5-hydroxymethyl-2-furoic acid, and

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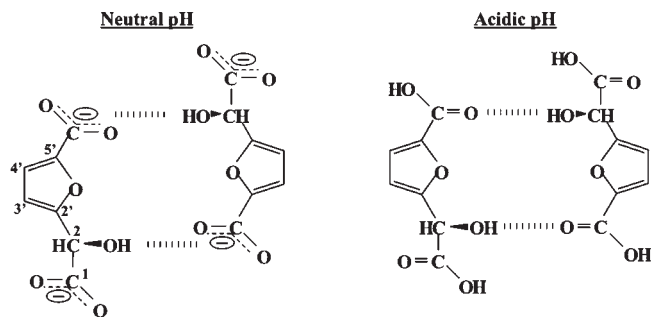
Figure 4. Estimated dimeric formation of (+)-osbeckic acid at neutral and acidic conditions. The estimated pK_a value of (+)-osbeckic acid was 3.01.
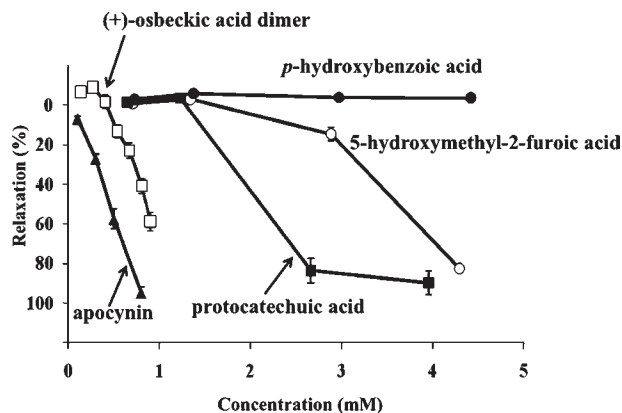
Figure 5. Vasorelaxation action induced by isolates identified from the acidic fraction of tartary buckwheat extract. The resting tension was set at 2 g before stimulation by each isolate. Each isolate collected from the acidic fraction was used for the experiment. 5-Hydroxymethyl-2-furoic acid, protocatechuic acid, *p*-hydroxybenzoic acid, and (+)-osbeckic acid dimer were individually added in a cumulative manner (0.5–2.5 mg/mL) to a 1.0 μM PE-contracted SD aorta ring. Apocynin was also evaluated in the same manner. The results are expressed as the mean $\pm$ SEM ($n = 3$).

protocatechuic acid exhibited a dose-dependent vasorelaxation, while no significant relaxation was observed for *p*-hydroxybenzoic acid (**Figure 5**). The effective concentration of (+)-osbeckic acid dimer, 5-hydroxymethyl-2-furoic acid, and protocatechuic acid producing 50% relaxation of maximal contractile response (EC_{50}) by 1.0 μM PE was 887, 3610, and 2160 μM (or 0.33, 0.51, and 0.33 mg/mL), respectively. Considering the vasorelaxation power of the acidic fraction of TBSP extract [EC_{50} of 0.25 mg/mL (*2*)] and the low content of (+)-osbeckic acid dimer in it (4.61%), the contribution of its vasorelaxation effect against the overall vasorelaxation effect of the acidic fraction would be low; in other words, any other vasoactive compounds may still remain in the acidic fraction of the TBSP extract. Alternatively, according to the report by Su et al. (*8*), (+)-osbeckic acid had an ability to enhance antioxidation power of α-tocopherol by its synergic effect. Therefore, the potent vasorelaxation effect of the acidic fraction of TBSP may be explained by (+)-osbeckic-acid-induced synergic antioxidation power, because the vasorelaxation effect of the acidic fraction was closely associated with endothelium-dependent NO/cGMP pathways (*2*). However, further studies must be needed to clarify whether the dimer elicits a synergic antioxidant effect as well. As shown in **Figure 5**,

half of the vasorelaxation power of (+)-osbeckic acid dimer was obtained in comparison to apocynin (EC_{50} of 452 μM). Interestingly, apocynin, which is a natural vasorelaxant NAD(P)H oxidase inhibitor (*10*), forms a dimer [a covalent dimeric form through redox reactions with superoxide anion radical ($O_2^{-\bullet}$)] to inhibit the assembly of the NAD(P)H oxidase complex (*11*). It also remains in question whether the dimeric (+)-osbeckic acid acts in a similar manner to apocynin at the vessel.

In conclusion, we demonstrated for the first time that a dimer of (+)-osbeckic acid [2*S*-hydroxy-2-(5-carboxy-2-furyl)acetic acid] was identified as a vasorelaxant compound in the tartary buckwheat extract. The dimer relaxed the PE-contracted aorta rings with an EC_{50} value of 887 μM. The candidate responsible for the effect should thus be addressed into bioactive compounds in tartary buckwheat, such as rutin, quercitrin, and quercetin.

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