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Isolation and Structural Elucidation of Antimicrobial Compounds from Buckwheat Hull

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Abstract Three new compounds were isolated from the methanol extracts of buckwheat (*Fagopyrum esculentum*) hull, and their structures were determined to be 6,7-dihydroxy-3,7-dimethyl-octa-2(*Z*),4(*E*)-dienoic acid (**1**), 6,7-dihydroxy-3,7-dimethyl-octa-2(*E*),4(*E*)-dienoic acid (**2**), and 4,7-dihydroxy-3,7-dimethyl-octa-2(*E*),5(*E*)-dienoic acid (**3**) by NMR and MS spectroscopic analyses. These compounds at 500 μg concentration showed antimicrobial activity against *Staphylococcus aureus*, demonstrated by the paper disc method.

Key words: Buckwheat hull, *Fagopyrum esculentum*, antimicrobial compound, 6,7-dihydroxy-3,7-dimethyl-octa-2,4-dienoic acid, 4,7-dihydroxy-3,7-dimethyl-octa-2,5-dienoic acid

Buckwheat (*Fagopyrum esculentum*, Polygonaceae) has been considered as a health food in terms of its nutritional value (essential amino acids, fatty acids, and vitamins B_1 and B_2) [7] and biological function (blood pressure lowering effect and antioxidative activity) [5, 9]. Several studies have been carried out on the biological activities in seeds, leaves, and hulls [4, 8, 11], and it has generally been suggested that the main role of seed hull is in the chemical and physical protection of seeds or grains from attack by insects and microorganisms [12]. Several compounds with antimicrobial and antioxidative activities have been isolated from plant hulls of rice [2], oat [13], and peanut [3]. Watanabe *et al.* [10] identified phenolic compounds such as quercetin, hyperin, protocatechuic acid, and 3,4-dihydroxybenzaldehyde from buckwheat hull as antioxidants, and Mukoda *et al.* [6] investigated antioxidative effects of buckwheat hull extract against various oxidative stresses *in vitro* and *in vivo*. However, a phytochemical study of antimicrobial substances from buckwheat hull has not yet been achieved.

In a previous study, we confirmed the antimicrobial activity of a methyl alcohol (MeOH) extract of buckwheat hull. In particular, the ethyl acetate (EtOAc)-soluble acidic fraction obtained by solvent fractionation had strong antimicrobial activity against various microorganisms. Therefore, we attempted to isolate antimicrobial compounds from the EtOAc-soluble acidic fraction, and the isolated compounds were identified as azelaic and 3,4-dihydroxybenzoic acids [1]. As a part of our ongoing investigation, we isolated three new monoterpenoids from the EtOAc-soluble acidic fraction from the MeOH extract of buckwheat hull and identified them as antimicrobial compounds. In this paper, we describe the isolation and structural elucidation of the compounds.

Materials and Methods

Plant Material

The buckwheat (*Fagopyrum esculentum* Möench) hull was cultivated in Bongpyoung, Korea. After harvesting, the seeds were sun-dried and dehulled. The moisture level of the hull was 13.20±0.07%.

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Extraction and Isolation of Antimicrobial Compounds

The hull (15 kg) was successively extracted with *n*-hexane, EtOAc, and MeOH (90 l, respectively) for 24 h at room temperature. The MeOH extracts were concentrated *in vacuo* at 38°C, and the MeOH extracts were partitioned between EtOAc and 0.2 M glycine-0.2 M HCl buffer solution at pH 3.0. The organic phase was fractionated between organic and aqueous layers with a buffer solution of 0.2 M NaH_2PO_4/0.2 M Na_2HPO_4 (pH 8.0). The pH of the aqueous layer was adjusted to 3.0 with 1.0 M HCl and extracted with EtOAc to obtain the EtOAc-soluble acidic fraction.

The EtOAC-soluble acidic fraction was chromatographed on a silica gel (160 g, Kieselgel 60, 70–230 mesh, Merck) column using a solvent system of EtOAc/MeOH (20:0, 20:1, 19:1, 18:2, 17:3, 16:4, v/v, stepwise; 1,600 ml each). The active fraction was refractionated by silica gel (60 g, Merck) column chromatography and eluting with *n*-hexane/EtOAc/MeOH (12:2:1, 10:4:1, 8:6:1, and 6:8:1, v/v, 600 ml each). The active fraction was subjected to Sephadex LH-20 (total volume 250 ml; 25–100 mesh; Pharmacia Fine Chemicals) column chromatography and elution was carried out with MeOH/$CHCl_3$ (4:1, v/v) as the solvent system. This active fraction was chromatographed on octadecylsilane (ODS, 7 g, 70230 mesh, YMC, Kyoto, Japan) column, which was stepwise eluted with gradients of 30, 40, 50, 60, 70, and 80% MeOH in H_2O (70 ml each). The fraction was finally purified by repeated HPLC under the following conditions: ODS column, Senshu pak (8 × 250 mm, 10 μm); mobile phase, MeOH/H_2O (3:7, v/v); flow rate, 1.5 ml/min (Model 510 solvent delivery system, Waters, U.S.A.); detection, UV detector (486 Tunable absorbance detector, Waters). Other fractions obtained from the silica gel column chromatography was purified by Sephadex LH-20 column chromatography, ODS column chromatography, and HPLC, under the same conditions described above.

Structural Determination of Antimicrobial Compounds

NMR (nuclear magnetic resonance) spectra were obtained through JEOL JNM 400 spectrometery (Jeol, Tokyo, Japan) in CD_3OD with tetramethylsilane (TMS, δ=0) as the internal standard. High resolution fast atomic bondard-mass spectrometry (HRFABMS) spectra were measured on a JMSD-300 spectrometer (Jeol). Optical rotations were measured on a JASCO CD-J600 digital polarimeter using a 5-cm cell, and UV spectra were measured on a DU-650 UV-VIS spectrophotometer (Buckman).

Microorganisms and Bioassay

The antimicrobial activity was measured using a paper disc (8 or 6 mm, Whatman, Maidstone, England) method [14]. The bioassay for purification of the antimicrobial substances was performed with *Staphylococcus aureus*, and benzoic acid (Hayashi Pure Chemical Industries, Osaka, Japan) was used as a positive control. To measure the antimicrobial activity, a paper disc treated with each sample was placed on a solid plate, and the diameter (mm) of inhibition was measured after 24 h of incubation at 30°C or 37°C. The media for microorganisms tested (16 strains) were BHI broth (Difco, Detroit, MI, U.S.A.) for *Enterococcus faecalis* KCTC 3195; Lactobacilli MRS broth (Difco) for Lactobacilli (*Lactobacillus plantarum* KCTC 3104, *Lactobacillus brevis* KCTC 3102), *Leuconostoc mesenteroides* KCTC 3100, and *Pediococcus cerevisiae* KCTC 1628; and Nutrient broth (Difco) for the remaining bacteria (*S. aureus* KCTC 1947, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Micrococcus luteus* ATCC 9341, *Escherichia coli* ATCC 10536, *E. coli* O157: H7 ATCC 43890, *Samonella typhi* ATCC 19214, and *Pseudomonas aeruginosa* ATCC 9027).

Results and Discussion

Extraction of Antimicrobial Compounds

Buckwheat hull (15 kg) was successively extracted with *n*-hexane, EtOAc, and MeOH.

Antimicrobial activity of each extract (2 g equivalent wt. hull) was determinated against *S. aureus* and *E. coli* (data not shown), and only the MeOH extract was found to have strong growth inhibitory activity against the microorganisms. Therefore, the MeOH extract (250 g) was partitioned between EtOAc and various buffers, and the EtOAc-soluble acidic fraction showed higher antimicrobial activity than the EtOAc-soluble neutral fraction against *S. aureus*, *B. subtilis*, *M. luteus*, *L. mesenteroides*, *E. coli* O157: H7, *S. typhi*, and *P. aeruginosa* (data not shown). In particular, the antimicrobial activity against *S. aureus* was stronger than that against the other microorganisms. Therefore, the EtOAc-soluble acidic fraction was subjected to further purification, which was guided by antimicrobial activity against *S. aureus*.

Purification and Isolation of Antimicrobial Compounds

The EtOAc-soluble acidic fraction (12.9 g) was chromatographed on a silica gel column (EtOAc/MeOH), and the active fraction (100% EtOAc, 5.3 g) was refractionated by the same silica gel column chromatography (*n*-hexane/EtOAc/MeOH). Of the two antimicrobial active fractions (*n*-hexane/EtOAc/MeOH, 12:2:1, v/v, 233.3 mg; 10:4:1, v/v, 272.4 mg), the less polar fraction (233.3 mg) was subjected to Sephadex LH-20 column chromatography by eluting with MeOH/$CHCl_3$ (4:1, v/v). This active fraction [V_e/V_t (elution volume/total volume) 0.72–0.86, 130 mg] was chromatographed on an ODS column (MeOH/H_2O, stepwise), and then the active fraction (50% MeOH, 67 mg) was rechromatographed on a Sephadex LH-20 column using

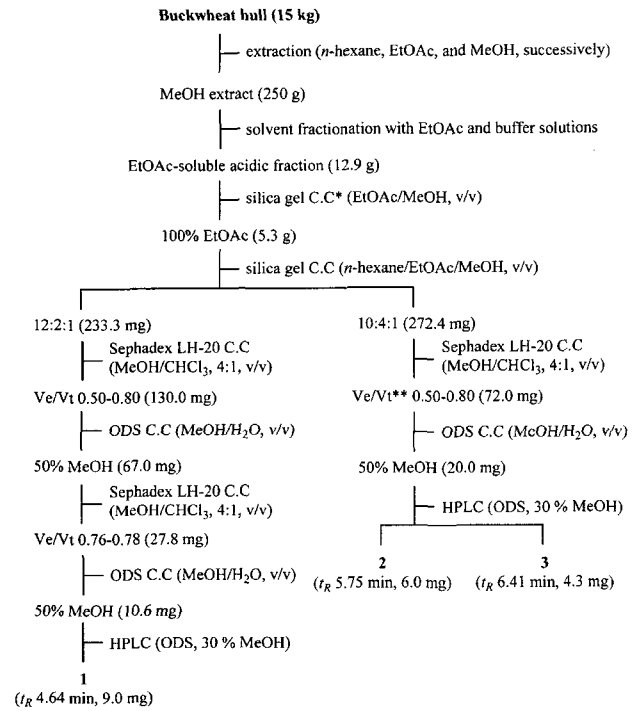

Fig. 1. Purification and isolation procedures of compounds **1–3** from MeOH extract of buckwheat hull. *: Column chromatography. **: V_e/V_t: the ratio of elution volume to total volume on Sephadex LH-20 column chromatography.

the same condition as described above. The active fraction (V_e/V_t 0.76–0.78, 27.8 mg) was further purified by ODS column chromatography ($MeOH/H_2O$, stepwise), and the resulting fraction (10.6 mg) was finally purified by repeated HPLC to yield compound **1** (t_R 4.64 min, 9.0 mg).

The more polar fraction (*n*-hexane/EtOAc/MeOH, 10:4:1, v/v, 272.4 mg) obtained from the silica gel column chromatography was subjected to Sephadex LH-20 column chromatography ($MeOH/CHCl_3$, 4:1, v/v). This active fraction (V_e/V_t 0.68–0.82, 72 mg) was further purified by ODS column chromatography ($MeOH-H_2O$, stepwise), and the active eluate (50% MeOH, 20.0 mg) was finally purified by HPLC to obtain compounds **2** (t_R 5.75 min, 6.0 mg) and **3** (t_R 6.41 min, 4.3 mg), by using the same condition as that for isolation of **1**. The isolation and purification procedures of these compounds are illustrated in Fig. 1.

Structural Elucidation of Antimicrobial Compounds

To elucidate the structure of antimicrobial compound isolated from the MeOH extract of buckwheat hull, NMR and HRFABMS analyses were performed. The spectroscopic data for antimicrobial compounds are as follows.

Compound **1**: white powder; $[\alpha]_D^{25}$ +3.0° (*c* 0.4, MeOH); UV (MeOH) λmax (log ε) 256 (4.00); ^{1}H- and ^{13}C-NMR (CD_3OD), Tables 1 and 2; HRFABMS (negative ion; matrix, Magic/Thio 12) *m/z* 199.0969 $[M-H]^-$ (calc. for $C_{10}H_{15}O_4$, -0.1 mmu)

Compound **2**: white powder; $[\alpha]_D^{25}$ +3.6° (*c* 0.11, MeOH); UV (MeOH) λmax (log ε) 251 (3.30); ^{1}H- and ^{13}C-NMR (CD_3OD), Tables 1 and 2; HRFABMS (negative ion; matrix, Magic/Thio 12) *m/z* 199.0974 $[M-H]^-$ (calc. for $C_{10}H_{15}O_4$, *m/z* 199.0974, +0.3 mmu)

Compound **3**: white powder; $[\alpha]_D^{25}$ -9.2° (*c* 0.13, MeOH); ^{1}H- and ^{13}C-NMR (CD_3OD), see Tables 1 and 2; HRFABMS (negative ion; matrix, Magic/Thio 12) *m/z* 199.0970 $[M-H]^-$ (calc. for $C_{10}H_{15}O_4$, +0.8 mmu)

The molecular formula of $C_{10}H_{16}O_4$ for **1** was determined by negative HRFABMS (matrix, Thio12) *m/z* 199.0969 (-0.1 mmu for $C_{10}H_{15}O_4$) together with other spectral data. The ^{1}H-NMR spectrum (Table 1) of **1** indicated the presence of three methyl protons (δ 1.15, 1.21, 2.26), three olefinic protons (δ 5.76, 6.38, and 6.26), and an oxygenated methine proton (δ 3.94). The ^{13}C-NMR spectrum (Table 2)

Table 1. ^{1}H-NMR data of compounds **1**, **2**, and **3** in CD_3OD.

Position	δ_H (mult, *J*)		
	1	**2**	**3**
1	-	-	-
2	5.76 (1H, s)	5.83 (1H, s)	5.94 (1H, t, *J*=1.2)
3	-	-	-
4	6.38 (1H, d, 15.9)	6.31 (1H, d, 15.6)	4.33 (1H, td, 1.2, 6.6)
5	6.26 (1H, dd, 6.2, 15.9)	6.26 (1H, dd, 6.6, 15.6)	5.58 (1H, dd, 6.6, 15.6)
6	3.94 (1H, d, 6.2)	3.90 (1H, dd, 6.6)	5.85 (1H, dd, 1.2, 15.6)
7	-	-	-
8	1.15 (3H, s)[a]	1.15 (3H, s)[b]	1.97 (3H, s)[c]
9	1.21 (3H, s)[a]	1.14 (3H, s)[b]	1.97 (3H, s)[c]
10	2.26 (3H, s)	2.17 (3H, s)	1.27 (3H, s)

[a-c]Shift may be interchangeable.

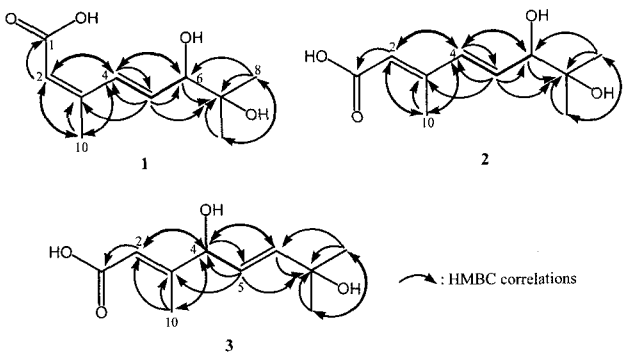

Fig. 2. The structures of compounds **1**–**3** and important correlations observed in their HMBC spectra.

of **1** showed 10 carbon signals, including one carbonyl carbon (δ 170.5), three methyl carbons (δ 25.8, 25.3, and 14.0), four olefinic carbons (δ 153.2, 136.7, 135.9, and 120.5), an oxygenated methine carbon (δ 79.9), and an oxygenated quaternary carbon (δ 73.7). Based on the HRFABMS and 1D-NMR (Tables 1 and 2) spectroscopic data, **1** was suggested to be octadienoic acid tetra-substituted with two hydroxyl and three methyl groups. The cross-peak between the olefinic proton signal (δ 5.76, H-2) and the carbonyl carbon signal (δ 170.5, C-1) in the HMBC spectrum (Fig. 2) indicates that the carboxyl carbon is conjugated with C-2 of **1**. Furthermore, the positions of two hydroxyl groups at C-6 and C-7 and two methyl groups at C-3 and C-7 were elucidated by HMBC experiment (Fig. 1). The coupling constant (15.9 Hz) of the olefinic proton signals [δ 6.38 (H-4) and 6.26 (H-5)] at the 4 position gave the configuration of the 4 position as an (*E*) geometry. In addition, to confirm the geometry of the double bond in the 2 position, **1** was analyzed by NOESY. The presence of correlation between δ 5.76 (H-2) and δ 2.26 (H-10) in the NOESY spectrum indicated that the configuration of the 2 position is a (*Z*) form. Consequently, the structure of **1** was determined to be 6,7-dihydroxy-3,7-dimethyl-octa-2(*Z*),4(*E*)-dienoic acid (Fig. 2).

The structure of **2** was determined in a similar manner as **1**. Compound **2** showed the same molecular formula ($C_{10}H_{16}O_4$, *m/z* 199.0974, +0.3 mmu for $C_{10}H_{15}O_4$) as that of **1** in the negative HRFABMS. The ^{1}H-NMR spectrum (Table 1) of **2** was almost identical to that of **1**, except for the coupling constants and chemical shifts of H-4 [δ 6.31 (d, *J*=15.6 Hz)], H-5 [δ 6.26 (dd, *J*=15.6, 6.6 Hz)], and H-6 [δ 3.90 (d, *J*=6.6 Hz)]. The double bond of the (*E*) form at the 4 position of **2**, corresponding to that of **1**, was also deduced from the coupling constants (15.6 Hz, respectively) of H-4 (δ 6.31) and H-5 (δ 6.26). The ^{13}C-NMR spectrum (Table 2) of **2** showed 10 carbon signals to be closely related to that of **1**. Comparing with the ^{13}C-NMR spectrum (Table 2) of **1**, the carbon signals of C-3 (δ 144.1) and C-4 (δ 132.9) of **2** were upfield-shifted at 10.9 and 3.0 ppm, respectively, while the carbonyl signal (C-1, δ 177.0) was downfield-shifted at 6.5 ppm. Other signals also exhibited the differential chemical shift at 0.6 ppm lower than those of **1**. The ^{1}H-^{1}H COSY and HMBC (Fig. 2) data of **2** also corresponded to those of **1**. The chemical shifts in the 1, 3, and 4 positions of **2** different from those of **1** suggested a mutually different geometry of the 2 position. In the NOESY analysis of **2** to determine the geometric structure, the correlation between δ 6.26 (H-5) and δ 2.17 (H-10) confirmed that the double bond in the 5 position of **2** was an (*E*) configuration. The cross-peak between δ 5.83 (H-2) and δ 6.31 (H-4) was also detected from the NOESY spectrum, showing that the partial structure in the 2 position of **2** was an (*E*) form. Consequently, the structure of **2** was assigned as 6,7-dihydroxy-3,7-dimethyl-octa-2(*E*),4(*E*)-dienoic acid (Fig. 2).

The ^{1}H-NMR spectrum (Table 1) of **3** showed the presence of three methyl protons (δ 1.97, 1.97, and 1.27), three olefinic protons (δ 5.94, 5.58, and 5.85), and an oxygenated methine proton (δ 4.33). In the ^{13}C-NMR spectrum (Table 2), four olefinic carbon signals (δ 122.7, 128.2, 140.9, and 150.8), including a quaternary carbon signal (δ 150.8), confirmed the partial structure of two double bonds. In addition, the presence of three methyl carbons (δ 29.8, 29.8, and 14.8) and an oxygenated methine carbon (δ 78.0) was also supported by the ^{13}C-NMR spectrum (Table 2). Furthermore, two carbon signals, an oxygenated quaternary carbon (δ 71.0) and a carbonyl carbon (δ 176.8), were also observed in the ^{13}C-NMR spectrum (Table 2). The molecular formula of **3** was deduced as $C_{10}H_{16}O_4$ by the negative HRFABMS (*m/z* 199.0970, +0.8 mmu for $C_{10}H_{15}O_4$). Comparing with the MS and 1D-NMR data of **1** and **2**, the structure of **3** appeared to be similar to those of **1** and **2**, but differs in the position and geometry of the hydroxyl and olefinic groups. In the HMBC spectrum (Fig. 2), the correlation of the hydroxyl methine proton (δ 4.33, H-4) with δ 122.7 (C-2),

Table 2. ^{13}C-NMR data of compounds **1**, **2**, and **3** in CD_3OD.

Position	δ_C (mult, *J*)		
	1	**2**	**3**
1	170.5, s	177.0, s	176.8, s
2	120.5, d	120.5, d	122.7, d
3	153.2, s	144.1, s	150.8, s
4	135.9, d	132.9, d	78.0, d
5	136.7, d	137.3, d	128.2, d
6	73.7, d	73.7, d	140.9, d
7	79.9, s	80.4, s	71.0, s
8	25.8, q[a]	25.4, q[b]	29.8, q[c]
9	25.3, q[a]	25.6, q[b]	29.8, q[c]
10	14.0, q	14.1, q	14.7, q

[a–c]Shift may be interchangeable.

Table 3. Antimicrobial activities of compounds **1**, **2**, and **3**.

Microorganisms	Inhibition (Clear zone, mm)			
	Compounds (500 μg)[a]			Benzoic acid[b] (250 μg)
	1	2	3	
Staphylococcus aureus KCTC 1927	9	9	9	11

[a]Size of paper disc for measurement of antimicrobial activity was 6 mm.
[b]Benzoic acid was used as a reference compound.

128.2 (C-5), 140.9 (C-6), and 14.7 (C-10) indicated that the hydroxyl group was substituted at C-4 of **3**. The complete planar structure of **3** was assigned as 4,7-dihydroxy-3,7-dimethyl-octa-2,4-dienoic acid. In addition, the coupling constant (15.6 Hz) of the olefinic proton signals [δ 5.88 (H-5) and 5.85 (H-6)] was deduced to be an (*E*) configuration of the 5 position. Moreover, the correlation between δ 4.33 (H-4) and δ 5.95 (H-2) was observed in the NOESY spectrum of **3**. This result suggests that the double bond at 2 position of **3** is an (*E*) configuration. Consequently, the structure of **3** was assigned to be 6,7-dihydroxy-3,7-dimethyl-octa-2(*E*),5(*E*)-dienoic acid (Fig. 2).

To the best of our knowledge, the compounds **1–3** have never before been reported.

Antimicrobial Activity of Novel Compounds

Antimicrobial assay for **1–3** was performed by the paper disc method against only one strain of *S. aureus*, because of the limited amount of the isolated materials. Although the antimicrobial activities of **1–3** were inferior to those of the antimicrobial compounds [1] (azelaic acid, 15.5± 0.4 mm; 3,4-dihydroxybenzoic acid, 11.4±0.7 mm; against *S. aureus* at the concentrations of 500 μg), they were comparable to those of the reported compounds and benzoic acid as a positive compound (Table 3). The antimicrobial activity as well as structural characterization of the compounds **1–3** should be elucidated against various microorganisms in future.

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