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Identification of novel saponins from edible seeds of Japanese horse chestnut (*Aesculus turbinata* BLUME) after treatment with wooden ashes and their nutraceutical activity

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Abstract

Natural seeds of Japanese horse chestnut (*Aesculus turbinata* BLUME) contain large amounts of mixed triterpenoidal saponins called escins. Recent studies have shown that escins have several biological activities including anti-inflammatory action and inhibitory effects on the absorption of ethanol and glucose. For the edible utilization of the seeds, natural seeds are usually treated with wooden ashes to remove harshness. Here, we found the novel compounds derived from escins in the edible seeds after the food processing with wooden ashes. The instrumental analyses revealed the chemical structures of escins and the derivatives. These compounds are identified as four types of deacetylescins Ia, IIa, Ib, and IIb as well as two types of desacylescins I and II. To determine their biological activity, the purified compounds were tested for their potential nutraceutical activity. The oral glucose tolerance test in mice revealed that a single oral administration of the isolated components of deacetylescins at a dose of 100 mg/kg was clearly effective in attenuating the elevation of blood glucose levels. The inhibitory effects of escins and their derivatives were in the order of escins > deacetylescins > desacylescins. Moreover, we found the inhibitory activity of those compounds on pancreatic lipase. Escins were the most potent in inhibiting the enzyme activity, and followed by desacylescins and then deacetylescins. Taken together, our results suggest the potential usefulness of novel saponins including deacetylescins and desacylescins from edible seeds as novel sources for nutraceutical foods with anti-obese effects.

Keywords: *Aesculus turbinata* BLUME; Japanese horse chestnut; Saponin; Escin; Deacetylescin; Desacylescin; ESI-MS/MS; NMR; Nutraceutical activity

1. Introduction

The seeds of Japanese horse chestnut (*Aesculus turbinata* BLUME) were used historically as emergency provisions in ancient times. Recently, the seeds have been utilized traditionally as components of rice cake and rice balls in Japan. The seeds are known to contain large amounts of saponins called escins, a mixture of triterpene oligoglycosides. For the edible utilization of the seeds, they are usually treated with alkaline wooden ashes to remove harshness which exhibits a highly bitter taste. Moreover, European horse chestnut seeds (*Aesculus hippocastanum* L.) also contain the same types of saponins. The extracts of European horse chestnut seeds have been useful for the utilization as commercial medicines and cosmetics for therapeutic applications to the inflammation and edema. Escins have been reported to show anti-inflammatory [1–3], anti-edematous

Abbreviations: ESI-MS/MS, electrospray ionization-mass spectrometry/mass spectrometry; NMR, nuclear magnetic resonance; RP-HPLC, reverse-phase high-performance liquid chromatography

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[3], and capillaro-protective activities [2,3] as well as inhibitory effects on the absorption of ethanol and glucose [4,5].

We are interested in the transformation of saponins in the edible seeds of Japanese horse chestnut after food processing to remove harshness with alkaline wooden ashes. This treatment would get rid of some of bitter substances from the edible seeds, but not all of them. Nevertheless, until now, the information on the content and composition of saponins in these edible seeds has been limited. From the point of finding nutraceutical activities of Japanese horse chestnuts, we attempt to identify the chemical structures of saponin constituents from the edible seeds after food processing, and to determine the biological activities of escins and their derivatives in terms of the inhibitory effects on the elevation of blood glucose levels in mice and inhibitory effects on pancreatic lipase activity in vitro.

Here, we provide the evidence that food processing of Japanese horse chestnuts with wooden ashes generates the novel compounds derived from escins, which have nutraceutical activities associated potentially with antidiabetic or anti-obese effects.

2. Materials and methods

2.1. Materials

The seeds of Japanese horse chestnut (*A. turbinata* BLUME) were collected in forests of northern Hyogo Prefecture in Japan where this species is the most predominant. We can easily distinguish the seeds of this species from other seeds of European horse chestnut (*A. hippocastanum* L.) because the fruits of the Japanese species have no thorns that are found in the European species. The identification of the seeds was finally done by the comparison with the known sample seeds in Tottori Prefectural Forest Experimental Station (Tottori, Japan). Wooden ashes were obtained from Makino Timber Industry (Okayama, Japan). Porcine pancreatic lipase (type II) and 4-methylumbelliferyl oleate were purchased from Sigma (St. Louis, MO, USA). β-Escin was obtained from Wako (Osaka, Japan). Glutestace R Kit was supplied by Sanwa Kagaku Kenkyusho (Nagoya, Japan). Diaion HP-20 and Chromatorex ODS 1024T for column chromatography were obtained from Nippon Rensui (Tokyo, Japan) and Fuji Silysia (Kasugai, Japan), respectively. Analytical and preparative columns of YMC-Pack ODS AM for high-performance liquid chromatography (HPLC) are the products of YMC (Kyoto, Japan). C18 Maxiclean cartridge column was from Alltech (Tokyo, Japan). Male ICR mice were supplied by Shimizu (Kyoto, Japan). All other chemicals were of reagent grade.

2.2. Preparation of edible seeds of Japanese horse chestnut by treatment with wooden ashes

After dried natural seeds were immersed for 4 days in tap water at room temperature, the outer layer of the seeds was removed away. The resulting seeds (1 kg) were boiled for 1 h in 3 L of tap water, mixed with 1.4 L of a suspension containing 1 kg of wooden ashes at 60 °C, and kept for 48 h at room temperature. The seeds were washed with tap water and used actually for edible sources of foods.

2.3. Extraction, fractionation, and isolation of saponins from natural and edible seeds

Extraction and purification of escins from natural and edible seeds of Japanese horse chestnut seeds were carried out by the modification of the method of Yoshikawa et al. [4]. The natural seeds (4 kg, 52.2% (w/w) water) were immersed in methanol for 1 week, and the resulting methanol extracts were filtrated and evaporated to dryness to give 154 g of the dried materials. The methanol extracts were applied to absorption column chromatography (500 mm × 60 mm i.d.) using Diaion HP-20 with 0.5-mm diameter particles (synthetic adsorbent). After the column was washed with 3 L of distilled water to remove sugars, the fraction containing saponins (56 g of dried materials) was obtained by eluting with 3 L of methanol. The resulting extracts corresponding to 10 g of dry materials were dissolved in 200 mL of 40% methanol and then applied to reverse-phase column chromatography with Chromatorex ODS 1024T (320 mm × 32 mm i.d.). The column was washed with 1 L of 40% methanol, and then followed by the elution with 1 L of 90% methanol to obtain purified saponins (8.45 g of dried materials) from the natural seeds. By repeating the above processes, 47 g of the purified saponins were obtained from 4 kg of natural seeds. Similarly, 11 g of the purified fraction including saponins and their derivatives were prepared from 4 kg of edible seeds (64.8% (w/w) water) as described above.

For the purification of individual components of saponins from natural and edible seeds, HPLC analysis was done on a Shimadzu LC-2010A system equipped with a preparative HPLC column of YMC-Pack ODS (150 mm × 10 mm i.d.). The preparative column was eluted at a flow rate of 3 mL/min with a mobile phase of methanol/10 mM sodium phosphate buffer (pH 7) (62:38, v/v). The elution of saponins was detected by monitoring the absorbance at 230 nm. For the analytical purpose, YMC-Pack ODS AM (150 mm × 6 mm i.d.) was eluted at a flow rate of 0.8 mL/min with the same mobile phase as above.

The conversion of escins to deacetylescins was performed by treatment with 5% potassium carbonate (pH 11.7) for 48 h at ambient temperature. After adjusting pH of the solutions to around pH 7 with 0.1 N HCl, the compounds were eluted twice with 5 ml of 70% methanol through a C18 Maxiclean cartridge column after washing twice with 5 ml of 40% methanol. The conversion was confirmed by HPLC using an analytical column of YMC-Pack ODS AM (150 mm × 6 mm i.d.) as described above. For the preparation of desacylescins, commercially available β-escins were hydrolyzed according to the method of Yoshikawa et al. [4].

2.4. Instrumental analyses

Chemical structures of saponins from natural and edible seeds were identified by electrospray ionization-mass spectrometry/mass spectrometry (ESI-MS/MS) at a positive ion mode (LCQ Deca XP, ThermoQuest, Waltham, MA, USA). In

addition, JNM-A400 FT-NMR (400 Hz) system (JEOL, Tokyo, Japan) was employed for the analyses of the compounds by ^{1}H nuclear magnetic resonance (NMR), and ^{13}C NMR. The samples were dissolved in pyridine-d_5 containing 0.05% TMS and subjected to the analyses. Water content was determined using a Shimadzu EB-340MOC infrared water analyzer (Shimadzu, Kyoto, Japan).

2.5. Glucose tolerance test in mice

Male 6-week ICR mice were housed at 23 °C on 12-h–12-h light–dark cycle, and had free access to conventional diets for two weeks. After the mice were fasted for 16 h, the blood was withdrawn from the tail vein and subjected to the assay of blood level of glucose. Then, saponin fractions from natural and edible seeds of Japanese horse chestnut or the purified components of escins, deacetylescins, and desacylescins were individually suspended in 0.3 mL of physiological saline. The resulting suspension was administered orally into the stomach of mice before a single oral injection of glucose (0.5 g/kg mouse) dissolved in 0.1 mL of physiological saline. Thereafter, the blood was withdrawn at 0.5, 1, and 2 h, and analyzed for the blood glucose levels using Glutestace R according to the manufacture's instructions. The elevation of blood glucose levels was calculated by subtracting the blood glucose levels prior to the administration of glucose from those after the administration of glucose.

2.6. Assay of pancreatic lipase activity

To determine the inhibitory activity of escins and their derivatives against porcine pancreatic lipase, the enzyme (0.066 units using triacetin as a substrate according to the manufacture) was incubated with 50 μM 4-methylumbellifery oleate as a substrate in the presence of increasing concentrations of the purified constituents of escins and their derivatives. The assay buffer (total 200 μL) consisted of 20 mM McIlvane buffer at pH 7.4 with 0.02% sodium deoxycholate [6,7]. The sample to be tested was dissolved in 70% methanol and included in the assay buffer to bring the concentration of methanol to 3.5%. After the enzyme reaction was conducted by the incubation at 37 °C for 20 min, the reaction was terminated by the addition of 1 mL of 0.1 M HCl and 2 mL of 0.1 M sodium citrate. The amount of 4-methylumbelliferone released by the lipase was determined fluorometrically at an excitation wavelength of 320 nm and an emission wavelength of 450 nm on a JASCO FP777 fluorometer (JASCO Co., Hachioji, Japan). Others were done essentially according to the procedures described elsewhere [6,7].

2.7. Statistical analysis

Data represent the mean ± standard error (S.E.M.) and were analyzed by one-way analysis of variance (ANOVA) and followed by Dunnett's post test for multiple comparisons. Differences were considered to be significant when the probability value was less than 0.05.

3. Results and discussion

3.1. Chemical conversion of saponins after treatment with wooden ashes

Saponins derived from natural and edible seeds of Japanese horse chestnut were extracted and fractionated by column chromatography using Diaion HP-20 and Chromatorex ODS 1024T. The amount of the fraction containing saponins and the related substances was calculated to be 0.78% of dried edible seeds while natural seeds contained 2.5% of the dried seeds. Therefore, edible seeds retained approximately 30% of saponins and the derivatives found in natural seeds.

When the saponin fractions obtained from natural seeds were analyzed by reverse-phase HPLC, four major peaks of **1**–**4** were

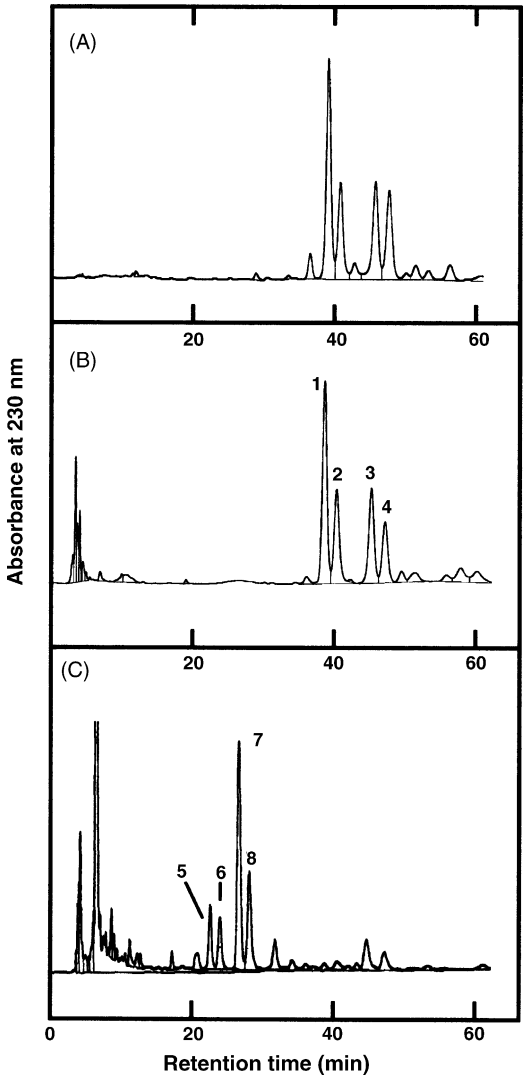


Fig. 1. Reverse-phase HPLC of saponins extracted from natural and edible seeds of Japanese horse chestnut. Extracted saponins were eluted with a mobile phase of methanol/10 mM sodium phosphate buffer (pH 2.7) (62:38, v/v) on a 5 μm YMC-Pack ODS AM column (150 mm × 6 mm i.d.) at a flow rate of 0.8 mL/min. The elution was monitored by detection at 230 nm. (A) Standard β-escin. (B) Saponins extracted from natural seeds. (C) Saponins extracted from edible seeds after treatment with wooden ashes.

detectable (Fig. 1B), which retention times coincide with those of four major peaks of commercially available standard β-escin from European horse chestnut seeds (Fig. 1A). On the other hand, the HPLC analysis revealed that the eluted peaks of edible saponins shifted to the positions of earlier retention time (Fig. 1C), suggesting the generation of four types of more polar compounds of **5**–**8**. Moreover, to determine the sources of the peaks of saponins from natural seeds for the generation of more polar saponins from edible seeds, each component of the peaks of saponins isolated from natural seeds was purified and subjected to the hydrolysis with 5% potassium carbonate (Fig. 2). After this treatment, the analysis by reverse-phase HPLC clearly demonstrated that the new peaks of **5**–**8** occurring in saponins from edible seeds were derived from the corresponding peaks of **1**–**4** found in saponins from natural seeds, respectively.

3.2. Identification of chemical structures of saponin derivatives formed by treatment with wooden ashes by instrumental analyses

Saponins and the related compounds derived from natural and edible seeds were isolated finally by reverse-phase HPLC. The isolated components were subjected to the analysis of ESI-MS/MS at a positive-ion mode (Fig. 3, Table 1). The analysis by ESI-MS revealed that mass spectra of the compounds **1** and **5** were characterized by quasimolecular ions $[M+Na]^+$ at *m/z* 1153.5 and *m/z* 1111.5, respectively (Fig. 3). The findings suggest the loss of an acetyl moiety of **1** to generate a hydroxyl group in **5**. Similarly, the compounds **2**–**4** appeared to lose an acetyl moiety to form the deacetylated derivatives **6**–**8**. Moreover, the analysis with ESI-MS/MS gave the characteristic fragment ions reflecting the parent molecules (Table 1).

To confirm the chemical structures of saponins and the derivatives, the structural analyses of those compounds were furthermore performed by ^{1}H NMR (Table 2) and ^{13}C NMR (Table 3).

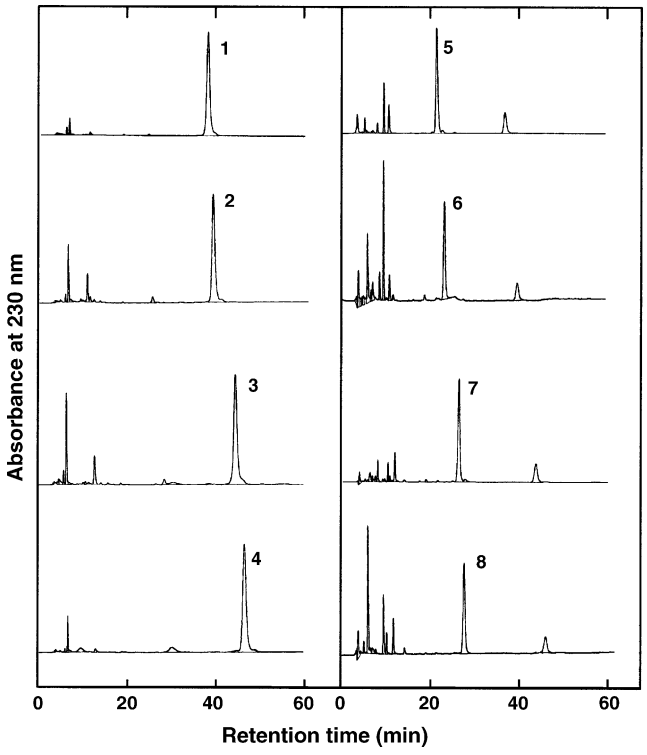


Fig. 2. Reverse-phase HPLC profiles of escins isolated from natural seeds and their hydrolytic products formed by mild alkaline treatment. Each component of escins from natural seeds was isolated by reverse-phase HPLC as described in Fig. 1. The purified components were treated with 5% potassium carbonate (pH 11.7). Then, the hydrolyzed products were extracted and analyzed by reverse-phase HPLC as detailed in Section 2. Panels show the HPLC profiles of escins Ia (**1**), IIa (**2**), Ib (**3**), and IIb (**4**) isolated from natural seeds, and their hydrolytic products (**5**–**8**), respectively.

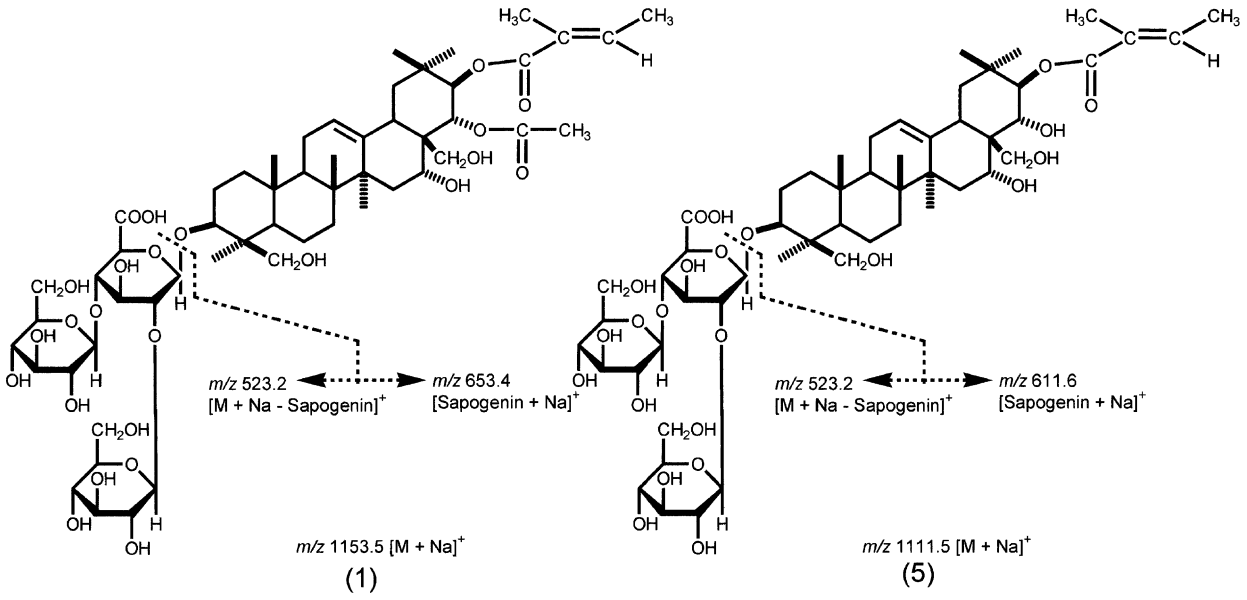


Fig. 3. ESI-MS/MS analysis of escin Ia (**1**) and its hydrolytic product (**5**) formed by treatment with wooden ashes. ESI-MS/MS analysis was performed in a positive-ion mode. Major ions characteristic for escin Ia (**1**) and its hydrolytic product (**5**) are represented in corresponding structural formulas.

Table 1
Mass spectral data for saponins isolated from natural and edible seeds of Japanese horse chestnut

Characteristic ion	*m/z* Value							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
$[M+Na]^+$	1153.5	1123.5	1153.5	1123.5	1111.5	1081.5	1111.5	1081.5
$[M+Na-Glc^a]^+$	991.5	961.5	991.4	961.5	949.4	919.3	949.4	919.3
$[M+Na-Xyl^b]^+$	–	991.5	–	991.3	–	949.5	–	949.4
$[Sapogenin+Na]^+$	653.4	653.4	653.4	653.4	611.6	–	–	611.5
$[M\text{-}Sapogenin+Na]^+$	523.2	493.2	523.1	493.2	523.2	493.2	523.1	493.2

Saponins from natural and edible seeds were separated by reverse-phase HPLC as described in Section 2. The resulting purified components were subjected to the analysis of ESI-MS/MS in a positive-ion mode. The mass spectral data provide *m/z* values of characteristic ions for escins (**1**–**4**) from natural seeds and their hydrolytic products (**5**–**8**) after treatment with wooden ashes.

[a] Glc, glucose.

[b] Xyl, xylose.

Table 2
^{1}H NMR data for acyl moieties at C-21 and an acetyl moiety at C-22 of saponins from natural and edible seeds of Japanese horse chestnut

Peak of HPLC	Chemical shift (δ)			
	Tigloyl or angeloyl moiety			Acetyl moiety, H2′′′′
	H3′′′′	H4′′′′	H5′′′′	
Escin Ia (1)	7.12 (1H, dq-like)	1.66 (3H, d, J = 7.1 Hz)	1.98 (3H, s)	1.92 (3H, s)
Escin IIa (2)	7.12 (1H, dq-like)	1.66 (3H, d, J = 7.1 Hz)	1.97 (3H, s)	1.92 (3H, s)
Escin Ib (3)	5.99 (1H, dq-like)	2.12 (3H, d, J = 7.1 Hz)	2.03 (3H, s)	1.92 (3H, s)
Escin IIb (4)	5.99 (1H, dq-like)	2.12 (3H, d, J = 7.1 Hz)	2.03 (3H, s)	1.93 (3H, s)
(5)	6.99 (1H, dq-like)	1.57 (3H, d, J = 7.1 Hz)	1.82 (3H, s)	–
(6)	6.99 (1H, dq-like)	1.56 (3H, d, J = 7.1 Hz)	1.86 (3H, s)	–
(7)	5.92 (1H, dq-like)	2.03 (3H, dd, J = 7.1, 1.5 Hz)	1.93 (3H, s)	–
(8)	5.91 (1H, dq-like)	2.02 (3H, dd, J = 7.1, 1.5 Hz)	1.93 (3H, s)	–

Saponins (**1**–**4**) and their derivatives (**5**–**8**) from natural and edible seeds were purified by reverse-phase HPLC, and then subjected to ^{1}H NMR analysis as described in Section 2. Values of chemical shift reflecting tigloyl, angloyl and acetyl moieties are shown for the corresponding compounds.

Table 3
^{13}C NMR data for saponins from natural and edible seeds of Japanese horse chestnut

Position	Chemical shift (δ_c)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sapogenol moiety								
C1	38.4	38.8	38.5	38.8	38.5	38.8	38.5	39.0
C2	26.5	26.6	26.6	26.6	26.6	26.6	26.5	26.6
C3	91.1	90.7	91.1	90.7	91.1	90.7	91.1	90.7
C4	43.6	44.3	43.7	44.3	43.7	44.3	43.6	44.3
C5	56.0	56.3	56.1	56.3	56.2	56.3	56.1	56.3
C6	18.5	18.7	18.5	18.7	18.6	18.7	18.5	18.7
C7	33.2	33.3	33.2	33.3	33.3	33.4	33.2	33.3
C8	39.9	40.0	39.9	40.1	40.0	40.0	39.9	40.0
C9	46.7	46.8	46.7	46.8	46.8	46.8	46.6	46.6
C10	36.4	36.5	36.4	36.5	36.5	36.5	36.4	36.5
C11	24.0	24.0	24.0	24.0	24.1	24.1	24.1	24.1
C12	122.9	123.1	123.0	123.0	123.5	123.5	123.4	123.5
C13	142.8	142.9	142.9	142.9	143.4	143.3	143.3	143.3
C14	41.6	41.7	41.7	41.7	41.9	41.9	41.9	41.9
C15	34.6	34.6	34.6	34.6	34.8	34.8	34.8	34.8
C16	68.0	68.1	68.0	68.0	68.2	68.1	68.1	68.1
C17	47.9	48.0	48.0	48.1	47.8	47.8	47.8	47.7
C18	40.0	40.1	40.1	40.2	41.0	41.0	40.9	40.9
C19	47.2	47.2	47.2	47.2	46.8	46.9	46.8	46.8
C20	36.3	36.5	36.3	36.3	36.4	36.4	36.4	36.4
C21	79.3	79.4	78.9	78.9	78.6	78.8	78.5	78.5
C22	74.2	74.3	74.3	74.5	73.7	73.6	73.6	73.7
C23	22.4	22.6	22.5	22.6	22.5	22.6	22.5	22.6

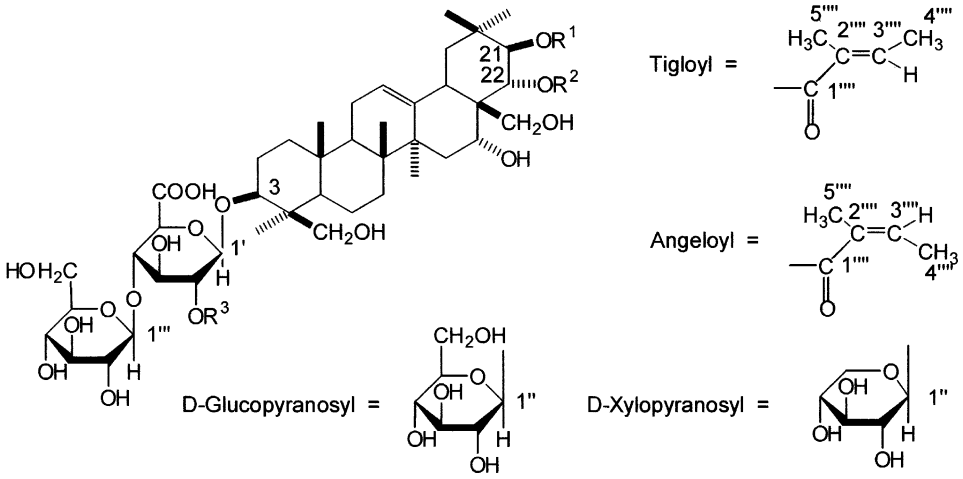
Table 3 (*Continued*)

Position	Chemical shift (δ_c)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
C24	63.3	62.9	63.3	62.9	63.3	62.9	63.3	62.8
C25	15.5	15.5	15.5	15.5	15.6	15.5	15.6	15.5
C26	16.6	16.8	16.7	16.8	17.0	17.0	16.9	17.0
C27	27.4	27.4	27.4	27.5	27.5	27.4	27.4	27.5
C28	63.7	63.7	63.8	63.8	66.9	66.6	66.6	66.6
C29	29.5	29.5	29.5	29.5	30.6	30.6	30.6	30.6
C30	20.1	20.1	20.3	20.3	19.4	19.3	19.4	19.4
Sugar moiety								
3-*O*-β-D-Glucuronopyranosyl moiety								
C1′	104.6	104.8	104.6	104.9	104.6	104.9	104.6	104.8
C2′	79.7	78.9	79.7	78.9	79.8	78.6	79.8	78.8
C3′	76.4	76.4	76.5	76.4	76.5	76.3	76.4	76.3
C4′	81.8	82.5	81.8	82.6	81.8	82.4	81.8	82.4
C5′	75.7	75.7	75.7	75.7	75.8	75.7	75.8	75.8
C6′	172.1	172.5	172.1	172.2	172.1	172.0	172.1	172.1
2′-*O*-β-D-Gluco- or xylopyranosyl moiety								
C1″	104.3	104.8	104.3	104.8	104.3	104.8	104.3	104.7
C2″	75.8	75.9	75.8	75.8	75.8	75.8	75.8	75.8
C3″	78.3	78.4	78.1	78.5	78.1	78.1	78.1	78.1
C4″	69.7	70.8	69.8	70.8	69.8	70.8	69.7	70.8
C5″	78.3	67.1	78.3	67.2	78.4	67.2	78.4	67.1
C6″	61.5	–	61.6	–	61.6	–	61.6	–
4′-*O*-β-D-Glucopyranosyl moiety								
C1‴	104.6	104.7	104.6	104.9	104.7	104.8	104.7	104.8
C2‴	74.8	74.9	74.9	74.9	74.9	74.9	74.9	74.9
C3‴	78.1	78.0	78.0	78.1	78.1	78.1	78.1	78.1
C4‴	71.4	71.4	71.5	71.5	71.5	71.5	71.5	71.5
C5‴	78.4	78.4	78.5	78.5	78.5	78.5	78.5	78.5
C6‴	62.3	62.3	62.4	62.4	62.4	62.4	62.4	62.4
Acyl moiety								
Tigloyl or angeloyl moiety								
C1⁗	168.0	168.1	167.9	167.9	167.9	167.8	167.7	167.7
C2⁗	129.4	129.5	129.0	129.0	129.2	129.2	128.3	128.3
C3⁗	136.8	136.9	137.2	137.2	137.0	137.0	138.3	138.2
C4⁗	14.2	14.2	15.9	15.9	14.2	14.1	15.9	15.9
C5⁗	12.4	12.4	21.0	21.0	12.3	12.3	21.0	20.9
Acetyl moiety								
C1⁗′	171.0	171.1	170.9	171.0	–	–	–	–
C2⁗′	20.9	20.9	20.9	20.9	–	–	–	–

Saponins (**1**–**4**) and their derivatives (**5**–**8**) from natural and edible seeds were purified by reverse-phase HPLC, and then subjected to ^{13}C NMR as described in Section 2. Values of chemical shift reflecting tigloyl, angloyl and acetyl moieties are shown for the corresponding compounds.

The structures of saponins **1**–**4** from natural seeds were identified as escins on the basis of data from ^{1}H NMR and ^{13}C NMR identical to those values that have been reported about the four components of commercially available β-escins derived from European horse chestnuts [4,5]. The NMR analyses of escins including **1**–**4** revealed the chemical shift values reflecting a tigloyl or angeloyl moiety at the C-21 position and an acetyl moiety at the C-22 position. Thus, saponins from natural seeds of Japanese horse chestnut predominantly consisted of escins Ia (**1**), IIa (**2**), Ib (**3**), and IIb (**4**). In support of these assignments, our analytical data by HPLC and MS on escins from natural seeds are consistent with HPLC profiles [8] and MS data [9] of the earlier studies on escins from European horse chestnuts.

On the other hand, the saponin derivatives formed by treatment with wooden ashes did not show the signals corresponding to the acetyl group found in escins as determined by the analysis of ^{1}H NMR and ^{13}C NMR. Taken together with the data of ESI-MS/MS and NMR, we conclude that four peaks of **5**–**8** from edible saponins detected in the profiles of reverse-phase HPLC are determined to be deacetylated forms of escins. Therefore, the treatment with wooden ashes to prepare edible seeds resulted in the hydrolysis of the common acetyl group at C-22 of escins Ia, IIa, Ib, and IIb to generate 21-*O*-tigloylprotoaescigenin-3-*O*-[β-D-glucopyranosyl-(1-2)] [β-D-glucopyranosyl-(1-4)]-β-D-glucuronopyranosyl acid as deacetylescin Ia (**5**), 21-*O*-tigloylprotoaescigenin-3-*O*-[β-D-xylopyranosyl-(1-2)] β-D-glucopyranosyl-(1-4)]-β-D-glucuronopyranosyl acid as deacetylescin IIa (**6**), 21-*O*-angeloylprotoaescigenin-3-*O*-[β-D-glucopyranosyl-(1-2)] [β-D-glucopyranosyl-(1-4)]-β-D-glucuronopyranosyl acid as dea-



Compounds	R^1	R^2	R^3
Escin Ⅰa (**1**)	Tigloyl	Acetyl	D-Glucopyranosyl
Escin Ⅱa (**2**)	Tigloyl	Acetyl	D-Xylopyranosyl
Escin Ⅰb (**3**)	Angeloyl	Acetyl	D-Glucopyranosyl
Escin Ⅱb (**4**)	Angeloyl	Acetyl	D-Xylopyranosyl
Deacetylescin Ⅰa (**5**)	Tigloyl	H	D-Glucopyranosyl
Deacetylescin Ⅱa (**6**)	Tigloyl	H	D-Xylopyranosyl
Deacetylescin Ⅰb (**7**)	Angeloyl	H	D-Glucopyranosyl
Deacetylescin Ⅱb (**8**)	Angeloyl	H	D-Xylopyranosyl
Desacylescin Ⅰ	H	H	D-Glucopyranosyl
Desacylescin Ⅱ	H	H	D-Xylopyranosyl

Fig. 4. Identified structural formulas of saponins isolated from natural and edible seeds of Japanese horse chestnut.

cetylescin Ib (**7**), 21-*O*-angeloylprotoaescigenin-3-*O*-[β-D-xylopyranosyl-(1-2)] [β-D-glucopyranosyl-(1-4)]-β-D-glucuronopyranosyl acid as deacetylescin IIb (**8**), respectively (Fig. 4).

Among four deacetylescins identified here, deacetylescins Ia and Ib have identical chemical structures with aesculiosides A and B isolated from Chinese horse chestnuts (*Aesculus chinenis*) [10]. Chinese horse chestnuts have been utilized as natural medicines called "Sha Luo Zi" for controlling the stomach and inducing analgesic action. It should be noted that deacetylescins IIa and IIb have not been reported until now as far as we know.

The present study showed more selective alkaline hydrolysis of an acetyl moiety at C-22 rather than acyl moieties at C-21 at milder alkaline condition using 5% potassium carbonate. This preferential cleavage of the acetyl moiety would be explained by the resistance of a tigloyl or angeloyl group to the hydrolysis because both acyl groups have a structure of α, β-conjugated carbonyl moiety which could prevent the nucleophilic attack of hydroxide anion. When the HPLC profiles of escins from natural seeds were compared with those of deacetylescins from edible seeds, the relative ratios of deacetylescins Ia and IIa were lower in the saponin fraction from edible seeds than in that of escins Ia and IIa from natural seeds. The observation suggests more efficient hydrolysis of escins Ia and IIa with tigloyl moiety than escins Ib and IIb with angeloyl moiety. Since an angeloyl moiety seems to cause more steric hindrance in structure than a tigloyl moiety, this effect might contribute to the reduced hydrolysis of escins Ib and IIb than other types.

3.3. Inhibitory effect of escins and their derivatives on the elevation of blood glucose levels

To determine a nutraceutical activity of escins and their derivatives from natural and edible seeds, the isolated constituents were tested for their inhibitory effect on the elevation of blood glucose levels by means of the oral glucose tolerance test in mice. A single oral administration of saponin fractions at a dose of 200 mg/kg mouse from natural and edible seeds was found to be efficacious to inhibit the increase in the blood glucose levels after 30 min of the oral administration of glucose (Table 4). The attenuating effect was more potent with saponins from natural seeds than with saponins from edible seeds, suggesting the higher inhibitory effect of escins than deacetylescins. To further confirm this suggestion, each of escins and deacetylescins was isolated from natural and edible seeds, and then separately tested

Table 4
Oral glucose tolerance test to monitor inhibitory effects of escins and their derivatives on the elevation of blood glucose levels in mice

Sample	Dose (mg/kg, p.o.)	Elevation of blood glucose level (mg/dL)		
		0.5 h	1 h	2 h
Control	0	112.0 ± 21.3 (100)	74.5 ± 14.3 (100)	44.0 ± 14.1 (100)
Saponin fraction from natural seeds	200	49.6 ± 9.8 (44)**	55.4 ± 13.5 (74)	48.2 ± 13.8 (110)
Saponin fraction from edible seeds	200	82.2 ± 15.1 (73)*	74.4 ± 20.8 (100)	44.8 ± 23.6 (102)
Desacylescin I + II	100	103.2 ± 7.3 (92)	78.2 ± 21.0 (105)	47.4 ± 16.7 (108)
Desacylescin I + II	200	74.2 ± 17.5 (66)**	48.6 ± 14.7 (65)	27.6 ± 13.8 (63)
Control	0	109.4 ± 12.5 (100)	52.2 ± 8.6 (100)	32.0 ± 5.4 (100)
Escin Ia	100	50.0 ± 29.9 (46)**	43.4 ± 26.2 (83)	33.4 ± 19.0 (104)
Escin IIa	100	57.4 ± 12.2 (53)**	61.8 ± 28.2 (118)	32.4 ± 20.6 (101)
Escin Ib	100	77.6 ± 24.7 (71)*	66.6 ± 11.9 (128)	36.6 ± 6.8 (114)
Escin IIb	100	50.8 ± 15.0 (46)**	56.8 ± 27.5 (109)	37.6 ± 22.6 (118)
Control	0	93.4 ± 4.5 (100)	64.8 ± 5.5 (100)	37.0 ± 11.0 (100)
Deacetylescin Ia	100	79.8 ± 6.7 (85)**	63.6 ± 5.0 (98)	23.6 ± 20.5 (64)
Deacetylescin IIa	100	78.4 ± 13.7 (84)*	54.0 ± 20.5 (83)	27.2 ± 5.6 (74)
Deacetylescin Ib	100	69.8 ± 9.3 (75)**	46.4 ± 13.7 (72)*	18.8 ± 18.2 (51)
Deacetylescin IIb	100	66.4 ± 17.7 (71)*	60.4 ± 24.1 (93)	17.2 ± 22.0 (46)

Escins and their derivatives were extracted, fractionated, and purified from natural and edible seeds of Japanese horse chestnut. After mice were fasted for 16 h, the blood was withdrawn from the tail vein and subjected to the determination of blood glucose levels. Then, various compounds at the indicated doses were administered orally into the stomach. After 30 min, glucose (0.5 g/kg mouse) was given similarly. Thereafter, blood levels were determined at the indicated time, and the data were expressed as the elevated blood glucose levels by subtracting the levels prior to the administration of glucose as described in Section 2. Data represent the mean ± S.E.M. ($n = 5$). Values in parenthesis represent % of control.

* $P < 0.05$.

** $P < 0.01$.

for its ability at a dose of 100 mg/kg to inhibit the elevation of blood glucose level. Although each of escins and deacetylescins showed the significant attenuating effect on the increased level of blood glucose, all components of deacetylescins had slightly less potent activity than those of the corresponding escins. Moreover, we examined the potency of desacylescins including the types I and II prepared by the complete hydrolysis of acyl and acetyl moieties of escins. Even though the oral administration of desacylescins at 100 mg/kg was not effective for the inhibition, higher dose of these compounds at 200 mg/kg was found to show a clearly inhibitory effect.

From our studies on the inhibitory effect of escins and their derivatives isolated from Japanese horse chestnuts on the elevation of blood glucose, we demonstrated that the potency of those compounds is in the order of escins > deacetylescins > desacylescins. This observation suggested that the acyl moieties of a tigloyl or angeloyl group at C-21 and an acetyl moiety at C-22 in escins are crucial for the expression of higher inhibitory activity on the elevation of blood glucose level. This idea is supported by the previous study about the inhibitory effect of escins isolated from European horse chestnut seeds (*A. hippocastanum* L.) on the elevation of plasma glucose level in rats [4,5]. They have also reported that desacylescins without both acyl moieties at C-21 and C-22 failed to show the inhibitory effect at a dose of 100 mg/kg. However, we were able to detect the inhibitory effect at a higher dose of 200 mg/kg even though a lower dose of 100 mg/kg was ineffective. Thus, deacetylescins and desacylesins included in edible seeds are potentially useful for expressing antidiabetic or anti-obese effects. When we examined the effect of escins and their derivatives on the digestive enzymes acting on sugars such as pancreatic α-amylase and α-glucosidase from the small intestine, the inhibition of those enzymes was not significantly evident in vitro assays (data not shown). Thus, as a possible mechanism of their action, escins and deacetylescins would predominantly inhibit the absorption of glucose in the small intestine.

3.4. Inhibitory effect of escins and their derivatives on pancreatic lipase

As another trial to find a nutraceutical activity of escins and their derivatives, we examined the inhibitory effect of those compounds on the enzyme activity of pancreatic lipase (Table 5). Escins Ia, IIa, Ib, and IIb inhibited the pancreactic lipase with IC_{50} values of 48, 61, 24, and 14 μg/mL, respectively. Hence, escins Ib and IIb with angeloyl moieties are more potent than escins Ia and IIa with tigloyl moieties. By contrast, all of deacetylescins exhibited much less potent activity than escins. Nevertheless, more inhibitory effect on the lipase activity was also found with deacetylescins Ib andIIb with an angelogyl group at C-21 than with those of Ia and IIa. This result is consistent with the above finding about more potent inhibitory activity of escins Ib and IIb with an angeloyl group at C-21. On the other hand, desacylescins I and II without both acyl moities at C-21 and C-22 showed higher inhibitory effects for the pancreatic lipase than deacetylescins with acyl moieties at C-21. Although the potency of desacylescins was slightly less efficient than escins, the inhibitory effect was clearly evident. Since desacylescins are available from edible seeds of Japanese horse chestnut by treatment with wooden ashes, these compounds would be potentially useful as constituents for nutraceutical

Table 5
Inhibitory effect of escins and their derivatives on pancreatic lipase activity

Compound	IC_{50} (μg/mL)[a]
Escin Ia	48
Escin IIa	61
Escin Ib	24
Escin IIb	14
Deacetylescin Ia	345
Deacetylescin IIa	>400
Deacetylescin Ib	170
Deacetylescin IIb	90
Desacylescin I	78
Desacylescin II	67

Escins and their derivatives were purified from natural and edible seeds of Japanese horse chestnut. Porcine pancreatic lipase was mixed with increasing amounts of either compound dissolved in 70% methanol, and allowed to react with 4-methylumbelliferyl oleate as a substrate for 20 min at 37 °C. The amount of 4-methylumbelliferone released by the lipase was determined fluorometrically as described in Section 2. The concentration required for 50% inhibition was represented as the value of IC_{50} (μg/mL).

[a] IC_{50}, concentration required to 50% inhibition.

foods aiming at an anti-obese effect. More recently, we have found that desacylescins are efficacious in the suppression of the obesity induced by high-fat diets in mice for 8 weeks (data not shown). Furthermore, we recently found much more reduced extent of a bitter taste with the saponin fraction from edible seeds as compared with the fraction from natural seeds when they were compared at the same concentrations (data not shown), implicating the advantage and significance of deacetylescins and desacylescins in the utilization as food constituents. A recent study has also reported the inhibitory effect of dietary teasaponins on the pancreatic lipase activity in vitro experiments as well as their anti-obese effects in mice treated with high-fat diets [6]. In addition, dioscin and its aglycone, diosgenin, a saponin family, isolated from the root of *Dioscorea nipponica* Makino, a perennial herb, have been reported to show anti-obese effects through the inhibition of pancreatic lipase in rodents [7]. Thus, efficacy of certain types of saponins is getting evident in vivo using animal model experiments. Although the safety and biological activity of those compounds in human remain to be determined, several types of saponins are expected to have promising potentials for exerting their anti-obese effects in vivo.

4. Conclusions

We identified novel types of saponin derivatives formed from edible seeds of Japanese horse chestnut by treatment with wooden ashes. Their chemical structures were determined by combined instrumental analyses using ESI-MS/MS and NMR. The edible seeds contain deacetylescins and desacylescins as novel saponin constituents. Deacetylescins Ia, IIa, Ib, and IIb as well as desacylescins I and II were found to be effective to cause the inhibitory effect on the elevation of blood glucose levels, indicating their antidiabetic activity. In addition, they exhibited the inhibitory action on pancreactic lipase, leading to anti-obese effects. Taken together, the saponin derivatives generated during the food processing of Japanese horse chestnuts would be promising because they retained nutraceutical activities with the reduction in a bitter taste.

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