Isolation of five proanthocyanidins from pear (*Pyrus pyrifolia* Nakai) fruit peels

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Abstract Five proanthocyanidins, two B-type dimers and three A-type trimers, were purified and isolated from the fruit peels of *Pyrus pyrifolia* Nakai cv. Chuhwangbae. The isolated compounds were identified as (–)-epicatechin gallate-(4β → 8)-(–)-epicatechin (Hahashi et al. in Ann Biol Res 3:3200–3207, 2012), (–)-epicatechin-(4β → 8)-(–)-epicatechin (procyanidin B2) (Tanrioven and Eksi in Food Chem 93:89–93, 2005), (–)-epicatechin-(4β → 8, 2β → O-7)-(–)-epicatechin-(4β → 8)-(–)-epicatechin (cin namtannins B1) (Salta et al. in J. Fun. Food 2: 153–157, 2010), (–)-epicatechin-(4β → 8)-(–)-epicatechin-(4β → 8, 2β → O-7)-(–)-epicatechin (aesculitannin A) (Challice and Westwood in Phytochemistry 11: 37–44, 1972), and (–)-epicatechin-(4β → 6)-(–)-epicatechin-(4β → 8, 2β → O → 7)-(–)-epicatechin (Es-Safi et al. in J Agric Food Chem 54: 6969–6977, 2006). Their structures were determined by nuclear magnetic resonance and mass spectrometry. The three A-type proanthocyanidin trimers were identified for the first time from pear.

Keywords *Pyrus pyrifolia* Nakai · Pear fruit · Proanthocyanidin · Trimeric A-type proanthocyanidin · Epicatechin

Introduction

Pear (*Pyrus* spp., Rosaceae) is one of the most highly consumed fruits in the world. Diverse pear species are cultivated in the mildly temperate regions of Asia and Europe [1]. However, despite the widespread of pear fruit, their biological effects and chemical constituents have not been studied sufficiently compared to other fruits such as apples, grapes, and citrus. Various phenolic compounds, including β-arbutin [2], chlorogenic acid derivatives [3, 4], catechin derivatives [4–6], as well as flavonols and their glycosides [7, 8] have been identified in pear fruits, where the studies was mainly focused on European pear species. Several studies suggest that both genetic and chemical characteristics are different between European and Asian pears [9, 10]. Recently, steroids, quercitrin, and triterpenoids were identified as anti-inflammatory compounds in *Pyrus bretschneideri*, which is one of the Chinese pear species [11, 12]. We also investigated the antioxidative properties of chemical constituents of the fruit peels of *P. pyrifolia* Nakai cv. Chuhwangbae, a highly consumed pear cultivar in Korea. Various chemical constituents such as phenolic compounds [13–15], hydroxycinnamoylmalic acids and their methyl esters [16], flavonoids [17], caffeoyl triterpenes [18], and sterol glucosides [15] have been isolated from them. We decided to further investigate the chemical constituents of pear fruits and then additionally isolated five proanthocyanidins, including two B-type dimers and three A-type trimers. In this study, we report the isolation and identification of these five proanthocyanidins from the fruit peels of *P. pyrifolia* Nakai cv. Chuhwangbae.

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Materials and methods

Materials and chemicals

The fresh fruits were collected from *P. pyrifolia* Nakai cv. Chuhwangbae growing in Naju city, South Korea. After hand peeling of the pear fruits at about 3 mm thickness, the fruit peel was immediately extracted. Deuterated methanol- d_4 (CD_3OD) containing tetramethylsilane as internal standard was purchased from Merck Co. (Darmstadt, Germany). The solvents used for the analyses were obtained from Fisher Scientific Korea (Seoul, Korea). Methanol (MeOH), acetonitrile (MeCN), and ethyl acetate (EtOAc) were obtained from Duksan (Ansan, Korea). All other chemicals were obtained from commercial sources.

Extraction and solvent fractionation

The procedures for the MeOH extraction and solvent fractionation of the fresh peels were described in our previous studies [13]. Briefly, the fresh peels (15 kg fresh wt.) were homogenized and extracted with MeOH. The MeOH extracts (3709 g) obtained after concentration in vacuo were suspended in a 0.2 M glycine-0.2 M HCl buffer (pH 3.0, 6 L) and partitioned with EtOAc (6 L, 3 times). The EtOAc layer was partitioned with a sodium phosphate buffer (pH 8.0, 6 L, 3 times). After adjusting the pH to 3.0 using 1.0 N HCl, the aqueous layer was partitioned with EtOAc (6 L, 3 times) to yield an EtOAc soluble acidic layer.

Isolation

The EtOAc soluble acidic layer (5.17 g) was loaded onto a Sephadex LH-20 column (3.3 cm $\times$ 82 cm) and eluted with 80% MeOH (2.6 L). All fractions were spotted on a silica gel thin-layer chromatographic plate (TLC; silica gel 60 F₂₅₄, 0.25 mm thickness; Merck Co.) and developed using *n*-BuOH/acetic acid/H₂O (4:1:1, v/v/v) as a mobile phase. Based on UV visualization and spraying of the TLC plate with a 1% cerium IV sulfate solution, eighteen fractions (A–R) were collected. Fractions D, K, M, and N were purified by octadecylsilane (ODS) high performance liquid chromatography (HPLC) using a Shim-pack Prep-ODS (H) Kit (5 μ m, 20 cm $\times$ 250 mm; Shimadzu, Kyoto, Japan; flow rate: 9.9 mL/min; wavelength: 280 nm). Fraction D (122.2 mg, Ve/Vt 1.28–1.39) was subjected to ODS-HPLC (gradient system, 25% MeOH, pH adjusted to 2.65 by TFA $\rightarrow$ 70% MeOH for 50 min) and ten peaks were observed in its HPLC chromatogram. Subfraction D2 (t_R 32.0–33.5 min, 15 mg) was suspended in a 1.0 N HCl aqueous solution (pH 3.0) and partitioned with EtOAc. The aqueous layer (3.2 mg) was subjected to ODS-HPLC under the same HPLC conditions used for fraction D to obtain **1**

(t_R 32.8 min, 0.18 mg). Compound **2** (t_R 35.7 min, 0.65 mg) was isolated from fraction K (78 mg, Ve/Vt 1.86–2.11) by purification using ODS-HPLC (gradient system: 100% H₂O, pH adjusted to 2.65 by TFA $\rightarrow$ 60% MeOH for 40 min). Fraction M (Ve/Vt 1.68–1.84, 44.5 mg) was injected in the ODS-HPLC (gradient system: 100% H₂O adjusted to pH 2.65 by TFA $\rightarrow$ 60% MeOH for 40 min) to yield a mixture of **5** and another compound (3:4 ratio, t_R 25.8 min, 6.26 mg). In addition, fraction N (31.3 mg, Ve/Vt 2.86–3.07) was injected in the ODS-HPLC (gradient system: 100% H₂O, pH adjusted to 2.65 by TFA $\rightarrow$ 60% MeOH for 40 min) to obtain a mixture of **3** and **4** (t_R 25.3 min, 4.23 mg). The isolation and purification procedures for **1**–**5** are shown in Fig. 1.

Structural analysis

The nuclear magnetic resonance (NMR) spectra of **1**–**5** were recorded using a ^{unity}INOVA 500 spectrometer (Varian, Walnut Creek, CA, USA). All mass spectra were recorded on a hybrid ion-trap time-of-flight mass spectrometer (LCMS-IT-TOF, Shimadzu) that was equipped with an electrospray ionization (ESI) source (ESI-MS).

Compound 1

¹H-NMR (500 MHz, CD_3OD) δ 4.71 (1H, d, J = 3.0 Hz, H-2), 4.24 (1H, m, H-3), 4.93 (1H, d, J = 4.5 Hz, H-4), 5.95 (1H, d, J = 2.0 Hz, H-6), 6.03 (1H, d, J = 2.0 Hz, H-8), 7.05 (1H, d, J = 2.0 Hz, H-10), 7.12 (1H, d, J = 8.5 Hz, H-13), 6.91 (1H, dd, J = 8.5, 2.0 Hz, H-14), 6.68 (2H, s, H-2', 6'), 4.76 (1H, d, J = 3.0 Hz, H-2''), 4.27 (1H, m, H-3''), 2.88–2.69 (2H, m, H-4''), 6.15 (1H, s, H-6''), 6.95 (1H, d, J = 2.0 Hz, H-10''), 6.77 (1H, d, J = 8.5 Hz, H-13''), 6.81 (1H, dd, J = 8.5, 2.0 Hz, H-14''); ESIMS (negative) m/z 729.1 [M–H][–].

Compound 2

¹H-NMR (500 MHz, CD_3OD) δ 4.92 (1H, br. s, H-2), 4.01 (1H, br. s, H-3), 4.56 (1H, br. s, H-4), 5.99 (1H, d, J = 2.0 Hz, H-6), 6.05 (1H, d, J = 2.0 Hz, H-8), 6.89 (1H, d, J = 2.0 Hz, H-10), 6.72 (1H, d, J = 8.5 Hz, H-13), 6.67 (1H, dd, J = 8.5, 2.0 Hz, H-14), 4.81 (1H, br. s, H-2'), 4.15 (1H, br. s, H-3'), 2.68 (1H, br. d, J = 9.0 Hz, H-4' α), 2.85 (1H, br. d, J = 9.0 Hz, H-4' β), 6.06 (1H, s, H-6'), 6.98 (1H, d, J = 2.0 Hz, H-10'), 6.75 (1H, d, J = 8.5 Hz, H-13'), 6.80 (1H, dd, J = 8.5, 2.0 Hz, H-14'); ESIMS (negative) m/z 577.1 [M–H][–].

Compound 3

¹H- and ¹³C-NMR data are shown in Table 1; ESIMS (negative) m/z 863.1 [M–H][–].

Fig. 1 Procedure used to isolate compounds **1–5** from the pear fruit peels

Compound 4

$^1\text{H-NMR}$ (500 MHz, CD_3OD) δ 4.96 (1H, br. s, H-2), 3.94 (1H, br. s, H-3), 4.67 (1H, br. s, H-4), 6.11 (1H, d, $J = 2.0$ Hz, H-6), 5.89 (1H, d, $J = 2.0$ Hz, H-8), 4.12 (1H, d, $J = 3.5$ Hz, H-3'), 4.44 (1H, d, $J = 3.5$ Hz, H-4'), 6.07 (1H, s, H-6'), 5.25 (1H, br. s, H-2''), 4.26 (1H, br. s, H-3''), 2.75–2.96 (2H, m, H-4''), 6.01 (1H, s, H-6''), 6.73–7.15 (9H, H-10, H-13, H-14, H-10', H-13', H-10'', H-13'', H-14''); ESIMS (negative) m/z 863.1 $[\text{M-H}]^-$.

Compound 5

$^1\text{H-NMR}$ (500 MHz, CD_3OD) δ 4.99 (1H, br. s, H-2), 3.99 (1H, br. s, H-3), 4.49 (1H, br. s, H-4), 6.01 (1H, d,

$J = 2.0$ Hz, H-6), 6.03 (1H, d, $J = 2.0$ Hz, H-8), 4.01 (1H, d, $J = 3.5$ Hz, H-3'), 3.77 (1H, d, $J = 3.5$ Hz, H-4'), 6.15 (1H, s, H-8'), 5.33 (1H, br. s, H-2''), 4.34 (1H, br. s, H-3''), 2.78–3.01 (2H, m, H-4''), 5.97 (1H, s, H-6''), 6.44–7.15 (9H, H-10, H-13, H-14, H-10', H-13', H-10'', H-13'', H-14''); ESIMS (negative) m/z 863.1 $[\text{M-H}]^-$.

Results and discussion

The MeOH extract (3708.67 g) of the pear fruit peels (15 kg fresh wt.) was solvent fractionated to afford various EtOAc-soluble layers. The collected EtOAc soluble acidic layers (5.17 g) were loaded onto a Sephadex LH-20 column (80% MeOH). The resulting 18 fractions (A–R) were

Table 1 ^1H -(500 MHz) and ^{13}C -(125 MHz) NMR data of **3** in CD_3OD

Position	δ_{H} (int., multi., J in Hz)	δ_{C}	Position	δ_{H} (int., multi., J in Hz)	δ_{C}	Position	δ_{H} (int., multi., J in Hz)	δ_{C}
2	—	98.6	2'	5.70 (1H, br. s)	77.5	2''	4.38 (1H, br. s)	78.9
3	3.27 (1H, d, 3.5)	65.8	3'	4.11 (1H, br. s)	71.6	3''	3.85 (1H, br. s)	66.2
4	4.14 (1H, d, 3.5)	27.4	4'	4.55 (1H, br. s)	36.9	4''	2.83 (2H, m)	28.5
4a	—	103.6	4a'	—	105.4	4a''	—	105.3
5	—	152.7	5'	—	150.4	5''	—	156.4
6	5.99 (1H, d, 2.0)	94.9	6'	5.90 (1H, s)	96.9	6''	5.79 (1H, s)	94.3
7	—	154.4	7'	—	154.7	7''	—	154.4
8	6.11 (1H, d, 2.0)	94.7	8'	—	105.1	8''	—	107.3
8a	—	149.7	8a'	—	155.4	8a''	—	154.2
9	—	131.8	9'	—	130.4	9''	—	131.1
10	7.02 (1H, d, 2.0)	114.3	10'	7.31 (1H, d, 2.0)	115.4	10''	6.82 (1H, d, 2.0)	114.6
11	—	145.3	11'	—	144.9	11''	—	144.5
12	—	144.4	12'	—	144.1	12''	—	144.0
13	6.83 (1H, d, 8.5)	114.7	13'	6.83 (1H, d, 8.5)	114.4	13''	6.81 (1H, d, 8.5)	114.1
14	6.82 (1H, dd, 8.5, 2.0)	118.1	14'	7.19 (1H, dd, 8.5, 2.0)	120.0	14''	6.72 (1H, dd, 8.5, 2.0)	118.5

separated by silica gel TLC analysis. We previously reported the isolation and identification of various compounds, including the new hydroxycinnamoylmalic acid and its methyl esters obtained from fractions E, H, I, K, and L of the EtOAc soluble acidic layer [13–16, 18, 19]. In the present study, we additionally identified five proanthocyanidins [1–5] from fractions D, K, M and N by ODS-HPLC purification (Fig. 1).

The ESI–MS (negative) spectrum of **1** exhibited a pseudomolecular ion peak at m/z 729.1 $[M-H]^-$, corresponding to a molecular weight of 730. The 1H -NMR spectrum of **1** (purity >95%) showed the presence of two trisubstituted benzene ring proton signals at δ 7.12–6.77, two meta-coupled benzene ring proton signals at δ 5.95 (1H, d, $J = 2.0$ Hz, H-6) and 6.03 (1H, d, $J = 2.0$ Hz, H-8), one benzene ring proton signal at δ 5.49 (1H, s, H-6''), and the galloyl proton signal at δ 6.68 (2H, s, H-2' and H-6'). In addition, four sp^3 methine proton signals at δ 4.93–4.24 and one sp^3 methylene carbon proton signal at δ 2.88–2.69 (2H, m, H-4'') were observed. The structure of **1** was suggested to be two catechins having a gallic acid moiety. The presence of one benzene ring proton signal at δ 6.15 (1H, s, H-6'') and one C-ring sp^3 methine proton signal at δ 4.93 (H-4) indicated procyanidin B2 having a 4 $\rightarrow$ 6 or 4 $\rightarrow$ 8 interflavonoid linkage [20]. A small coupling constant value ($J = 3.0$ Hz) of the C ring proton signals at δ 4.71 (H-2) and 4.76 (H-2'') was indicative of two epicatechin units. The MS and 1H -NMR results suggest that **1** is procyanidin B2-3-*O*-gallate and procyanidin B2-3'-*O*-gallate having a 4 $\rightarrow$ 6 or 4 $\rightarrow$ 8 interflavonoid linkage. Compound **1** was identified as (–)-epicatechin gallate-(4 β $\rightarrow$ 8)-(-)-epicatechin (procyanidin B2-3-*O*-gallate) by comparison of the MS and 1H -NMR data with those of the compound previously reported in the literature (Fig. 2) [21, 22].

The ESI–MS (negative) spectrum of **2** showed a pseudomolecular ion peak at m/z 577.1 $[M-H]^-$, which was indicative of a molecular weight of 578. The 1H -NMR spectrum of **2** (purity >95%) was closely related to that of **1**, except for the galloyl group. The broad singlets of the C ring proton signals at δ 4.92 (1H, br. s, H-2), 4.01 (1H, br. s, H-3), 4.81 (1H, br. s, H-2'), and 4.15 (1H, br. s, H-3') indicated two (–)-epicatechin units. The presence of one benzene ring proton signal at δ 6.06 (H-6') and one C-ring sp^3 methine proton signal at δ 4.56 (H-4) assigned procyanidin B2 having a 4 β $\rightarrow$ 6 or 4 β $\rightarrow$ 8 interflavonoid bond [20]. Compound **2** was identified as (–)-epicatechin-(4 β $\rightarrow$ 8)-(-)-epicatechin (procyanidin B2) by comparison of the MS and 1H -NMR data with those of the compound previously reported in the literature (Fig. 2) [20, 23].

The ESI–MS (negative) spectrum of the mixture containing **3** and **4** exhibited a pseudomolecular ion peak at m/z 863.1 $[M-H]^-$, corresponding to a the molecular weight of 864. The 1H -

and ^{13}C -NMR spectra indicated the presence of two structurally related compounds. These compounds were trimeric A-type procyanidins having one C–O–C interflavonoid linkage, although the linkage between the catechin units in their structures differed. The 1H -NMR spectrum showed unequally intensities of the C-ring proton signals with a ratio of **3**/**4** = 5:1. The 1H -NMR spectrum of **3** showed three trisubstituted benzene ring proton signals at δ 7.16–6.48, two doublet proton signals relative to the meta-coupled benzene ring at δ 6.11 (1H, d, $J = 2.0$ Hz, H-6) and 5.99 (1H, d, $J = 2.0$ Hz, H-8), and two singlet proton signals of the benzene ring at δ 5.90 (1H, s, H-6') and 5.79 (1H, s, H-6''). In addition, seven sp^3 methine proton signals at δ 5.70–3.27 and one sp^3 methylene proton signal at δ 2.83 (2H, m, H-4'') were observed (Table 1). The presence of two benzene ring singlet proton signals at δ 5.90 (H-6') and 5.79 (H-6'') and two C-ring sp^3 methine proton signals at δ 4.11 (C-4) and 4.55 (H-4') established **1** to be a procyanidin trimer. The 1H -NMR spectrum of **3** showed broad singlets for the C ring proton signals at δ 5.70 (1H, br. s, H-2'), 4.11 (1H, br. s, H-3'), 4.38 (1H, br. s, H-2''), and 3.85 (1H, br. s, H-3''), which were indicative of the epicatechin units. Additionally, two C-ring sp^3 methine proton signals of the AB system at δ 3.27 (H-3) and 4.14 (H-4) were observed, suggesting that the partial structure of **3** was epicatechin-(4 $\rightarrow$ 8, 2 $\rightarrow$ O $\rightarrow$ 7)-epicatechin. The trimeric A-type procyanidin structure was also confirmed by the presence of 45 carbon signals including one acetal carbon at δ 98.6 (C-2) in the ^{13}C -NMR spectrum (Table 1). Furthermore, the complete structure of **3** was determined by 1H - 1H COSY, HSQC, and HMBC experiments. The correlations of H-3 and H-4 to C-8' and C-2 in the HMBC spectrum confirmed the structure of epicatechin-(4 $\rightarrow$ 8, 2 $\rightarrow$ O $\rightarrow$ 7)-epicatechin (Fig. 3). The observation of the 1H - 1H COSY correlations of H-2'/H-3'/H-4' and the HMBC correlations of H-3' to C-8'' and H-4' to C-7'', C-8'', and C-8a'' indicated a linkage between the C-4' and C-8'' carbon atoms. The MS and NMR data suggest that **1** is (–)-epicatechin-(4 $\rightarrow$ 8', 2 $\rightarrow$ O-7')-(-)-epicatechin-(4' $\rightarrow$ 8'')-(-)-epicatechin. Therefore, compound **3** was identified as (–)-epicatechin-(4 β $\rightarrow$ 8, 2 β $\rightarrow$ O-7)-(-)-epicatechin-(4 β $\rightarrow$ 8)-(-)-epicatechin by comparison of the MS and 1H - and ^{13}C -NMR data with reported in the literature for the same compound previously isolated from *Pavetta owariensis* and *Ixora coccinea* (Fig. 2) [24, 25].

The 1H -NMR spectrum of **4** (purity 20%) was closely related to that of **3**, suggesting a triflavanol moiety (trimeric A-type proanthocyanidin). When compared to the 1H -NMR spectrum of **3**, the chemical shifts of the A ring proton signals of the three epicatechin moieties at δ 5.25–3.85 were different. In particular, the H-2 at higher field (δ 4.96) and the H-2'' at lower field (δ 5.25) were observed in the 1H -NMR spectrum of **4**. In addition, the proton signals of the AB system at δ 4.12 (H-3') and 4.44 (H-4') were observed in the 1H -NMR spectrum of **4**,

Fig. 2 Chemical structures of the isolated compounds **1–5**

confirming the presence of epicatechin-(4 $\rightarrow$ 8, 2 $\rightarrow$ *O*-7)-epicatechin. Compound **4** was suggested to be an A-type trimeric proanthocyanidin where the C-4 of epicatechin is connected with the C-6' or C-8' of epicatechin-(4 β $\rightarrow$ 8, 2 β $\rightarrow$ *O*-7)-epicatechin. The MS and $^1\text{H-NMR}$ spectra of **4** were consistent with those of (–)-epicatechin-(4 β $\rightarrow$ 8)-(–)-epicatechin-(4 β $\rightarrow$ 8, 2 β $\rightarrow$ *O*-7)-(–)-epicatechin (aesculitannin A), which was previously reported from *Aesculus hippocastanum* [26]. Therefore, compound **4** was identified as (–)-epicatechin-(4 β $\rightarrow$ 8)-(–)-epicatechin-(4 β $\rightarrow$ 8, 2 β $\rightarrow$ *O*-7)-(–)-epicatechin (Fig. 2).

The ESI-MS (negative) spectrum of **5** revealed a pseudomolecular ion peak at m/z 863.1 $[\text{M-H}]^-$, corresponding to a molecular weight of 864, which suggested a trimeric A-type procyanidin possessing one C–O–C interflavonoid linkage in its structure. The MS result was supported by the $^1\text{H-NMR}$ spectrum of **5** (purity 75%), which was very closely related to those of **3** and **4**. In particular, compounds **4** and **5** were suggested to be isomeric compounds. The MS and $^1\text{H-NMR}$ results suggest that **5** is an A-type trimeric procyanidin having a 4 β $\rightarrow$ 6 or 4 β $\rightarrow$ 6' interflavonoid bond in epicatechin-(4 β $\rightarrow$ 8, 2 β $\rightarrow$ *O*-7)-

epicatechin. The MS and $^1\text{H-NMR}$ spectra of **5** were consistent with those of (–)-epicatechin-(4 β $\rightarrow$ 6)-(–)-epicatechin-(4 β $\rightarrow$ 8, 2 β $\rightarrow$ *O*-7)-(–)-epicatechin previously isolated from cranberry [27]. Therefore, compound **5** was identified as (–)-epicatechin-(4 β $\rightarrow$ 6)-(–)-epicatechin-(4 β $\rightarrow$ 8, 2 β $\rightarrow$ *O*-7)-(–)-epicatechin (Fig. 2).

Proanthocyanidins have been found in numerous food sources, such as fruits, nuts, and beans [28]. The two B-type proanthocyanidin dimers (**1** and **2**) isolated in this study have been already found in pear fruits [21, 29]. However, to the best of our knowledge, the three A-type proanthocyanidin trimers **3–5** were identified for the first time in pear fruits. It has been reported that proanthocyanidins exhibit various biological effects, including antioxidative, antimicrobial, antiallergic, anticancer, and antiobesity activities [28, 30–32]. Furthermore, the A-type proanthocyanidin trimers **3–5** exert antioxidative, antibacterial, and anticancer properties [25–27, 33]. Therefore, three A-type proanthocyanidin trimers identified in this study might partially contribute to health beneficial effects of the pear fruits. As mentioned above, we elucidated various chemical constituents, including steroid, phenolic

Fig. 3 Important correlations of **3** in ^1H – ^1H COSY (bold lines) and HMBC (arrows) experiments

derivatives, and hydroxycinnamoylmalic acids, flavonoids, and caffeoyl triterpenes in pear fruits [13–19]. Therefore, on molecular level these investigations may additionally provide very useful information for establishing the chemical profiles of pear fruits.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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