antioxidants

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Supplementary Files

Effects of Phenolic-rich *Pinus densiflora* Extract on Learning, Memory, and Hippocampal Long-term Potentiation in Scopolamine-induced Amnesic Rats

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1. Materials and methods

1.1. Determination of total phenolic and flavonoid content

The total phenolic content of the *Pinus densiflora* Sieb. et Zucc. bark extract (PBE) was measured using the colorimetric method with Folin-Ciocalteu's phenol reagent [1]. Two hundred microliters of PBE (100 mg/L) Two hundred microliters of PBE (100 mg/L) was mixed with 2.6 mL of deionized water. Then, 200 μL of Folin-Ciocalteu's phenol reagent was added to the mixture. At 6 min, 2 mL of 7% (*w*/*v*) Na_2CO_3 solution was added; at 90 min, the absorbance was measured at 750 nm using a spectrophotometer (SPECTRONIC 200; Thermo Fisher Scientific Inc., Waltham, MA, USA). The content of total phenolics was expressed as mg gallic acid equivalents (GAE)/g extract.

The total flavonoid content of the PBE was measured using the method of [2]. Five hundred microliters of PBE (100 mg/L) was mixed with 3.2 mL of deionized water. Then, 150 μL of 5% (*w*/*v*) $NaNO_2$ solution was added. After 5 min, 150 μL of 10% (*w*/*v*) $AlCl_3$ was added. At 6 min, 1 mL of 1 M NaOH was added, and absorbance was measured immediately using a spectrophotometer (SPECTRONIC 200; Thermo Fisher Scientific Inc.). The content of total flavonoids was expressed as mg catechin equivalents (CE)/g extract.

1.2. Determination of antioxidant capacity

The antioxidant capacity of PBE was determined using the ABTS and DPPH radicals [3] and expressed in mg vitamin C equivalents (VCE)/g extract. The ABTS radical solution was adjusted to an absorbance of 0.650 ± 0.020 at 734 nm using a spectrophotometer (SPECTRONIC 200; Thermo Fisher Scientific Inc.). The reaction between ABTS radicals and the PBE (100 mg/L) was allowed to proceed at 37°C for 10 min, and then the decrease in absorbance of the resulting solution was measured at 734 nm using a spectrophotometer (SPECTRONIC 200; Thermo Fisher Scientific Inc.). For the assay using DPPH radicals, the absorbance of DPPH radicals in 80% (*v*/*v*) aqueous methanol was set to 0.650 ± 0.020 at 517 nm using a spectrophotometer (SPECTRONIC 200; Thermo Fisher Scientific Inc.). DPPH radicals and PBE (100 mg/L) were reacted together at 23°C for 30 min. The absorbance of the resulting solution was monitored at 517 nm using a spectrophotometer (SPECTRONIC 200; Thermo Fisher Scientific Inc.).

2. Results and discussion

2.1. Total phenolic and flavonoid content

The total phenolic content of PBE was approximately 433.4 mg GAE/g (Table S1), which is similar to those of other bark extracts of *Pinus* species; 360.8 mg GAE/g for *P. pinaster* (maritime pine) [4], 550 mg GAE/g for *P. radiata* [5], and 299.3 mg GAE/g for *P. cembra* [6].

The total flavonoid content of PBE was approximately 299.4 mg CE/g (Table S1), which is similar to those of other bark extracts of the *Pinus* species; 379 mg CE/g for *P. durangensis* [7], 403 mg CE/g for *P. pinaster* [8], and 125.3 mg CE/g for *P. cembra* [6].

2.2. Antioxidant capacity

The antioxidant activities of PBE were approximately 540.5 and 402.6 mg VCE/g in the ABTS and DPPH assays, respectively (Table S1). A previous study showed that the bark of *P. densiflora* had a higher radical scavenging activity than the bark of *P. thunbergii* and *P. pinaster* (Pycnogenol®) in the ABTS assay [9]. Also, the results in this study were similar to those of another extract of *P. densiflora* bark extract (PineXol®) showing 697.3 mg VCE/g in the ABTS assay and 521.8 mg VCE/g in the DPPH assay [10]. The antioxidant capacity of PBE was due to phenolics in PBE such as procyanidins and their building blocks [(+)-catechin and (-)-epicatechin], and taxifolin and its derivatives, which were reported to have strong antioxidant capacities [11-14].

Table S1

Total phenolic and flavonoid content, and antioxidant capacity of *Pinus densiflora* Sieb. et Zucc. bark extract (PBE)

	Total phenolic content (mg gallic acid equiv./g)	**Total flavonoid content (mg catechin equiv./g)**	**Antioxidant capacity (mg vitamin C equiv./g)**	
			ABTS [a]	**DPPH** [b]
PBE	433.4 ± 9.0 [c]	299.4 ± 11.3	540.5 ± 8.2	402.6 ± 22.9

[a] 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay

[b] 2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay

[c] Data are experssed as means ± standard deviations (n = 3)

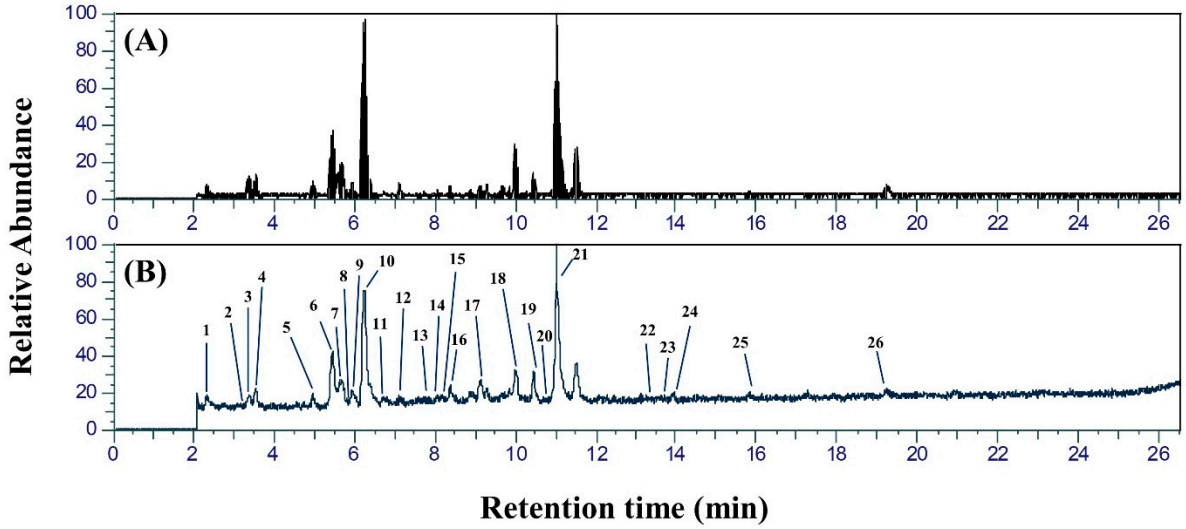

Figure S1. (A) Total ion chromatogram and (B) base peak chromatogram (filtered with MS^1) of *Pinus densiflora* Sieb. et Zucc. bark extract (PBE). Peaks: 1, procyanidin trimer; 2, hydroxymandelic acid; 3, syringaldehyde; 4, protocatechuic acid; 5, unknown; 6, 3-*p*-coumaroylquinic acid; 7, procyanidin B1; 8, procyanidin B3; 9, procyanidin trimer; 10, (+)-catechin; 11, procyanidin trimer; 12, caffeic acid; 13, 5-*p*-coumaroylquinic acid; 14, (−)-epicatechin; 15, procyanidin trimer; 16, vanillyl ethyl ester hexoside; 17, procyanidin B2; 18, taxifolin 3-*O*-glucoside; 19, unknown; 20, luteolin; 21, taxifolin; 22, taxifolin isomer; 23, quercetin 3-*O*-rhamnoside; 24, dehydroxyltaxifolin; 25, quercetin 7-*O*-glucoside; 26, quercetin.

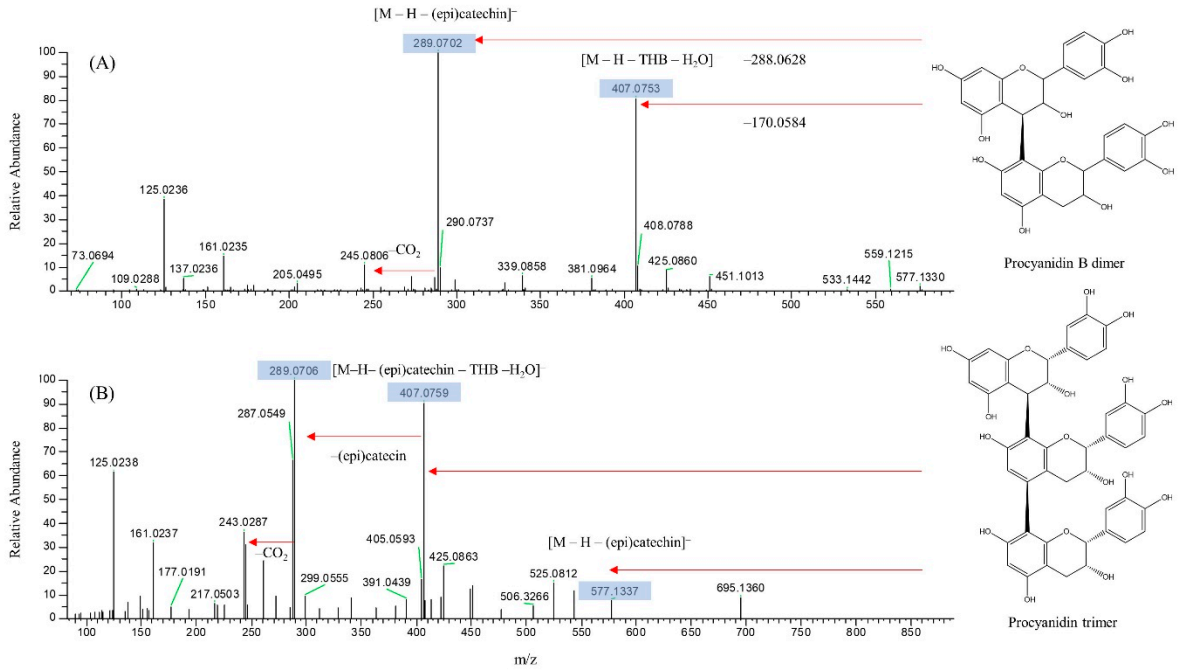

Figure S2. Typical fragmentation patterns for procyanidins (A) dimer and (B) trimer. The highlight *m*/*z* is characteristic MS² fragment ions.

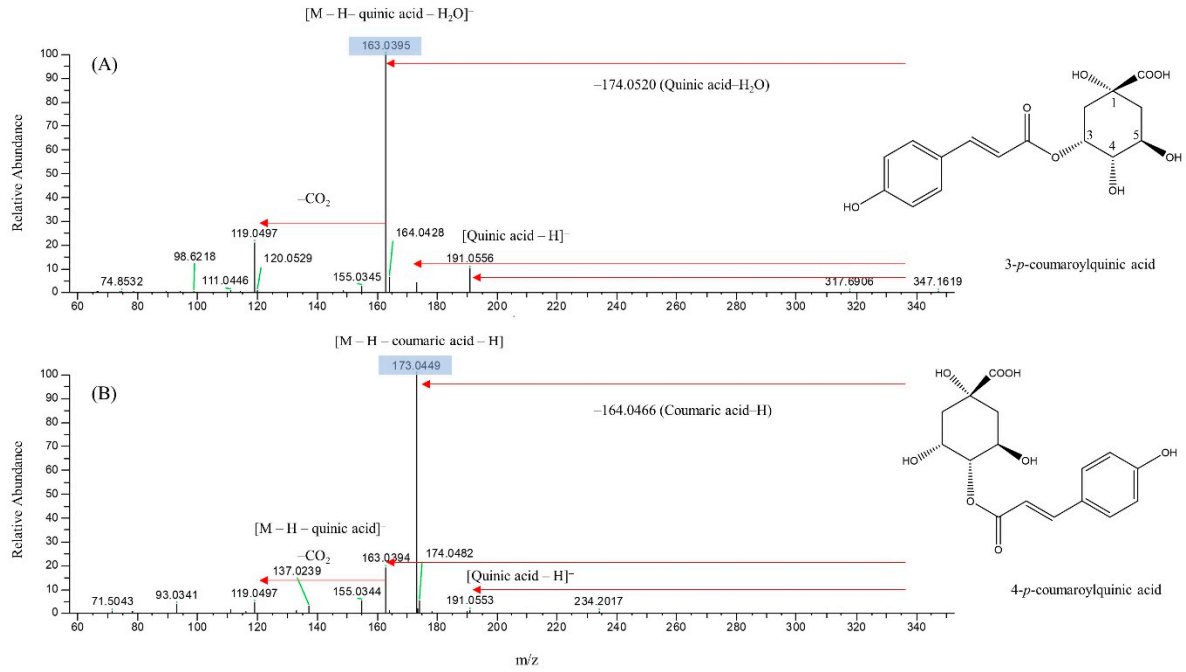

Figure S3. Typical fragmentation patterns for (A) 3-*p*-coumaroylquinic acid and (B) 5-*p*-coumaroylquinic acid. The highlight *m*/*z* is characteristic MS² fragment ions.

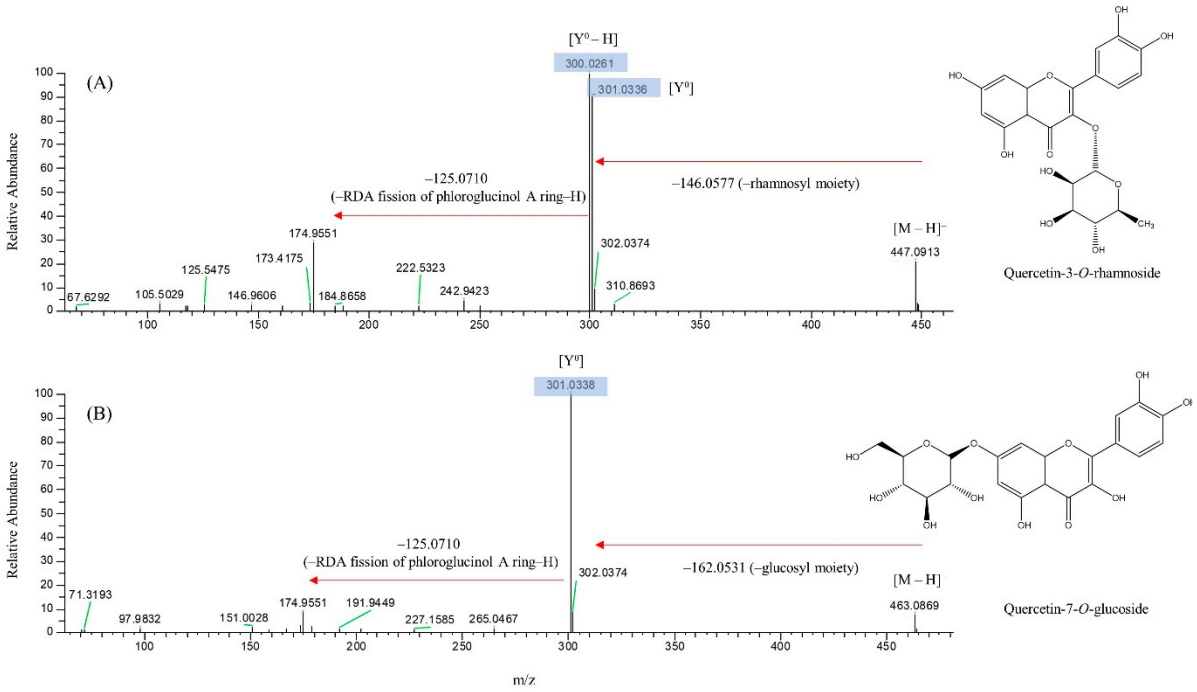

Figure S4. Typical fragmentation patterns for (A) quercetin 3-*O*-rhamnoside and (B) quercetin 7-*O*-glucoside. The highlight *m*/*z* is characteristic MS^2 fragment ions.

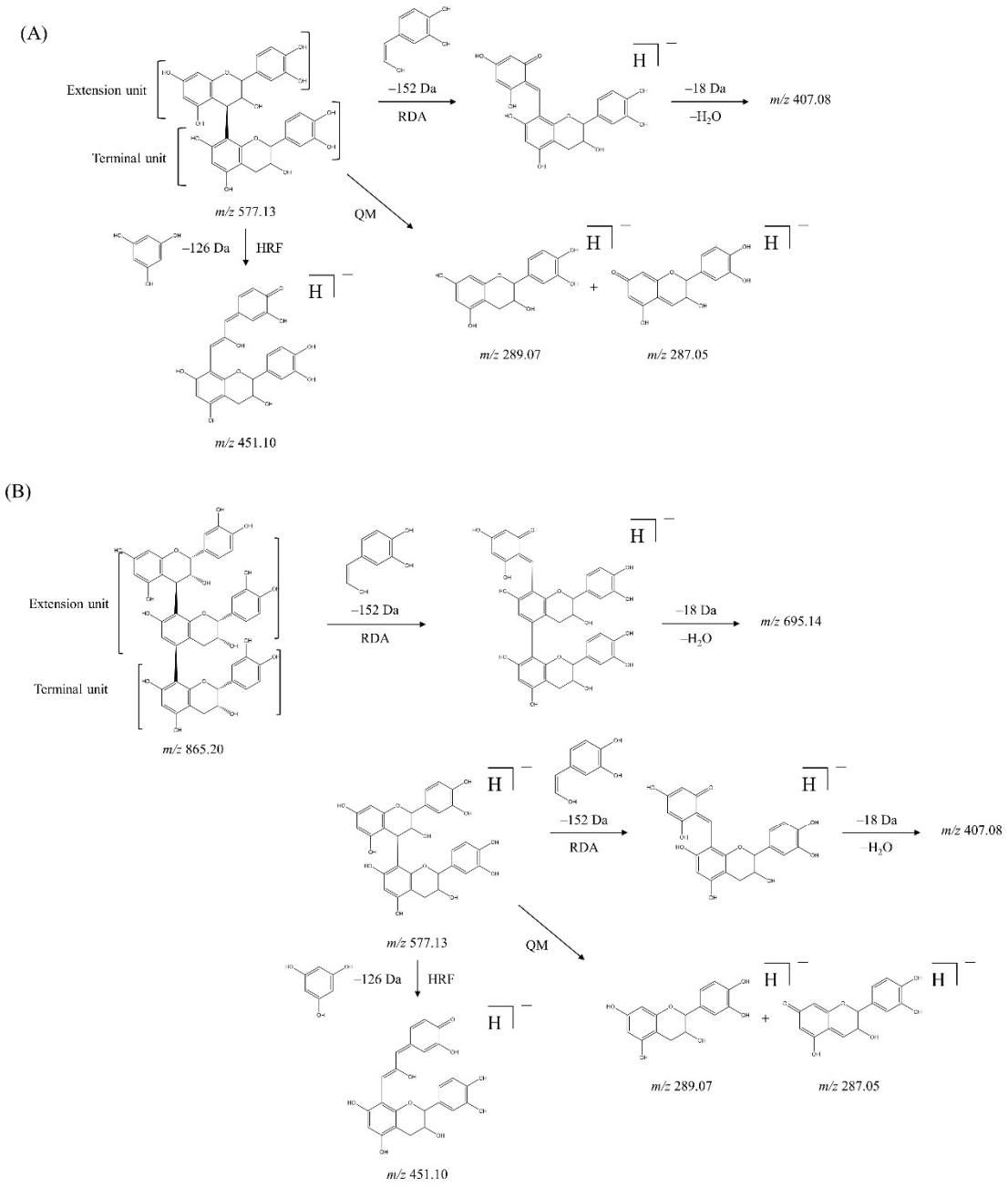

Figure S5. Typical fragmentation patterns for (A) procyanidin B type dimer and (B) procyanidin trimer via retro-Diels-Alder (RDA), heterocyclic ring fission (HRF), and quinone methide (QM) reactions.

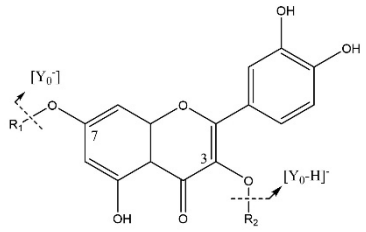

Figure S6. MS^2 characteristics of O-glycosylated hydroxyflavonol.

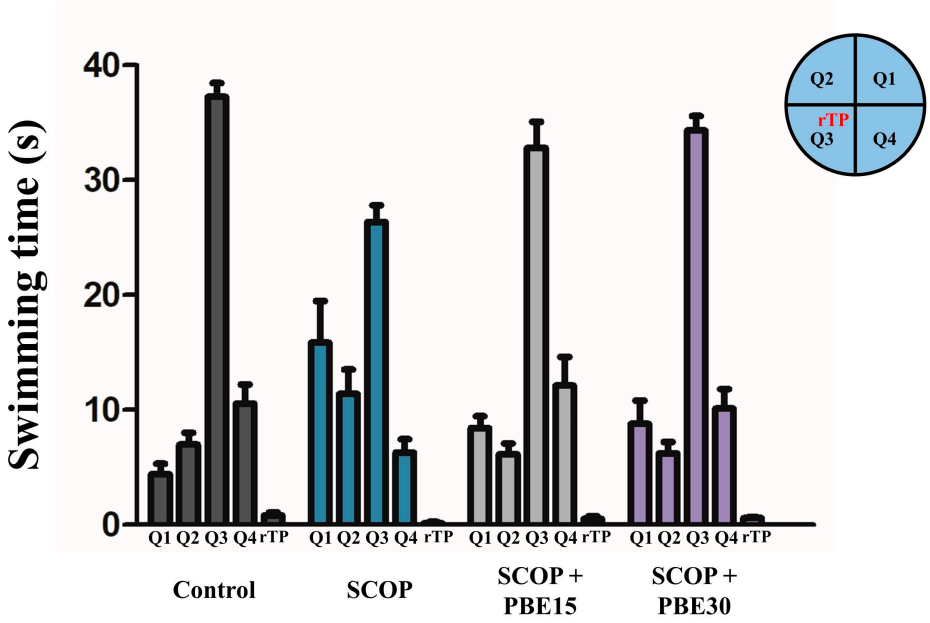

Figure S7. Swimming time in each quadrant of trial acquisition in the Morris water maze test. The removed target platform (rTP) is located in target quadrant 3. Quadrants: Q1, quadrant 1; Q2, quadrant 2; Q3, quadrant; Q4 quadrant 4.