CHEMICAL CONSTITUENTS FROM THE FRUIT OF *CITRUS UNSHIU* AND THEIR INHIBITORY EFFECTS ON ACETYLCHOLINESTERASE

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Acetylcholinesterase (AChE) inhibition has been considered a promising temporary treatment for Alzheimer's disease. The present study was conducted to isolate chemical constituents from the fruit of *Citrus unshiu* Markovich (Rutaceae) and evaluate their inhibitory effects on AChE. Phytochemical investigation of *C. unshiu* fruit led to the isolation of methyl nomilinate (**1**), limocitrunshin (**2**), nobiletin (**3**), kaempferol 3-*O*-rutinoside (**4**), limocitrin 3-glucoside (**5**), kaempferol 3-(2^G-rhamnosylrutinoside) (**6**), didymin (**7**), (2*S*)-narirutin 4'-*O*-glucoside (**8**) and naringenin 7-*O*-rutinoside (**9**). The structures were identified with ^{1}H and ^{13}C NMR spectroscopic data and ESI-MS, then comparison of their spectroscopic and physical data with those previously reported, as well as by measurement of their specific rotations. A limonoid, methyl nomilinate (**1**), inhibited AChE activity by more than 50% at a concentration of 30 µg/ml. The findings in this study provide the first experimental evidence that a limonoid compound **1** from *C. unshiu* fruit acts as a natural AChE inhibitor.

Keywords: *Citrus unshiu*; Rutaceae; limonoid; acetylcholinesterase

ХЕМИСКИ СОСТАВ НА СОСТОЈКИТЕ ОД ПЛОДОТ НА *CITRUS UNSHIU* И НИВНОТО ИНХИБИТОРНО ДЕЈСТВО ВРЗ АЦЕТИЛХОЛИНЕСТЕРАЗАТА

Инхибицијата на ацетилхолинестеразата (AChE) претставува потенцијален привремен третман за Алцхајмеровата болест. Цел на ова истражување беше да се изолираат хемиските состојки на *Citrus unshiu* Markovich (Rutaceae) и да се евалуира нивното инхибиторно дејство на AChE. При фитохемиското истражување на плодот на *C. unshiu* беа изолирани метил номилинат (**1**), лимоцинтрушин (**2**), нобилетин (**3**), кампферол 3-*O*-рутинозид (**4**), лимоцитрин 3-глукозид (**5**), кампферол 3-(2^G-рамнозилрутинозид) (**6**), дидимин (**7**), (2*S*)-нарирутин 4'-*O*-глукозид (**8**) and нарингенин 7-*O*-рутинозид (**9**). Структурите беа идентификувани преку податоци добиени од ^{1}H и ^{13}C NMR спектроскопија и ESI-MS, кои беа споредени со претходно објавени податоци, како и со мерење на аголот на нивните специфични ротации. Лимоноидот метил номилинат (**1**) ја инхибираше активноста на AChE за повеќе од 50% при концентрација од 30 µg/ml. Резултатите од овие испитувања се прв експериментален доказ дека лимоноидно соединение **1** од плодот на *C. unshiu* дејствува како природен инхибитор на AChE.

Клучни зборови: *Citrus unshiu*; Rutaceae; лимоноид; ацетилхолинестераза

1. INTRODUCTION

Alzheimer's disease (AD) is a chronic, neurodegenerative disorder characterized by progressive dementia [1, 2]. AD will become a serious problem in an aging society because it usually occurs in the elderly. Therefore, research into its causes and practical treatments is becoming necessary. Many factors are involved in AD including aberrant protein processing, genetic effects, oxida-

tive stress, cholinergic deficiency, inflammation and excitotoxicity. However, definitive AD therapies and diagnosis remain uncertain [3, 4]. One of the most promising approaches for AD treatment is acetylcholinesterase (AChE) inhibition, which can reduce acetylcholine levels in the brain [5]. There are only a few drugs that are approved by the Food and Drug Administration (FDA) for AChE inhibition, including tacrine, donepezil, rivastigmine and galantamine [6]. Thus, the search for new AChE inhibitors is highly necessary to broaden the selection of drugs available to treat AD. The modern pharmaceutical field has focused its attention on natural products that have been used for centuries throughout the world. Thus, research on discovering new AChE inhibitors has been focused on compounds derived from natural sources.

Citrus unshiu Markovich (Rutaceae), also known as the satsuma mandarin, is cultivated in countries in cool subtropical regions including Korea, and is consumed in a variety of forms such as fresh juice, citrus-based drinks, jam, salad dressing and tea [7, 8]. Due to its sweet and fresh flavor, it is one of the most popular fruits in Korea. In addition, for centuries it has been consumed not only as food but also as a medicine. The peel of *C. unshiu*, well-known as "Jin-pee", has been used for traditional herbal medicines against the common cold, exhaustion and cancer [7, 9]. Previous studies have demonstrated that the peel has antitumor properties in a tumor-bearing murine model [9] as well as antioxidant activity [10–12]. The peel contains compounds associated with antioxidant and free radical scavenging effects such as ascorbic acid, flavonoids and phenolic compounds. In particular, phenolic compounds from the peel including flavonoids and hydroxyl cinnamic acids have a number of biological and pharmacological effects including anti-inflammatory, antimicrobial, antithrombotic, antiallergenic, cardioprotective and vasodilatory effects, along with hypocholesterolemic, hypoglycemic and antitumor activity [11, 12].

In our ongoing search for bioactive constituents from Korean natural resources, we have investigated bioactive compounds from the fruit of *C. unshiu*. In our recent studies, we reported the isolation of a novel cyclic peptide, citrusin XI, and its anti-inflammatory effects in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells [13]. In addition, we discovered a new flavonoid glycoside, limocitrunshin, together with other flavonoids, and evaluated their inhibitory effects on renal cell damage and nitric oxide (NO) production [7]. NO is an important pro-inflammatory mediator that can accelerate the appearance of some neurodegenerative disorders such as AD [14]. Thus, the fruit of *C. unshiu* containing compounds with NO inhibitory properties may be a promising candidate for providing therapeutic agents for AD. In our continued interest in discovering bioactive compounds of *C. unshiu* fruit, we further investigated such for possible new natural AChE inhibitors from the fruit of *C. unshiu*. Nine compounds designated as **1–9** including a limonoid and eight flavonoids were isolated, and the inhibitory effects of the isolates on AChE activity were evaluated using a micro-well plate AChE inhibition assay.

2. EXPERIMENTAL

2.1. *General experimental procedures*

Optical rotations were measured on a Jasco P-1020 polarimeter (Jasco, Easton, MD, USA). Infrared (IR) spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer (Bruker, Karlsruhe, Germany). Ultraviolet (UV) spectra were acquired on an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). High-resolution (HR)-electrospray ionization (ESI) mass spectra were recorded on a Waters UPLC-QTOF Xevo G2-S mass spectrometer (Waters Corporation, Milford, CT, USA). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE III 700 NMR spectrometer operating at 700 MHz (^{1}H) and 175 MHz (^{13}C), with chemical shifts given in ppm (δ) (Bruker). Preparative high-performance liquid chromatography (HPLC) purification was carried out on an Agilent 1100 or 1200 Series HPLC system (Agilent Technologies) equipped with a photo diode array detector. LC-MS analysis was performed on an Agilent 1200 Series HPLC system equipped with a diode array detector and a 6130 Series ESI mass spectrometer using an analytical Kinetex® 5 μm C18 100 Å column (5 μm, 2.1 × 100 mm, Phenomenex, Torrance, CA, USA). Column chromatography was performed with a silica gel 60 (Merck, Darmstadt, Germany; 230–400 mesh) and an RP-C$_{18}$ silica gel (Merck, 230–400 mesh). The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Merck precoated silica gel F$_{254}$ plates and reverse-phase (RP)-18 F$_{254s}$ plates (Merck) were used for thin-layer chromatography (TLC). Spots were detected on TLC under UV light or by heating after spraying with anisaldehyde–sulfuric acid.

2.2. *Plant material*

Fully ripe fruit of the "Miyagawa-wase" variety of satsuma mandarin (*C. unshiu* Marc.) were obtained from the National Institutes of Horticultural and Herbal Science, Jeju, Korea, in August 2012. Plant material was identified by one of the authors (K. H. Kim). A voucher specimen (SKKU-CU 2012-8) has been stored in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

2.3. *Extraction and isolation*

The unpeeled fruit of *C. unshiu* (1.0 kg) was extracted twice with 100% EtOH (2 × 2 days) at room temperature. The resultant extract was then filtered and concentrated under vacuum pressure to yield crude extracts. EtOH extracts (89.0 g) were suspended in distilled water (10 liters) and successively partitioned with hexane, EtOAc and *n*-BuOH, yielding residues weighing 230 mg, 3.0 g and 15.0 g, respectively. The EtOAc-soluble fraction (3.0 g) was subjected to fractionation with silica gel column chromatography using a gradient solvent system of *n*-hexane-EtOAc (1:1), $CHCl_3$–MeOH (50:1, 10:1, 5:1, 2:1 and 1:1) and 100% MeOH to give thirty fractions (E1–E30) according to TLC analysis. Fraction E7 (260 mg) was applied to column chromatography on an RP-C_{18} silica gel column using a solvent system of MeOH–H_2O (2:1) to yield 10 sub-fractions (E7a–E7j). Fraction E7b (52 mg) was further purified by semi-preparative RP HPLC (250 mm × 10 mm i.d., 10 μm, Phenomenex Luna C18(2) column, flow rate 1.5 ml/min) using a solvent system of MeOH–H_2O (2:1) to afford compounds **1** (8.0 mg), **3** (12.0 mg) and **5** (6.4 mg). The *n*-BuOH soluble fraction (15.0 g) was subjected to fractionation with HP-20 column chromatography using a gradient solvent system that increased MeOH in H_2O from 0% to 100% to yield six fractions (B1–B6) according to TLC analysis. Fraction B2 (4.5 g) was further subjected to silica gel column chromatography using a solvent system of $CHCl_3$–MeOH–H_2O (7:3:1) to obtain 32 sub-fractions (B2(1)–B2(32)). Fraction B2(11) (122 mg) was purified by preparative RP HPLC (250 mm × 20 mm i.d., 10 μm, YMC-Pack ODS-AM C18(2) column, flow rate 8.0 ml/min) using a 40% MeOH solvent system to obtain compound **9** (38.0 mg). Fraction B2(31) (277 mg) was further separated by preparative RP HPLC (250 mm × 20 mm i.d., 10 μm, YMC-Pack ODS-AM C18(2) column, flow rate 10.0 ml/min) using a 40% MeOH solvent system to yield compound **6**

(9.0 mg), along with subfraction B2(31)A (87.0 mg), which was further purified by semi-preparative RP HPLC (250 mm × 10 mm i.d., 10 μm, Phenomenex Luna C18(2) column, flow rate 2.0 ml/min) with a 30% MeOH solvent system to furnish compound **8** (30.0 mg). Fraction B4 (2.8 g) was applied to a silica gel column using a gradient of $CHCl_3$–MeOH–H_2O (8:3:1, 6:3:1 and 6:4:1) and 100% MeOH to give 29 sub-fractions (B4(1)–B4(29)). Fraction B4(9) (44 mg) was purified by semi-preparative RP HPLC (250 mm × 10 mm i.d., 10 μm, Phenomenex Luna C18(2) column, flow rate 2.0 ml/min) using a 40% MeOH solvent system to afford compound **7** (5.2 mg). Fraction B4(15) (168 mg) was further separated by semi-preparative RP HPLC (250 mm × 10 mm i.d., 10 μm, Phenomenex Luna C18(2) column, flow rate 2.0 ml/min) with a 40% MeOH solvent system to yield compound **2** (21.5 mg). Finally, fraction B6 (3.2 g) was subjected to silica gel column chromatography using a gradient of $CHCl_3$–MeOH–H_2O (8:3:1) and 100% MeOH to give 20 sub-fractions (B6(1)–B6(20)). Fraction B6(10) (383 mg) was purified by semi-preparative RP HPLC (250 mm × 10 mm i.d., 10 μm, Phenomenex Luna C18(2) column, flow rate 3.0 ml/min) using a 40% MeOH solvent system to obtain compound **4** (6.0 mg).

2.3.1. *Methyl nomilinate (**1**)*

^{1}H NMR (400 MHz, acetone-d_6) δ: 1.12 (s, 3H, H–18), 1.17 (s, 3H, H–25a), 1.21 (s, 3H, H–25b), 1.22 (s, 3H, H–24), 1.27 (s, 3H, H–19), 2.10 (s, 3H, C$\underline{H}_3$COO), 2.53 (dd, 1H, *J* = 5.0, 14.0 Hz, H–6a), 2.60 (dd, 1H, *J* = 10.5, 14.0 Hz, H–2a), 2.61 (br d, 1H, *J* = 14.0 Hz, H–6b), 2.72 (br d, 1H, *J* =14.0 Hz, H–2b), 3.57 (OMe), 3.76 (s, 1H, H–15), 5.23 (br d, 1H, *J* = 10.5 Hz, H–1), 5.66 (s, 1H, H–17), 6.44 (br s, 1H, H–22), 7.41 (br s, 1H, H–23), 7.74 (br s, 1H, H–21); ^{13}C NMR (100 MHz, acetone-d_6) δ: 16.0 (C–19), 16.8 (C–18), 17.4 (C–11), 20.7 ($\underline{C}H_3$COO), 20.8 (C–24), 23.1 (C–25b), 31.9 (C–12), 33.4 (C–25a), 35.3 (C–6), 37.4 (C–13), 38.9 (C–2), 44.2 (C–9), 45.7 (C–10), 51.2 (OMe), 51.8 (C–5), 52.5 (C–8), 52.8 (C–15), 65.7 (C–14), 73.2 (C–4), 76.8 (C–1), 77.8 (C–17), 110.2 (C–22), 120.3 (C–20), 141.4 (C–23), 143.2 (C–21), 166.9 (C–16), 169.5 (CH$_3\underline{C}$OO), 171.3 (C–3), 209.8 (C–7); ESI-MS *m/z* 547.2 [M+H]$^+$.

2.3.2. *Limocitrunshin (**2**)*

^{1}H NMR (400 MHz, CD$_3$OD) δ: 1.23 (s, 3H, H–6‴), 2.52 (m, 4H, H–2‴, 4‴), 3.33 (m, 1H, H–4″), 3.39 (m, 1H, H–3″), 3.50 (m, 1H, H–5″), 3.53

(m, 1H, H–2″), 3.94 (s, 3H, 8–OMe), 3.98 (s, 3H, 3′–OMe), 4.14 (dd, 1H, J = 3.0, 11.0 Hz, H–6″b), 4.25 (dd, 1H, J = 1.0, 11.0 Hz, H–6″a), 5.36 (d, 1H, J = 7.5 Hz, H–1″), 6.29 (br s, 1H, H–6), 6.94 (d, 1H, J = 8.0 Hz, H–5′), 7.73 (br d, 1H, J = 8.0 Hz, H–6′), 7.97 (br s, 1H, H–2′); ^{13}C NMR (100 MHz, CD$_3$OD) δ: 26.9 (C–6‴), 45.1 (C–4‴), 45.2 (C–2‴), 55.8 (3′–OMe), 61.2 (8–OMe), 63.6 (C–6″), 69.7 (C–3‴), 70.8 (C–4″), 74.9 (C–2″), 75.0 (C–5″), 77.0 (C–3″), 99.3 (C–6), 103.0 (C–1″), 104.7 (C–10), 113.3 (C–2′), 115.2 (C–5′), 122.1 (C–1′), 123.1 (C–6′), 128.2 (C–8), 134.4 (C–3), 147.5 (C–3′), 149.4 (C–9), 150.1 (C–4′), 157.1 (C–5), 157.6 (C–2), 157.9 (C–7), 171.3 (C–1‴), 171.5 (C–5‴), 178.5 (C–4); ESI-MS *m/z* 653.2 [M+H]$^+$.

2.3.3. *Nobiletin (3)*

^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.96 (s, 3H, 8–OMe), 3.97 (s, 3H, 6–OMe), 3.98 (s, 3H, 4′–OMe), 4.00 (s, 3H, 3′–OMe), 4.03 (s, 3H, 5–OMe), 4.11 (s, 3H, 7–OMe), 6.73 (s, 1H, H–3), 7.00 (d, 1H, J = 9.0 Hz, H–5′), 7.42 (d, 1H, J = 2.0 Hz, H–2′), 7.59 (dd, 1H, J = 2.0, 9.0 Hz, H–6′); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 55.6 (6–OMe), 55.7 (8–OMe), 61.4 (4′–OMe), 61.5 (3′–OMe), 61.8 (7–OMe), 61.9 (5–OMe), 106.3 (C–3), 108.9 (C–2′), 111.9 (C–5′), 114.3 (C–10), 119.3 (C–6′), 123.1 (C–1′), 137.6 (C–6), 143.5 (C–5), 147.1 (C–9), 147.5 (C–8), 149.0 (C–3′), 150.9 (C–7), 151.8 (C–4′), 160.2 (C–2), 175.8 (C–4); ESI-MS *m/z* 403.1 [M+H]$^+$.

2.3.4. *Kaempferol 3-O-rutinoside (4)*

^{1}H NMR (400 MHz, CD$_3$OD) δ: 1.11 (d, 3H, J = 6.0 Hz, H–6‴), 3.24 (t, 1H, J = 9.0 Hz, H–4″), 3.27 (t, 1H, J = 9.5 Hz, H–4‴), 3.32 (ddd, 1H, J = 1.0, 6.0, 9.0 Hz, H–5″), 3.37 (dd, 1H, J = 6.0, 12.5 Hz, H–6″b), 3.40 (t, 1H, J = 9.0 Hz, H–3″), 3.43 (dd, 1H, J = 7.5, 9.0 Hz, H–2″), 3.44 (dq, 1H, J = 6.0, 9.5 Hz, H–5‴), 3.51 (dd, 1H, J = 3.0, 9.5 Hz, H–3‴), 3.62 (dd, 1H, J = 1.5, 3.0 Hz, H–2‴), 3.80 (dd, 1H, J = 1.0, 12.5 Hz, H–6″a), 4.51 (d, 1H, J = 1.5 Hz, H–1‴), 5.12 (d, 1H, J = 7.5 Hz, H–1″), 6.21 (d, 1H, J = 2.0 Hz, H–6), 6.40 (d, 1H, J = 2.0 Hz, H–8), 6.89 (d, 2H, J = 9.0 Hz, H–3′, 5′), 8.05 (d, 2H, J = 9.0 Hz, H–2′, 6′); ^{13}C NMR (100 MHz, CD$_3$OD) δ: 17.9 (C–6‴), 68.6 (C–6″), 69.7 (C–5‴), 71.5 (C–4″), 72.1 (C–2″), 72.3 (C–3‴), 73.9 (C–4‴), 75.8 (C–2″), 77.2 (C–5″), 78.2 (C–3″), 95.0 (C–8), 99.9 (C–6), 102.4 (C–1‴), 104.6 (C–1″), 105.7 (C–10), 116.1 (C–3′, 5′), 122.8 (C–1′), 132.4 (C–2′, 6′), 135.5 (C–3), 158.6 (C–9), 159.4 (C–4′), 161.5 (C–2), 163.0 (C–5), 166.0 (C–7), 179.4 (C–4); ESI-MS *m/z* 595.2 [M+H]$^+$.

2.3.5. *Limocitrin 3-glucoside (5)*

^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.08 (m, 1H, H–5″), 3.10–3.40 (m, 1H, H–3″), 3.40 (m, 1H, H–6″a), 3.57 (br d, 1H, J = 11.5 Hz, H–6″b), 3.81 (s, 3H, 8–OMe), 3.90 (s, 3H, 3′–OMe), 5.46 (d, 1H, J = 7.0 Hz, H–1″), 6.57 (s, 1H, H–6), 6.86 (d, 1H, J = 8.0 Hz, H–5′), 7.63 (br s, 1H, H–2′), 7.65 (dd, 1H, J = 2.0, 9.0 Hz, H–6′), 12.48 (s, 1H, 5–OH); ^{13}C NMR (100 MHz, DMSO-d_6,) δ: 56.5 (3′–OMe), 60.9 (C–6″), 61.1 (8–OMe), 69.9 (C–4″), 74.0 (C–2″), 76.4 (C–3″), 77.5 (C–5″), 95.7 (C–6), 100.9 (C–1″), 104.2 (C–10), 115.2 (C–2′), 116.0 (C–5′), 121.2 (C–1′), 121.7 (C–6′), 128.3 (C–8), 133.2 (C–3), 144.9 (C–3′), 147.7 (C–4′), 148.7 (C–9), 156.3 (C–2, 5), 158.0 (C–7), 177.7 (C–4); ESI-MS *m/z* 509.1 [M+H]$^+$.

2.3.6. *Kaempferol 3-(2^G-rhamnosylrutinoside) (6)*

^{1}H NMR (400 MHz, CD$_3$OD) δ: 0.98 (d, 3H, J = 6.0 Hz, H–6⁗), 1.07 (d, 3H, J = 6.0 Hz, H–6‴), 3.23 (t, 1H, J = 9.0 Hz, H–4″), 3.23 (t, 1H, J = 9.5 Hz, H–4‴), 3.33 (ddd, 1H, J = 1.0, 6.0, 9.0 Hz, H–5″), 3.33 (t, 1H, J = 9.5 Hz, H–4⁗), 3.37 (dd, 1H, J = 6.0, 12.5 Hz, H–6″b), 3.41 (dq, 1H, J = 6.0, 9.5 Hz, H–5‴), 3.47 (dd, 1H, J = 3.5, 9.5 Hz, H–3‴), 3.53 (t, 1H, J = 9.0 Hz, H–3″), 3.57 (dd, 1H, J = 1.5, 3.5 Hz, H–2‴), 3.59 (dd, 1H, J = 7.5, 9.0 Hz, H–2″), 3.78 (dd, 1H, J = 3.5, 9.5 Hz, H–3⁗), 3.81 (dd, 1H, J = 1.0, 12.5 Hz, H–6″a), 3.99 (dd, 1H, J = 1.5, 3.5 Hz, H–2⁗), 4.05 (dq, 1H, J = 6.0, 9.5 Hz, H–5⁗), 4.49 (d, 1H, J = 1.5 Hz, H–1‴), 5.22 (d, 1H, J = 1.5 Hz, H–1⁗), 5.59 (d, 1H, J = 7.5 Hz, H–1″), 6.18 (d, 1H, J = 2.0 Hz, H–6), 6.37 (d, 1H, J = 2.0 Hz, H–8), 6.89 (d, 2H, J = 9.0 Hz, H–3′, 5′), 8.01 (d, 2H, J = 9.0 Hz, H–2′, 6′); ^{13}C NMR (100 MHz, CD$_3$OD) δ: 17.6 (C–6⁗), 17.8 (C–6‴), 68.3 (C–6″), 69.8 (C–5‴), 69.9 (C–5⁗), 72.0 (C–4″), 72.1 (C–2″), 72.3 (C–3‴, 3⁗), 72.4 (C–2⁗), 73.8 (C–4‴), 74.1 (C–4⁗), 77.1 (C–5″), 78.9 (C–3″), 79.9 (C–2″), 94.8 (C–8), 99.8 (C–6), 100.5 (C–1‴), 102.3 (C–1″), 102.6 (C–1⁗), 105.9 (C–10), 116.1 (C–3′, 5′), 123.2 (C–1′), 132.1 (C–2′, 6′), 134.3 (C–3), 158.5 (C–9), 159.0 (C–4′), 161.2 (C–2), 163.2 (C–5), 165.6 (C–7), 179.3 (C–4); ESI-MS *m/z* 741.2 [M+H]$^+$.

2.3.7. *Didymin (7)*

^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.07 (d, 3H, J = 6.2 Hz, H–6‴), 2.77 (dd, 1H, J = 2.5, 17.0 Hz, H–3a), 3.77 (s, 3H, 4′–OMe), 4.52 (br s, 1H, H–1‴), 4.98 (d, 1H, J = 7.5 Hz, H–1″), 5.58 (dd, 1H, J = 3.0, 12.5 Hz, H–2), 6.13 (s, 2H, H–6, 8), 6.98 (d, 2H, J = 8.0 Hz, H–3′, 5′), 7.47 (d, 2H, J =

8.5 Hz, H–2′, 6′), 12.02 (s, 1H, 5–OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 18.0 (C–6‴), 42.0 (C–3), 55.3 (4′-OMe), 66.1 (C–6″), 68.5 (C–5‴), 69.8 (C–4″), 70.4 (C–3‴), 70.8 (C–2‴), 72.2 (C–4‴), 73.1 (C–2″), 75.7 (C–3″), 76.4 (C–5″), 78.5 (C–2), 95.6 (C–8), 96.6 (C–6), 99.5 (C–1‴), 100.8 (C–1″), 103.4 (C–10), 114.1 (C–3′, 5′), 128.5 (C–6′), 128.7 (C–2′), 130.5 (C–1′), 159.7 (C–4′), 162.7 (C–9), 163.2 (C–5), 165.3 (C–7), 197.2 (C–4); ESI-MS *m/z* 595.2 [M+H]+.

2.3.8. *(2S)-Narirutin 4′-O-glucoside (8)*

^{1}H NMR (400 MHz, CD3OD) δ: 1.21 (d, 3H, *J* = 6.0 Hz, H–6‴), 2.81 (dd, 1H, *J* = 3.0, 17.0 Hz, H–3), 4.71 (d, 1H, *J* = 1.0 Hz, H–1‴), 5.49 (dd, 1H, *J* = 3.0, 12.0 Hz, H–2), 6.22 (s, 2H, H–6, 8), 7.17 (d, 2H, *J* = 8.0 Hz, H–3′, 5′), 7.46 (d, 2H, *J* =8.0 Hz, H–2′, 6′), 11.70 (s, 1H, 5- OH); ^{13}C–NMR (100 MHz, CD3OD) δ: 18.1 (C–6‴), 43.5 (C–3), 62.3 (C–6⁗), 67.6 (C–6″), 69.9 (C–5‴), 71.5 (C–4″, 4⁗), 72.2 (C–2″), 72.5 (C–3‴), 74.2 (C–4‴), 74.8 (C–2″), 75.0 (C–2⁗), 77.3 (C–5″), 78.0 (C–3″), 78.1 (C–5⁗), 78.3 (C–3⁗), 80.3 (C–2), 97.3 (C–8), 98.2 (C–6), 101.3 (C–1‴), 102.3 (C–1″, 1⁗), 105.1 (C–10), 118.0 (C–3′, 5′), 129.1 (C–2′, 6′), 134.06 (C–1′), 159.4 (C–4′), 164.5 (C–9), 165.1 (C–5), 167.0 (C–7), 198.4 (C–4); ESI-MS *m/z* 743.2 [M+H]+.

2.3.9. *Naringenin 7-O-rutinoside (9)*

^{1}H NMR (400 MHz, CD3OD) δ: 2.68 (dd, 1H, *J* = 3.0, 17.0 Hz, H–3b), 3.09 (dd, 1H, *J* = 12.0, 17.0 Hz, H–3a), 5.29 (dd, 1H, *J* = 3.0, 12.0 Hz, H–2), 6.14 (d, 2H, *J* = 1.0 Hz, H–6, 8), 6.81 (d, 2H, *J* = 8.0 Hz, H–3′, 5′), 7.27 (d, 2H, *J* = 8.0 Hz, H–2′, 6′); ^{13}C–NMR (100 MHz, CD3OD) δ: 17.9 (C–6‴), 43.9 (C–3), 67.4 (C–6″), 69.7 (C–5‴), 71.2 (C–4″), 71.9 (C–2″), 72.3 (C–3‴), 74.0 (C–4‴), 74.5 (C–2″), 77.0 (C–5″), 77.7 (C–3″), 80.4 (C–2), 97.2 (C–8), 98.0 (C–6), 101.0 (C–1″), 102.0 (C–1‴), 104.9 (C–10), 116.4 (C–3′, 5′), 129.0 (C–2′, 6′), 130.7 (C–1′), 158.8 (C–9), 164.3 (C–4′), 164.7 (C–5), 166.7 (C–7), 198.3 (C–4); ESI-MS *m/z* 581.2 [M+H]+.

2.4. *AChE inhibition activity*

An Acetylcholinesterase Activity Colorimetric Assay Kit was purchased from BioVision (Milpitas, CA, USA) and AChE inhibition activity was measured using the AChE assay kit according to the manufacturer's protocol. In brief, 50 μl of sample (30 μg/ml) and assay buffer was added to each well in a 96-well plate, followed by the addition of 50 μl of the reaction mixture. After incubation at 37 °C for 20 min, absorbance at 570 nm was measured using a microplate reader (PowerWave XS; Bio-Tek Instruments, Winooski, VT, USA). Aricept (10 μM) was used as a positive control.

2.5. *Statistical analysis*

Statistical significance was determined with analysis of variance (ANOVA) followed by a multiple comparison test with Bonferroni adjustment. *P* values less than 0.05 were considered statistically significant. Analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1. *Isolation and structural elucidation of compounds*

The unpeeled *C. unshiu* fruit was extracted with 100% EtOH to give crude EtOH extracts (89.0 g) that were solvent-partitioned with hexane, EtOAc and *n*-BuOH. Phytochemical investigation of the EtOAc and *n*-BuOH soluble fractions using repeated column chromatography and HPLC purification resulted in isolation and identification of nine compounds, **1–9** (Fig. 1). The compounds were identified as methyl nomilinate (**1**) [15], limocitrunshin (**2**) [7], nobiletin (**3**) [16], kaempferol 3-*O*-rutinoside (**4**) [17], limocitrin 3-glucoside (**5**) [18], kaempferol 3-(2^G-rhamnosylrutinoside) (**6**) [17], didymin (**7**) [19], (2*S*)-narirutin 4′-*O*-glucoside (**8**) [20] and naringenin 7-*O*-rutinoside (**9**) [21], respectively, based on comparison of their spectroscopic and physical data with those previously reported as well as by measurement of their specific rotations.

3.2. *Evaluation of AChE inhibition activity of the isolates*

Compounds **1–9** were evaluated for inhibitory effects on AChE to verify their potential health benefits. The inhibition effects of compounds **1–9** on AChE activity were examined using a microwell plate AChE inhibition assay [22].
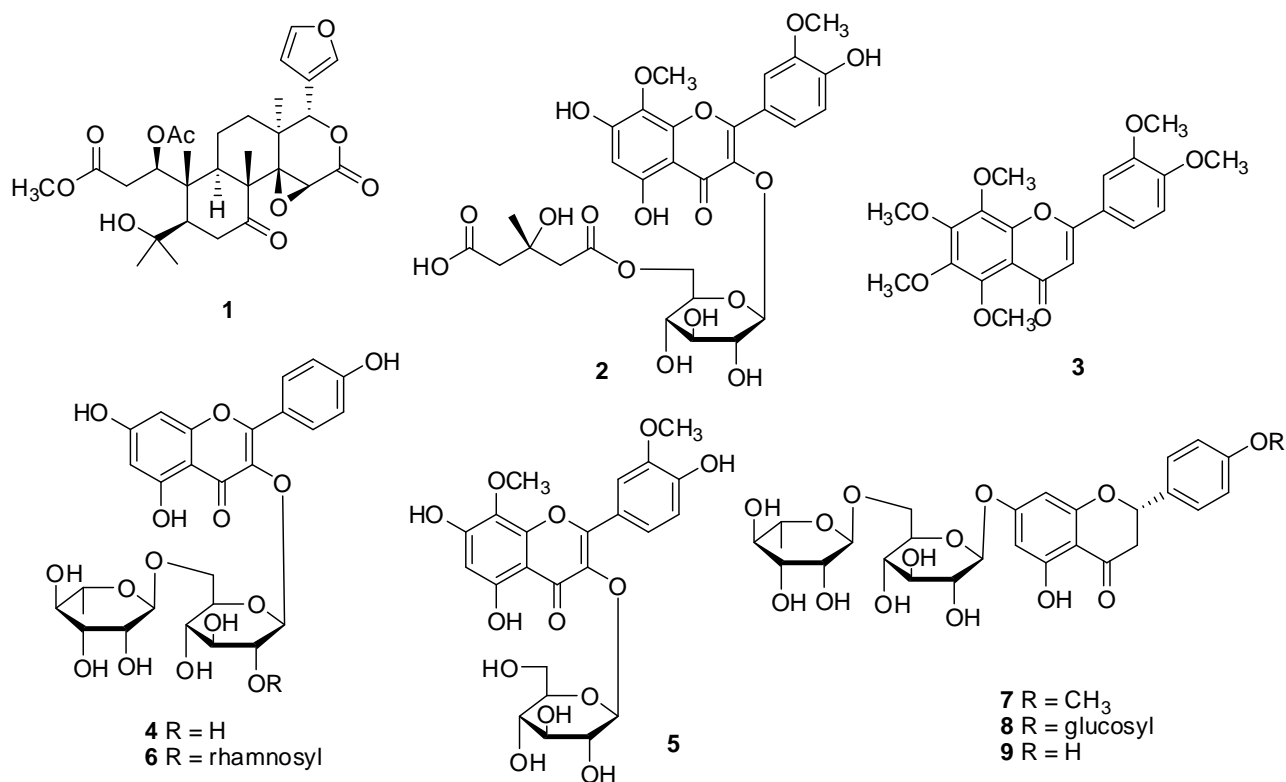
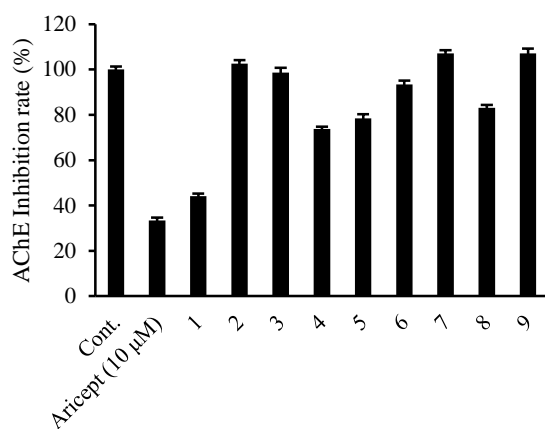**Fig. 1.** Chemical structures of compounds **1**–**9**

Fig. 2. Acetylcholinesterase inhibition rate (%) comparison for compounds **1**–**9**. Fifty µl of sample (30 µg/ml) and assay buffer was added to each well of a 96-well plate, followed by the addition of 50 µl of the reaction mixture. After incubation at 37 °C for 20 min, the absorbance at 570 nm was measured using a microplate reader. Aricept (10 µM) was used as a positive control.

Inhibition of AChE activity leading to an increase in acetylcholine concentration in the brain is recognized as a potential treatment for mild to moderate AD and various other memory impairment disorders [23−27]. At a concentration of 30 µg/ml, compound **1** inhibited AChE activity by more than 50% (Fig. 2). Compared with the control, compound **1** showed a 44.11 ± 1.1% AChE inhibition. This effect was similar to the 34.40 ± 1.2% inhibition seen with a 10 µM concentration of Aricept (positive control). However, the other compounds showed weak (compounds **4**, **5**, **6** and **8**) to no inhibition (compounds **2**, **3**, **7** and **9**) of AChE activity when compared to the control (Table 1).

T a b l e 1

*The acetylcholinesterase inhibition rate (%) for compounds **1**–**9** compared to the control*

Compound	AChE inhibition rate (%) at 30 µg/ml
1	44.11 ± 1.1
2	102.62 ± 1.5
3	98.69 ± 2.1
4	73.78 ± 1.0
5	78.42 ± 1.9
6	93.44 ± 1.7
7	107.04 ± 1.5
8	83.11 ± 1.3
9	107.11 ± 2.1
Aricept [a]	33.40 ± 1.2

[a] Aricept was used as a positive control for AChE inhibition activity and its AChE inhibition rate (%) was measured at a concentration of 10 µM.

3.3. *Limonoid as a potential AChE activity inhibitor*

In the micro-well plate AChE inhibition assay of compounds **1**–**9**, compound **1** was the most potent AChE activity inhibitor. Compound **1** is a highly oxygenated limonoid compound with a prototypical structure consisting of four six-membered rings and a furan ring. This type of limonoid compound is abundant in citrus fruit and other plants from the families Rutaceae and Meliaceae [14]. Limonoids have been reported to exhibit diverse bioactivity including insecticidal, antibacterial, antifungal, antiviral and anticancer effects [28]. There have been widespread efforts to identify new AChE inhibitors from natural sources, but only limited work has focused on limonoids. Recently, three limonoids isolated from *Trichilia welwitschii* were reported to show weak to moderate inhibitory effects on AChE activity [14, 29]. Our present findings suggest for the first time that another limonoid, methyl nomilinate (**1**), is a potential AChE activity inhibitor.

In our previous study, we found that methyl nomilinate showed an anti-inflammatory effect by inhibiting NO production by LPS-activated RAW 264.7 macrophages and significantly abrogated cisplatin-induced nephrotoxicity in LLC-PK1 cells [7]. In addition, methyl nomilinate was reported to inhibit the proliferation of MCF-7 ER+ human breast cancer cells with an IC_{50} of 0.01 µg/ml [30]. Recently, methyl nomilinate from citrus was shown to inhibit cell proliferation in the SW480 human colon cancer cell line through the modulation of G1 cell cycle regulators [31].

4. CONCLUSION

Phytochemical investigation of the EtOH extract of *C. unshiu* fruit led to the isolation and identification of nine compounds including one limonoid and eight flavonoids. AChE inhibition has been considered one of the promising temporary treatments for AD. The inhibitory effect of these isolates on AChE activity was evaluated using a micro-well plate AChE inhibition assay, which indicated that the limonoid, methyl nomilinate (**1**), exhibited significant AChE inhibitory activity. This is the first study to identify the limonoid methyl nomilinate from *C. unshiu* fruit as a natural AChE inhibitor. Additionally, our findings suggest that *C. unshiu* fruit, one of the most popular fruits in Korea, can serve as a valuable natural, functional food source of therapeutic agents for AD.

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