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Cholinesterase Inhibitory Constituents from *Capsosiphon fulvescens*

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Abstract – Nine compounds (**1 - 9**), α-linolenic acid (**1**), *cis*-5,8,11,14,17-elcosapentaenoic acid (**2**), phytol (**3**), loliolide (**4**), uridine (**5**), thymidine (**6**), deoxyadenosine (**7**), adenine (**8**), and adenosine (**9**), were isolated from the *n*-hexane, methylene chloride, ethyl acetate and *n*-butanol fractions of *Capsosiphon fulvescens*. The structures of these compounds were elucidated on the basis of spectroscopic evidence. Compounds **1 - 9** exhibited acetylcholinesterase (AChE) inhibitory activities with IC_{50} values ranging from 114.91 to 252.40 μM, whereas **2 - 4** showed butyrylcholinesterase (BChE) inhibitory activities with IC_{50} values ranging from 251.18 to 499.16 μM.
Keywords – *Capsosiphon fulvescens*, DPPH, Acetylcholinesterase, Peroxynitrite, Butyrylcholinesterase.

Introduction

Capsosiphon fulvescens, a green alga, grows in copious amounts along the Southwestern coast of Korea and widely throughout the world (Park *et al*., 2005). Although *C. fulvescens* has been traditionally used in Korea as a functional food for centuries, the uses and potential values as a bioactive material have not been well studied. Many reports have been published highlighting the variety of biological activities of *C. fulvescens*, including antioxidant, antitumor, and anti-cancer activities (Hwang *et al*., 2008; Kwon *et al*., 2007), as well as immuno-stimulation (Na *et al*., 2010), prevention of osteoporosis (Kim *et al*., 2006), reduction of skin irritation (Kim *et al*., 2003), inhibition of melanogenesis (Mun *et al*., 2005), hangover reduction (Choi *et al*., 2002), and hepatoprotection (Park *et al*., 1997). However, limited studies are available on the constituents of this maritime plant, except for the studies on polysaccharides.

Alzheimer's disease (AD) is a chronic neurodegenerative disorder that damages the brain and results in cognitive impairment, including memory loss and learning disturbancs commonly seen in the elderly (Scarpini *et al*., 2003; Perry *et al*., 1978). Many different theories have been postulated for the etiology of AD and one of the well-established theories suggests the involvement of a cholinergic pathway, indicating that a progressive decline in the levels of acetylcholine, a neurotransmitter, as the culminating process for the development of AD (Arendt *et al*., 1992). AD is also characterized pathologically by the presence of amyloid plaques and tauassociated neurofibrillary tangles which are commonly related to the states of oxidative stress, such as exposure to peroxynitrite ($ONOO^-$) and superoxide anion ($\cdot O_2^-$) (Buttertield *et al*., 2001). Thus, this research was done to determine effectiveness as cholinesterases (ChE) inhibitors by alleviating the cholinergic deficits in improving neurotransmission and utilizing its antioxidant property to ameliorate oxidative stress.

Since there has been limited research on the anticholinesterase and antioxidant activities of *C. fulvescens* or its chemical constituents, this work focused on the isolation and characterization of bioactive constituents from *C. fulvescens* and evaluated their inhibitory activities against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) as well as their scavenging activities for DPPH radicals and $ONOO^-$.

C. fulvescens was extracted with 95% ethanol (EtOH) and concentrated in vacuo. The EtOH extract of C. fulvescens was subsequently fractionated into five parts: *n*-hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) and H_2O. Anticholinesterase and antioxidant activities of these fractions were examined via inhibitory activities against AChE and BChE as well as scavenging activities for DPPH radicals and $ONOO^-$. The results showed that the *n*-hexane, CH_2Cl_2 and EtOAc fractions had strong AChE and BChE inhibitory activities (Table 1). Chromatographic separations of the *n*-hexane,

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Table 1. Anticholinesterase and antioxidant activities of *C. fulvescens*

Samples	IC_{50}			
	AChE (μM)	BChE (μM)	DPPH (μg/mL)	$ONOO^-$ (μg/mL)
EtOH ex.	6.23 ± 1.63	13.55 ± 4.25	> 400.00	> 50.00
Hexane fr.	5.37 ± 1.07	12.78 ± 2.24	> 400.00	> 50.00
CH_2Cl_2 fr.	5.72 ± 2.54	10.59 ± 1.87	> 400.00	> 50.00
EtOAc fr.	6.17 ± 2.01	13.45 ± 2.04	> 400.00	> 50.00
BuOH fr.	98.22 ± 5.21	129.07 ± 3.94	> 400.00	> 50.00
H_2O fr.	135.56 ± 6.23	159.24 ± 2.87	> 400.00	> 50.00
berberine	0.23 ± 0.01	11.97 ± 0.12		
L-ascorbic acid			0.33 ± 0.02	
L-penicillamine				0.35 ± 0.01

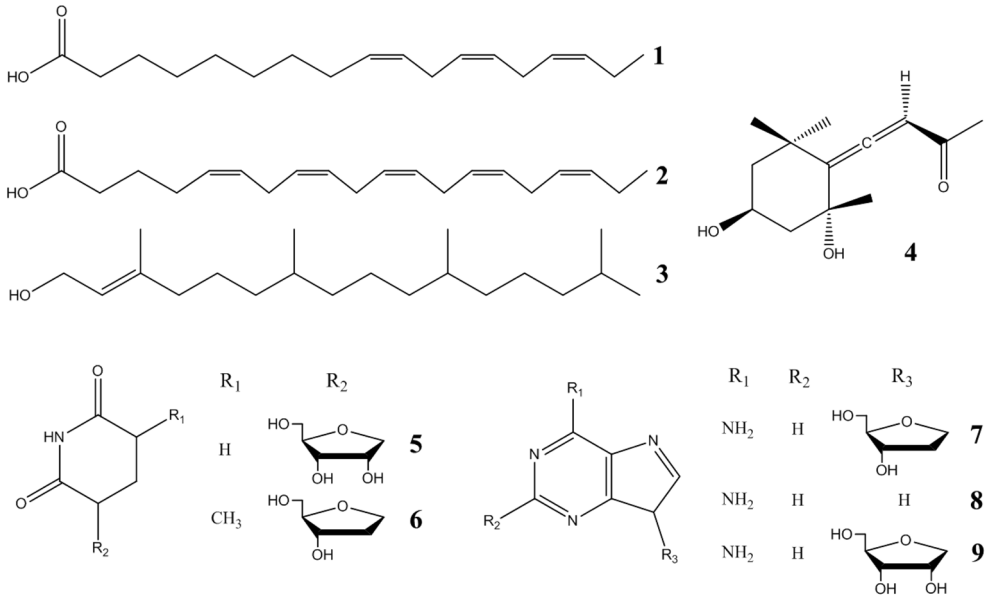

Fig. 1. The structures of compounds isolated from *Capsosiphon fulvescens.*

CH_2Cl_2 and EtOAc fractions by combinations of RP-C18 and silica gel, Sephadex LH-20 column, and HPLC chromatographies yielded the following nine compounds: α-linolenic acid (**1**) (Cherif *et al*., 1975) and *cis*-5,8,11,14,17-elcosapentaenoic acid (**2**) (Yasuda *et al*., 1991) from the *n*-hexane fraction, phytol (**3**) (Sato *et al*., 1967) and loliolide (**4**) (Jakupovic *et al*., 1991) from the CH_2Cl_2 fraction, uridine (**5**) (Notaro *et al*., 1992), deoxyadenosine (**7**) (Yokoyama *et al*., 2007) and adenosine (**9**) (Narlawar *et al*., 2010) from the EtOAc fraction, and thymidine (**6**) (Magdalena *et al*., 1998) and adenine (**8**) (Manna *et al*., 2010) from the *n*-BuOH fraction (Fig. 1).

Experimental

Instruments and reagents – Melting points were measured using a Yanaco micro melting point apparatus (Kyoto, Japan). Optical rotation was measured using a Jasco DIP-370 digital polarimeter (Tokyo, Japan). UV spectra were measured on a Shimadzu UV-160A spectrometer (Kyoto, Japan). IR (KBr disk) spectra were measured on Mattson Genesis II (Madison, WI, USA) and Jasco-300E FT-IR spectrophotometers (Tokyo, Japan). EIMS and HRFABMS were performed with a Quattro II spectrometer (Micromass, Altrincham, UK). NMR spectra were recorded in CD_3OD, $CDCl_3$ and C_5D_5N on a Varian OXFORD-AS400 MHz instrument (Palo Alto, CA, USA).

Plant material – *C. fulvescens* was collected from the southern coastal area of Wando, Korea in February 2009. This plant was verified by Professor Byung Sun Min, College of Pharmacy, Catholic University of Daegu. A

voucher specimen (CUDP 200901) has been deposited at the College of Pharmacy, Catholic University of Daegu, Korea.

Extraction and isolation – *C. fulvescens* (200 kg) was freeze-dried, cut into small pieces and extracted with 95% EtOH at 80 °C to yield 3.61 kg of an EtOH extract. This extract was then suspended in distilled H_2O and successively partitioned with *n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH to yield *n*-hexane (819.2 g), CH_2Cl_2 (364.4 g), EtOAc (4.70 g), and *n*-BuOH (21.58 g) fractions, respectively, as well as a H_2O residue (1.3 kg). A portion (150 g) of the *n*-hexane soluble fraction was subjected to flash column chromatography over silica gel (2.0 kg) and eluted with *n*-hexane-acetone (100 : 0 to 0 : 100, gradient) followed by acetone-methanol (100 : 0 to 1 : 1, gradient). Fractions CF-HE-A to CF-HE-N were collected and pooled according to their similar TLC patterns. CF-HE-B (3.8 g) was chromatographed on a silica-phase column (4.2 × 46 cm) with *n*-hexane-acetone (gradient from 50 : 1 to 1 : 2). The subractions (CF-HE-B-1 to CF-HE-B-10) were collected and pooled according to their similar TLC patterns. CF-HE-B-8 (150 mg) was chromatographed on reverse-phase column (1.8 × 45 cm, RP-C18) with MeOH-H_2O (96 : 4) to afford α-linolenic acid (**1**, 90 mg). CF-HE-B-9 (350 mg) was chromatographed on a reverse-phase column (2.5 × 48 cm, RP-C18) with MeOH-H_2O (94 : 6) to afford CF-HE-B-9-2. CF-HE-B-9-2 (160 mg) was chromatographed on silica-phase column (2.2 × 45 cm) with *n*-hexane-acetone (50 : 1) to afford *cis*-5,8,11,14,17-elcosapentaenoic acid (**2**, 98 mg).

The CH_2Cl_2-soluble fraction (4.7 g) was subjected to open column chromatography over silica gel (2.0 kg) eluted with *n*-hexane-acetone (200 : 1 to 0 : 100, gradient), and followed by acetone-methanol (100 : 0 to 1 : 2, gradient). Fractions CF-MC-A to CF-MC-H were collected and pooled according to their similar TLC patterns. CF-MC-B (3.8 g) was chromatographed on a silica-phase column (3.6 × 45 cm) with *n*-hexane-acetone (gradient from 50 : 1 to 1 : 1). The subfractions (CF-MC-B-1 to CF-MC-B-10) were collected and pooled according to their similar TLC patterns. CF-MC-B-9 (180 mg) was chromatographed on a reverse-phase column (1.5 × 45 cm, RP-C18) with a MeOH-H_2O (95 : 5) to afford CF-MC-B-9-1 (100 mg). CF-MC-B-9-1 was purified with a silica-phase column (1.4 × 45 cm) with *n*-hexane-acetone (20 : 1) to afford phytol (**3**, 61 mg).

CF-MC-F (3.6 g) was chromatographed on a on a silica-phase column (3.6 × 48 cm) with CH_2Cl_2-MeOH (gradient form 30:1 to 1:1). The subfractions (CF-MC-F-1 to CF-MC-F-10) were collected and pooled according to their similar TLC patterns. CF-MC-F-2 (45 mg) was chromatographed on a reverse-phase column (1.2 × 40 cm, RP-C18) with MeOH-H_2O (50 : 50) to afford loliolide (**4**, 15 mg).

The EtOAc soluble fraction (4.7 g) was subjected to open column chromatography over silica gel (60 g) eluted with *n*-hexane-acetone (50 : 1 to 100 : 0, gradient). Fractions (CF-EA-A to CF-EA-J) were collected and pooled according to their similar TLC patterns. Subfraction CF-EA-E (101 mg) was chromatographed on a reverse-phase column (2.0 × 50 cm, RP-C18) with MeOH-H_2O (gradient from 35 : 65 to 60 : 40) to afford CF-EA-E-4 and CF-EA-E-5. CF-EA-E-4 (55 mg) was purified with a reverse-phase column (1.2 × 45 cm, RP-C18) with MeOH-H_2O (35 : 65) to afford uridine (**5**, 25 mg). CF-EA-E-5 (40 mg) was purified with reverse-phase column (1.2 × 45 cm, RP-C18) with MeOH-H_2O (30 : 70) to afford deoxyadenosine (**7**, 20 mg). Fraction CF-EA-D (102 mg) was chromatographed on a reverse-phase column (1.8 × 40 cm, RP-C18) with MeOH-H_2O (gradient from 55 : 45 to 80 : 20) to fractionate CF-EA-D-1 to CF-EA-D-6. Subfraction CF-EA-D-1 (70 mg) was purified with a reverse-phase column (1.5 × 45 cm, RP-C18) with MeOH-H_2O (40 : 60) to afford adenosine (**9**, 50 mg).

The BuOH soluble fraction (21.5 g) was subjected to open column chromatography over silica gel (60 g) eluted with *n*-hexane-acetone (20 : 1 to 100 : 0, gradient), followed by acetone-MeOH (100 : 0 to 1 : 4, gradient). Fractions CF-Bu-A to CF-Bu-G were collected and pooled according to their similar TLC patterns. Subfraction CF-Bu-C (60 mg) was chromatographed on a reverse-phase column (1.2 × 45 cm, RP-C18) with MeOH-H_2O (25 : 75) to afford GF-Bu-C-6. Subfraction CF-Bu-C-6 (52 mg) was chromatographed on a Sephadex column (1.5 × 70 cm, LH-20) with MeOH-H_2O (30 : 70) to afford thimidine (**6**, 28 mg). CF-Bu-E (1.13 g) was chromatographed on a reverse-phase column (2.8 × 50 cm, RP-C18) with MeOH-H_2O (gradient from 40 : 60 to 70 : 30). The subfractions (CF-Bu-E-1 to CF-Bu-E-8) were collected and pooled according to their similar TLC patterns. CF-Bu-E-5 (60 mg) was chromatographed on a reverse-phase column (1.2 × 45 cm, RP-C18) with MeOH-H_2O (50 : 50) to afford CF-Bu-E-5-1. CF-Bu-E-5-1 (39 mg) was purified with a silica-phase column (1.2 × 40 cm) with *n*-hexane-acetone (5 : 3) to afford adenine (**8**, 15 mg).

α-Linolenic acid (**1**) – IR (KBr) cm^{-1}: 3169 (OH), 2929 (C-H), 1710 (C=O), 1412 (C=C). EI-MS m/z: 278.2 $[M]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Cherif *et al*., 1975).

***cis*-5,8,11,14,17-Elcosapentaenoic acid (2)** – IR (KBr) cm^{-1}: 3132 (OH), 2927 (C-H), 1709 (C=O), 1413 (C=C); EI-MS m/z: 302.1 $[M]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Yasuda *et al*., 1991).

Phytol (3) – IR (KBr) cm^{-1}: 3333 (OH), 2927 (C-H), 1081 (C-O); EI-MS m/z: 296 $[M]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Sato *et al*., 1967).

Loliolide (4) – IR (KBr) cm^{-1}: 3440 (OH), 2917 (C-H), 1730 (C=O), 1056 (C-O); FAB-MS m/z: 197 $[M + H]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Jakupovic *et al*., 1991).

Uridine (5) – IR (KBr) cm^{-1}: 3420 (OH), 2917 (C-H), 1699 (C=O), 1056 (C-O); FAB-MS m/z: 245 $[M + H]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Notaro *et al*., 1992).

Thymidine (6) – IR (KBr) cm^{-1}: 3273 (OH), 2949 (C-H), 1673 (C=O), 1066 (C-O); FAB-MS m/z: 229 $[M + H]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Magdalena *et al*., 1998).

Deoxyadenosine (7) – IR (KBr) cm^{-1}: 3382 (OH), 2936 (C-H), 1692, 1613 (C=O), 1070 (C-O); FAB-MS m/z: 252 $[M + H]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Yokoyama *et al*., 2007).

Adenine (8) – IR (KBr) cm^{-1}: 3303 (OH), 2982 (C-H), 1056 (C-O); FAB-MS m/z: 136 $[M + H]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Manna *et al*., 2010).

Adenosine (9) – IR (KBr) cm^{-1}: 3330 (OH), 2960 (C-H), 1072 (C-O); FAB-MS m/z: 268 $[M + H]^+$; This compound exhibited comparable spectroscopic data (^{1}H- and ^{13}C-NMR) to published values (Narlawar *et al*., 2010).

Measurement of DPPH radical scavenging activity – The DPPH radical scavenging effect was evaluated using the method reported by Blois with suitable modifications (Bios *et al*., 1958). The reduction of DPPH was followed by monitoring the decrease in absorbance at a characteristic wavelength during the reaction. In its radical form, DPPH absorbed at 520 nm, but after reduction by an antioxidant or a radical species, the absorption disappeared. 160 μL of MeOH solution (final concentration 400 μg/mL) was added to 40 μL DPPH methanol solution (1.5×10^{-4} M). After the mixture was gently mixed and allowed to stand at room temperature for 30 min, the optical density was measured at 530 nm using a microplate reader spectrophotometer VERSAmax (Molecular Devices, CA, USA). L-Ascorbic acid was used as a positive control. The antioxidant activity of each sample was expressed in terms of IC_{50} (μg/mL required to inhibit DPPH radical formation by 50%), which was calculated from the log-dose inhibition curve.

Measurement of the $ONOO^-$ scavenging activity – $ONOO^-$ scavenging was measured by monitoring the oxidation of dihydrorhodamine 123 (DHR 123) using the method described by Kooy and Royall et al with suitable modifications (Kooy *et al*., 1994). DHR 123 (5 mM) in dimethylformamide, which was purged with nitrogen, was stored at −80 ºC as a stock solution. This solution was then placed on ice and protected from direct light until just prior to the study. The samples were dissolved in 10% DMSO (final concentration, 50 μg/mL). The buffer consisted of 90 mM sodium chloride, 50 mM sodium phosphate, 5 mM potassium chloride at pH 7.4, and 100 μM diethylenetriaminepentaacetic acid (DTPA), each of which was prepared with high quality deionized water and was purged with nitrogen. The final concentration of DHR 123 was 5 μM. The background and final fluorescent intensities were measured 5 min after treatment with and without adding authentic $ONOO^-$. DHR 123 was oxidized rapidly by authentic $ONOO^-$, and its final fluorescent intensity remained unchanged over time. The fluorescence intensity of oxidized DHR 123 was measured with a microplate fluorescence reader (FL 500, Bio-Tek Instruments) at the excitation and emission wavelengths of 480 nm and 530 nm, respectively. Results are expressed as mean ± SEM (n = 3) for the final fluorescence intensity minus background fluorescence. The effects are expressed as the percent inhibition of oxidation of DHR 123. l-Penicillamine was used as a positive control.

Measurement of cholinesterase inhibitory activity – The inhibitory activities against ChEs were measured using the spectrophotometric method developed by Ellman *et al* (Ellman *et al*., 1961). ACh (acetylcholine) and BCh (butyrylcholine) were used as substrates to assay the inhibition of AChE and BChE, respectively. The reaction mixture contained 140 μL of sodium phosphate buffer (pH 8.0), 20 μL of the test sample solution and 20 μL of either AChE or BChE and was then incubated for 15 min at room temperature after mixing. The reactions were initiated by adding of 10 μL of dithiobisnitrobenzoate (DTNB) and 10 μL of either ACh or BCh, respectively. The hydrolysis of ACh or BCh was monitored at 412 nm by the formation of yellow 5-thio-2-nitrobenzoate anion from the reaction of DTNB with thiocholine, which was

Table 2. Anticholinesterase and antioxidant activities (n = 3) of **1 - 9**

Compounds	IC_{50}			
	AChE (μM)	BChE (μM)	DPPH (μg/mL)	$ONOO^-$ (μg/mL)
1	127.37 ± 13.86	> 359.7	> 400.00	> 50.00
2	114.97 ± 3.07	251.18 ± 25.71	> 400.00	> 50.00
3	148.94 ± 7.47	268.44 ± 2.47	> 400.00	> 50.00
4	192.90 ± 7.19	499.16 ± 4.66	> 400.00	> 50.00
5	156.27 ± 6.85	> 409.84	> 400.00	> 50.00
6	156.25 ± 9.87	> 413.22	> 400.00	> 50.00
7	209.68 ± 7.11	> 662.25	> 400.00	> 50.00
8	252.40 ± 4.53	> 740.74	> 400.00	> 50.00
9	128.95 ± 2.87	> 374.53	> 400.00	> 50.00
berberine	0.23 ± 0.01	11.97 ± 0.12		
L-ascorbic acid			0.33 ± 0.02	
L-penicillamine				0.35 ± 0.01

released by enzymatic hydrolysis of either ACh or BCh, respectively. The test samples and the positive control (berberine) were dissolved in 10% analytical grade ethanol. All reactions were performed in triplicate in 96-well microplates with VERSA max (Molecular Devices, CA, USA). The percentage (%) inhibition was calculated from (E – S) / E × 100, where E and S are the enzyme activities without and with the test sample, respectively. The ChE inhibitory activity of each sample was expressed in terms of the IC_{50} value (μg/mL required to inhibit the hydrolysis of the substrate, ACh or BCh, by 50%), as calculated from the log-dose inhibition curve.

Results and Discussion

The compounds (**1 - 9**) were isolated from the 95% EtOH extract of *C. fulvescens* using a combination of various column chromatographies. Based on the ^{1}H-, ^{13}C-NMR and MS data, together with their physical constants, the isolates were characterized as α-linolenic acid (**1**), *cis*-5,8,11,14,17-elcosapentaenoic acid (**2**), phytol (**3**), loliolide (**4**), uridine (**5**), thymidine (**6**), deoxyadenosine (**7**), adenine (**8**), and adenosine (**9**). All of these compounds were isolated for the first time from this plant. The anticholinesterase and antioxidant activities of **1 - 9** from *C. fulvescens* were evaluated in AChE and BChE inhibitory assays as well as in DPPH radical and $ONOO^-$ scavenging assays (Table 2). All of the compounds **1 - 9** exhibited weak inhibitory activities in the AChE assay, with respective IC_{50} values ranging from 114.91 to 252.40 μM, compared with berberine (IC_{50} = 0.23 μM). In the BChE assay, only **2 - 4** showed inhibitory activities, with respective IC_{50} values of 251.18, 268.44 and 499.16 μM, compared with berberine (IC_{50} = 11.97 μM). In the antioxidant assays, none of the compounds revealed scavenging activity for DPPH up to 400 μg/mL, as compared with L-ascorbic acid (IC_{50} = 0.33 μg/mL). Moreover, all of the compounds did not show $ONOO^-$ scavenging activity, as compared with L-penicillamine (IC_{50} = 0.35 μg/mL).

In conclusion, nine known compounds (**1 - 9**) were isolated for the first time from *C. fulvescens*. Compounds **1 - 9** exhibited AChE inhibitory activities with IC_{50} values ranging from 114.91 to 252.40 μM, whereas **2 - 4** showed BChE inhibitory activities with IC_{50} values ranging from 251.18 to 499.16 μM.

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