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Antioxidant and Cholinesterase Inhibitory Activities of Antarctic Krill *Eupausia superba*

Na Young Yoon[1]*, Chengliang Xie[2], Kil-Bo Shim[1], Yeon-Kye Kim[1], Doo-Seog Lee[1] and Ho-Dong Yoon[1]

[1]Food and Safety Research Division, National Fisheries Research and Development Institute, Busan 617-705, Korea
[2]Department of Food Science and Technology, Pusan National University, Busan 609-735, Korea

Abstract

The antioxidant and cholinesterase inhibitory activities of methanol, pretanol, and acetone extracts of *Eupausia superba* were investigated and their bioactivities compared. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid] ($ABTS^+$) radical-scavenging activities and reducing power assays were used to determine antioxidant activities, and Ellman's colorimetric methods were applied to evaluate cholinesterase inhibitory activity. Although all extracts were positive, Acetone extract of *E. superba* showed the highest activities. However, these showed moderate or no inhibitory activity against butyrylcholinesterase. Moreover, the total carotenoid contents of the organic solvent extracts followed the same order as their antioxidant and acetylcholinesterase inhibitory activities. These results suggest that *E. superba* is a potential source of natural antioxidants and cholinesterase inhibitors.

Key words: Krill, *Eupausia superba*, Antioxidant, Cholinesterase

Introduction

Alzheimer's disease (AD), the most common type of senior dementia, is characterized by the progressive degeneration of neurological function (Nie et al., 2009). The pathogenesis of AD is associated with a reduction in cholinergic neurotransmitter levels in the basal forebrain, resulting in memory loss and reduced cognitive ability (Felder et al., 2000). AD can be prevented by cholinergic agents that recover the cholinergic functions through the inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which hydrolyze neurotransmitters such as acetylcholine (ACh) and butyrylcholine (BCh) (Schneider, 2001).

Oxidative stress caused by free radicals and reactive oxygen species (ROS) contributes to oxidation of biomolecules and cellular damage (Zhu et al., 2004). Recently, oxidative stress was related to the pathological changes in AD (Praticò and Delanty, 2000). Interest in the discovery of natural antioxidants from marine sources is growing because such compounds prevent oxidative damage and neurodegenerative diseases (Fusco et al., 2007).

The Antarctic krill, *Eupausia superba* Dana, is a crustacean with a large biomass and it is a primary species in the Southern Ocean. It is a good source of protein and polyunsaturated fatty acids, such as eicosapentaenoate (C20:5) and docosahexaenoate (C22:6), and it has potential as a food source (Bottino, 1975; Phleger et al., 2002). Recently, interest in krill has increased due to developments in processing technology, including those in aquaculture feed and krill-based products for human consumption (Nicol et al., 2000; Smetacek and Nicol, 2005).

In this study, the antioxidant and cholinesterase (ChEs) inhibitory activities of *E. superba* solvent extracts were investigated *in vitro* by determining 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid] ($ABTS^+$) radical scavenging activities, reducing power,

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***Corresponding Author**
E-mail: dbssud@nfrdi.go.kr

and inhibition of AChE and BChE. In addition, the association of the total carotenoid content of extracts with the above-mentioned activities was evaluated.

Materials and Methods

Materials

E. superba (average total length of 3-4 cm) was obtained from Dong Won Co. (Busan, Korea) in May 2011. Astaxanthin, butylhydroxytoluene (BHT), L-ascorbic acid, DPPH, $ABTS^+$, trolox, potassium persulfate, potassium ferricyanide, trichloroacetic acid, ferric chloride, AChE, BChE, acetylthiocholine, butylthiocholine, 5,5'-dithiobis(2-nitribenzoic acid) [DTNB], and eserine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and methanol (MeOH), acetone, and pretanol (Pretanol-A, 95% alcohol) were obtained from Duksan Chemical Co. (Seoul, Korea). All other reagents were of the highest grade available.

Preparation of sample

Whole *E. superba* was freeze-dried and stored at -20°C until use. Lyophilized *E. superba* (10 g) was extracted three times with 50 mL MeOH, pretanol, or acetone.

DPPH radical-scavenging activity

The DPPH radical-scavenging activity was measured by modifying the method of Blois (1958). An aliquot (160 μL) of sample in MeOH was added to 40 μL of 0.15 mM DPPH solution. After mixing and leaving for 30 min at room temperature, the absorbance at 520 nm was measured using a spectrophotometer (Powerwave XS; BioTex, Inc., Houston, TX, USA). The DPPH radical-scavenging activity of each sample was expressed as an IC_{50} value, indicating the concentration required for scavenging 50% of the absorbance of the DPPH radical. L-Ascorbic acid was used as a positive control.

$ABTS^+$ radical-scavenging activity

$ABTS^+$ radical-scavenging activity was determined by modifying the method of Arnao et al. (2001). The stock solutions were 7.4 mM $ABTS^+$ and 2.6 mM potassium persulfate. The working solution was prepared by mixing the two stock solutions in equal quantities. The mixture was allowed to react for 12 h at room temperature in the dark, followed by dilution by mixing 1 mL $ABTS^+$ solution with 50 mL MeOH to obtain an absorbance at 734 nm of 1.10 ± 0.02, as determined using a spectrophotometer (BioMate 5; Thermo Electron, Waltham, MA, USA). Fresh $ABTS^+$ solution was prepared for each assay. Sample (150 μL) was mixed with 2.85 mL $ABTS^+$ solution and the mixture was left in the dark for 2 h. The absorbance at 734 nm was then measured using a spectrophotometer. A standard curve of trolox ranging from 9.4 to 37.5 μg/mL was prepared and the results were expressed as trolox equivalents per gram of extract.

Reducing power assay

Reducing power was evaluated by the method of Oyaizu (1986). Various sample concentrations (2.5 mL) were mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. After incubation at 50°C for 20 min, 2.5 mL of 10% trichloroacetic acid (w/v) was added. The mixture was then centrifuged at 2,000 *g* for 10 min, and 5 mL of the upper layer was mixed with deionized water and 1 mL of 0.1% ferric chloride. The absorbance at 700 nm was measured using a spectrophotometer (BioMate 5). L-Ascorbic acid was used as a positive control.

ChEs inhibitory activity assay

ChEs inhibition was measured using the spectrophotometric method of Ellman et al. (1961). The reaction mixture contained 140 μL of 100 mM sodium phosphate buffer (pH 8.0), 20 μL of sample, and 20 μL of either AChE (0.36 U/mL) or BChE (0.36 U/mL). The solution was placed in a 96-well microplate and mixed. After incubation at room temperature for 15 min, 10 μL of the DTNB solution and 10 μL of ACh or BCh, respectively, were added. The absorbance of all reactions was measured using a spectrophotometer (Powerwave XS). Eserine was used as a positive control.

Total carotenoid contents

A spectrophotometric method was used to evaluate the total carotenoid contents following the modified method of Tolasa et al. (2005). Astaxanthin standard (3.0 mg) and BHT (100 mg) were dissolved in 10 mL of dichloromethane. Subsequently, 1 mL of this stock solution was diluted to 10 mL with *n*-hexane, and the absorbance was measured in a UV-visible spectrophotometer (BioMate 5) at a wavelength between 350 and 600 nm. The maximum absorbance was observed at 472 nm and the concentration of astaxanthin in the solution was measured and corrected according to the following formula:

$$C_{astaxanthin}\ (\mu g/mL) = A \times 10{,}000/E,$$

where $C_{astaxanthin}$ is the total carotenoid content, A is the absorbance at 472 nm, E = 2100 is the extinction coefficient, and 10,000 is the scale factor.

To prepare the standard curve, 0.1, 0.25, 0.50, 0.75, 1.0, 1.25, and 1.5 mL of stock solution were placed in separate 10 mL flasks using a solvent dispensing pipette and made up to the appropriate volume with *n*-hexane. The absorbance at 472 nm was measured using *n*-hexane as the blank. The standard

curve was prepared in triplicate under yellow light and low temperature.

Results and Discussion

Antioxidant activity

Oxidative stress is associated with age-related neurodegenerative diseases (Mount and Downton, 2006). ROS oxidize and damage nucleic acids, lipids, and proteins. These reactions contribute to brain aging and age-associated neurodegenerative diseases such as AD, likely because of the imbalance between antioxidant defenses and intracellular generation of ROS. Antioxidants play a crucial role in reducing unsaturated fatty acid oxidation in the brain and in preventing the neuronal death associated with the pathology of neurodegenerative disorders (Ramassamy, 2006; Kamatou et al., 2008).

DPPH and $ABTS^+$ radical-scavenging activities and reducing power were used to determine *in vitro* antioxidant activities of *E. superba* organic solvent extracts (Tables 1 and 2). As shown in Table 1, the *E. superba* extracts exhibited potent DPPH and $ABTS^+$ radical-scavenging activities. DPPH radical-scavenging activities were acetone ex. (IC_{50} = 1.16 ± 0.02 mg/mL) > MeOH ex. (IC_{50} = 1.24 ± 0.02 mg/mL) > pretanol ex. (IC_{50} = 1.45 ± 0.04 mg/mL). $ABTS^+$ radical-scavenging activities of the extracts were acetone ex. (158.9 ± 9.628 mg trolox eq/g extract) > MeOH ex. (153.8 ± 10.92 mg trolox eq/g extract) > pretanol ex. (113.6 ± 11.94 mg trolox eq/g extract). As summarized in Table 2, the reducing power of *E. superba* extracts increased in a dose-dependent manner. The order of the absorbance for the extracts at a concentration of 2.3 mg/mL was acetone ex. (0.35 ± 0.05) > MeOH ex. (0.18 ± 0.02) > pretanol ex. (0.17 ± 0.03). The acetone extract showed the most potent radical-scavenging activities and reducing power. However, the DPPH radical-scavenging activity and reducing power of *E. superba* extracts were lower than those of the L-ascorbic acid used as a positive control.

ChEs inhibitory activities

AChE, a substrate-specific enzyme, exists in nerve synapses and catalyzes the cleavage of ACh in the synaptic cleft, which plays an important role in the initial stage of AD. BChE is a less-specific enzyme located in plasma and tissues, and lingers as the major ChE in the late-stage AD brain (Ballard et al., 2005; Silman and Sussman, 2005). Thus, inhibition of ChEs shows promise as an anti-AD therapy, and it has been shown to reverse the reduced cognition and behavioral functions associated with AD in clinical studies (Giacobini, 2004).

The ChEs inhibitory activity of *E. superba* extracts was evaluated by AChE and BChE inhibition assays (Table 3). The MeOH and pretanol extracts of *E. superba* exhibited selective AChE inhibitory activities (IC_{50} = 0.13 ± 0.00 mg/mL and 0.13 ± 0.00 mg/mL, respectively), whereas the acetone extract inhibited both enzymes, with IC_{50} values of 0.11 ± 0.00 mg/mL

Table 1. DPPH and $ABTS^+$ radical-scavenging activities of the extracts of *Eupausia superba*

Samples	DPPH (IC_{50}, mg/mL)	$ABTS^+$ (mg trolox eq/g extract)
MeOH ex.	1.24 ± 0.02	153.8 ± 10.92
Pretanol ex.	1.45 ± 0.04	113.6 ± 11.94
Acetone ex.	1.16 ± 0.02	158.9 ± 9.628
L-Ascorbic acid	0.002 ± 0.000	

The values of DPPH and $ABTS^+$ radical-scavenging activities were expressed as the means ± SD of three experiments. L-Ascorbic acid was used as a positive control of DPPH radical-scavenging activity assay, respectively.

Table 3. Cholinesterase inhibitory activity of the extracts of *Eupausia superba*

Samples	AChE (IC_{50}, mg/mL)	BChE (IC_{50}, mg/mL)
MeOH ex.	0.13 ± 0.00	>0.50
Pretanol ex.	0.13 ± 0.00	>0.50
Acetone ex.	0.11 ± 0.00	0.32 ± 0.03
Eserine	0.00003 ± 0.00000	0.00006 ± 0.00000

The values were expressed as the mean ± SD of three experiments. Eserine was used as a positive control.
AChE, acetylcholinesterase; BChE, butyrylcholinesterase.

Table 2. The reducing power of the extracts of *Eupausia superba*

Samples (μg/mL)	Reducing power					
	90	450	2300	0.025	0.05	0.1
MeOH ex.	0.01 ± 0.00	0.05 ± 0.00	0.18 ± 0.02			
Pretanol ex.	0.01 ± 0.00	0.05 ± 0.01	0.17 ± 0.03			
Acetone ex.	0.03 ± 0.00	0.11 ± 0.01	0.35 ± 0.05			
L-Ascorbic acid				0.25 ± 0.01	0.47 ± 0.01	0.96 ± 0.03

The absorbance of reducing power was expressed as the means ± SD of three experiments. L-Ascorbic acid was used as a positive control of reducing power assay.

Table 4. Total carotenoid contents of the extracts of *Eupausia superba*

Samples	Total carotenoid contents (mg/g)
MeOH ex.	1.21 ± 0.05
Pretanol ex.	0.64 ± 0.02
Acetone ex.	1.96 ± 0.03

and 0.32 ± 0.03 mg/mL, respectively, for AChE and BChE.

This selective AChE inhibitory activity may be due to the characteristics of enzyme-substrate binding (Silman and Sussman, 2005). As with antioxidant activities, the ChEs inhibitory activity of *E. superba* extracts was lower than that of eserine, which was used as a positive control.

Total carotenoid contents

The carotenoids, a class of hydrocarbons with cyclic or acyclic end groups, exist as a pigment in crustaceans and exert biological effects such as antioxidant activity and prevention of cardiovascular disease and cancer (Britton, 1995; Kohlmeier and Hastings, 1995; Stahl et al., 1998; Fraser and Bramley, 2004). The total carotenoid content of the *E. superba* extracts is shown in Table 4. The highest total carotenoid content was identified in the acetone ex. (1.96 ± 0.03 mg/g), followed by the MeOH ex. (1.21 ± 0.05 mg/g) and pretanol ex. (0.64 ± 0.02 mg/g).

Thus, the order of total carotenoid content was similar to those of the antioxidant and ChEs inhibitory activities. Thus, these activities may be attributable to carotenoids. More detailed investigations are necessary to isolate and identify the active ingredients from extracts and to clarify their mechanism of action.

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