Antioxidant activities of crude and fractionated extracts from the ascidian *Styela clava*

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***Abstract*—The antioxidant activities of crude and fractionated extracts from the ascidian *Styela clava* were determined using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) system, reducing power assay and β-carotene method. Results indicated that the viscera extract showed the highest activities among three different crude extracts obtained from *S. clava*. In addition，the ethyl acetate-soluble fraction isolated from viscera extract exhibited obvious antioxidant activities, which EC_{50} were 54.91 and 59.86 μg/mL on DPPH radicals scavenging effect and β-carotene bleaching assay, respectively. Moreover, its reducing power was 152.0 mg/g dry weight of AscAE.**

Keywords- Styela clava; Ascidian; Antioxidant activity

I. INTRODUCTION

Oxidative stress generated by reactive oxygen species (ROS), such as hydroxyl radicals and hydrogen peroxide, is thought to be implicated in pathological conditions such as inflammation and cancer, as well as neurodegenerative disorders [1]. Antioxidants have been widely used as food additives to provide protection against the attack of ROS and delaying the progress of certain chronic diseases [2]. Several synthetic antioxidants such as tert-butylated hydroxyquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commercially available and widely used. However, these antioxidants have been restricted for use in foods as they are suspected to be carcinogenic [3, 4]. For this reason, governmental authorities and consumers are concerned about the safety of the food and also about the potential harmful effects of synthetic additives on health. Recently there has been a growing interest in the search for natural antioxidants.

Much attention has been focused on the use of marine natural antioxidants because of the increasing research on marine natural products [5]. Marine animals such as sea cucumber and jellyfish are well recognized as resources of natural antioxidants [6-8]. The solitary ascidain *Styela clava* is a dominant member of the benthic community in Bohai Sea and Western Yellow Sea. Until recently, there is some lack of knowledge about the antioxidant activity of *Styela clava*. In this paper, antioxidant activities of extracts from body, tunica and viscera of *Styela clava*, as well as the fractions derived from viscera were investigated. Furthermore, the reducing power of all samples was also determined. This research also provides information on potential application of *S. clava* as a food additive or as a health-promoting commodity.

II. MATERIALS AND METHODS

A. Materials

Styela clava was collected from the inertial zone of Yantai, Shandong Province, People's Republic of China, in August, 2008.

Linoleic acid, β-carotene, gallic acid (GA), butylated hydroxytoluene (BHT), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), and Tween-40 (polyoxyethylene sorbitan monopalmitate) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ascorbic acid (AscA) was purchased from Shanghai Chemical Reagents Co. (Shanghai, China). Other chemicals were all analytical grade.

B. Sample preparation

The specimens were divided into three parts: body, tunica and viscera before being lyophilized and powdered. Each sample was extracted with methanol: chloroform (2:1) in a Soxhlet extractor for 6 h. The extraction was repeated twice. The combined organic solution was evaporated under vacuum at 40 °C to dryness. The crude extracts obtained from three samples were used directly for antioxidant analysis.

The crude extract of viscera which showed the highest activities in the subsequent antioxidant capacity assays was then sequentially partitioned with petroleum ether, ethyl acetate, and n-butanol, respectively. Four resulting fractions were evaporated to dryness in vacuum, to yield the petroleum ether- (PER), ethyl acetate- (ETH), n-butanol-soluble (BUT) fractions, and aqueous residue (AQU), respectively.

C. DPPH scavenging assay

The scavenging effects of samples for DPPH radical were determined according to reference [9] with a slight modification. Sample (1.5 ml) in different concentration was added to 1.5 ml of 0.2 mM DPPH in ethanol. The mixture was

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mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm. The ability to scavenge the DPPH radical was calculated using the follow equation:

Scavenging effect (%) = [1- (A_{sample} - $A_{sample\ blank}$)/ $A_{control}$] × 100

Where $A_{control}$ is the absorbance of the control (DPPH solution without sample), A_{sample} is the absorbance of the test sample (DPPH solution plus test sample), and $A_{sample\ blank}$ is the absorbance of the sample only (sample without DPPH solution). BHT was used as positive control.

D. β-Carotene-Linoleate Model System

The antioxidant activities of samples in a β-carotene-linoleate model system were measured according to reference [10] with minor modification. Briefly, 0.5 mL of β-carotene (1 mg/mL) dissolved in chloroform was pipetted into a 50 mL round-bottom flask. After chloroform was removed under vacuum at room temperature, 20 mg linoleic acid, 200 mg Tween 40 emulsifier, and 50 mL aerated distilled water were added to the flask with vigorous agitation to form an emulsion. Emulsion was freshly prepared for each experiment. A 96-well microtitre plate (polystyrene) was charged with 50 µL test sample and 200 µL the emulsion, and the absorbance was measured at 450 nm, immediately, against a blank consisting of the emulsion without β-carotene. The plate was stood at room temperature, and the absorbance measurements were conducted again at 20 min intervals up to 320 min. All determinations were carried out in triplicate. The antioxidant activity (AA) of the extracts was evaluated in terms of bleaching of β-carotene using the following formula:

$AA = [1- (A_0 - A_t)/ (A'_0 - A'_t)] \times 100$

Where A_0 and A'_0 are the absorbance of values measured at zero time of the sample and the control, respectively, and A_t and A'_t are the absorbencies measured in the test sample and the control, respectively.

E. Reducing Power Activity

The reducing power of the test samples was determined according to reference [11]. Generally, 1 mL of each sample dissolved in methanol was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixtures were incubated for 20 min at 50 °C. At the end of the incubation, 2.5 mL of 10% trichloroacetic acid was added to the mixtures, followed by centrifugation at 650 × g for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride and the absorbance was measured at 700 nm. The reducing power tests were run in triplicate. The data were presented as ascorbic acid equivalent (AscAE) in milligrammes of ascorbic acid per gramme of sample. A greater value of the AscAE related to greater reducing power of the sample.

F. Statistical analysis

Values expressed are means of three replicate determinations ± standard deviation. All statistical analyses were carried out using SPSS 11.01 for Windows. Significant differences between means were calculated by one-way analysis of variance (ANOVA). Differences at $P < 0.05$ (95% confidence level) were considered to be significant.

III. RESULTS AND DISCUSSION

The crude extract derived from viscera had the highest yield (24.51% dry weight), followed by the crude extract obtained from body (15.23% dry weight) and tunica (3.57% dry weight). The three different extracts were then tested for the antioxidant activity by various assays.

A. DPPH scavenging assay

The DPPH free radicals scavenging method is a colorimetric assay and can be used to evaluate in a short time [12]. As can be seen in Table 1, the viscera extract showed the highest activity among the three different crude extracts obtained from *S. clava*, followed by body and tunica extracts. The activity of viscera extract was observed to be similar to that of the positive control, BHT, at all concentration levels.

Table 2 shows the DPPH radical-scavenging activities of different fractions derived from the crude extract of viscera. Among the four different polarity fractions, the petroleum ether (PER)- and n-butanol-soluble (BUT) fractions, as well as the aqueous residue (AQU), revealed no or only moderate activities in this assay system, while the ethyl acetate-soluble (ETH) fraction showed the highest activity and indicated that compounds with strongest radical-scavenging activity in viscera of *S. clava* were of medium polarity. The activity of ethyl acetate-soluble fraction (EC_{50}=54.91 µg/mL) was observed to be significantly higher than that of BHT (EC_{50}=75.42 µg/mL) at all concentration levels ($P < 0.05$).

B. Antioxidant Activity with β-Carotene-Linoleate Assay System

The mechanism in β-carotene assay is a free-radical-mediated phenomenon resulting from the hydroperoxides

Table 1. DPPH radical-scavenging activity of different crude extracts obtained from *S. clava*

Crude extract	Antioxidant activity ± SD (%)			
	50 (µg/mL)	100 (µg/mL)	150 (µg/mL)	200 (µg/mL)
Body	19.2 ± 0.37b	30.12 ± 0.60b	37.9 ± 0.64b	42.6 ± 0.88b
Viscera	36.32 ± 0.27c	58. 1 ± 0.97c	70.8 ± 0.72c	81.6 ± 0.12c
Tunica	4.6 ± 0.08a	10.23 ± 0.12a	15.1 ± 0.32a	20.5 ± 0.14a
BHT	39.45 ± 0.78c	60.2 ± 0.20c	72.3 ± 0.46c	85.21 ± 0.42c

Each value is presented as mean ± SD (n=3). Means with each column with different letters (a-c) differ significantly ($P < 0.05$)

Table 2. DPPH radical-scavenging activity of different fractions derived from the crude extract of viscera

Fraction	Antioxidant activity ± SD (%)			
	50 (µg/mL)	100 (µg/mL)	150 (µg/mL)	200 (µg/mL)
Viscera	36.32 ± 0.27c	58.1 ± 0.97d	70.8 ± 0.72d	81.6 ± 0.12d
PER	5.3 ± 0.02a	14.4 ± 0.22b	22.32 ± 0.44b	30.15 ± 0.36b
ETH	47.8 ± 0.56d	70.2 ± 0.44e	85.11 ± 0.72e	93.1 ± 0.62e
BUT	15.0 ± 0.31b	32.62 ± 0.24c	46.79 ± 0.38c	60.5 ± 1.21c
AQU	2.1 ± 0.04a	3.25 ± 0.05a	4.38 ± 0.08a	4.99 ± 0.04a
BHT	39.45 ± 0.78c	60.2 ± 0.20d	72.3 ± 0.46d	85.21 ± 0.42d

Each value is presented as mean ± SD (n=3). Means with each column with different letters (a-e) differ significantly (P <0.05)

Table 3. Antioxidant activity of different crude extracts from *S. clava* in the β-carotene-linoleate assay system

Crude extract	Antioxidant activity ± SD (%)			
	10 (μg/mL)	50 (μg/mL)	100 (μg/mL)	200 (μg/mL)
Body	12.0 ± 0.21b	20.23 ± 0.46b	38.6 ± 0.12c	50.2 ± 0.41c
Viscera	20.2 ± 0.22c	42.6 ± 0.52c	61.2 ± 0.38d	73.7 ± 0.54d
Tunica	6.5 ± 0.17a	10.38 ± 0.76a	17.4 ± 0.88a	25.59 ± 0.51a
GA	21.8 ± 0.46c	42.8 ± 0.65c	60.6 ± 1.12d	71.1 ± 0.64d
AscA	10.5 ± 0.12ab	20.7 ± 0.31b	32.9 ± 0.18b	46.6 ± 0.32b

Each value is presented as mean ± SD (n=3). Means with each column with different letters (a-d) differ significantly (P <0.05)

Table 4. Antioxidant activity of different fractions derived from the crude extract of viscera in the β-carotene-linoleate assay system

Fraction	Antioxidant activity ± SD (%)			
	10 (μg/mL)	50 (μg/mL)	100 (μg/mL)	200 (μg/mL)
Viscera	20.2 ± 0.22c	42.6 ± 0.52d	61.2 ± 0.38d	73.7 ± 0.54e
PER	6.22 ± 0.12ab	14.8 ± 0.25b	22.0 ± 0.43b	31.9 ± 0.38b
ETH	27.3 ± 0.64d	45.7 ± 0.21e	67.5 ± 0.55e	80.8 ± 0.60f
BUT	9.5 ± 0.31b	18.4 ± 0.56bc	30.7 ± 0.41c	40.18 ± 0.30c
AQU	2.54 ± 0.05a	4.18 ± 0.08a	6.9 ± 0.13a	8.38 ± 0.17a
GA	21.8 ± 0.46c	42.8 ± 0.65d	60.6 ± 1.12d	71.1 ± 0.64e
AscA	10.5 ± 0.12b	20.7 ± 0.31c	32.9 ± 0.18c	46.6 ± 0.32d

Each value is presented as mean ± SD (n=3). Means with each column with different letters (a-f) differ significantly (P < 0.05)

formed from linoleic acid. In the absence of antioxidants, the β-carotene molecules lose their double bonds by oxidation as well as the characteristic orange color, which can be monitored spectrophotometrically. The presence of different antioxidants can hinder the extent of β-carotene-bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system [10]. As shown in Table 3, the viscera extract also showed the highest activity among the three different crude extracts obtained from *S. clava*, followed by body and tunica extracts. The viscera extract exhibited higher antioxidant activity than AscA at all concentration levels. In addition, at concentrations of 100 and 200 μg/mL, the antioxidant activity of viscera extract was higher than that of GA.

The antioxidant activities of different fractions derived from the crude extract of viscera were shown in Table 4. Again, among the four different fractions as well as positive controls (GA and AscA), the ethyl acetate-soluble fraction presented the highest activity, which indicated that compounds with strongest antioxidant activity in the β-carotene-linoleate assay system were also of medium polarity. Compared to GA (EC_{50}=70.22 μg/mL), the ethyl acetate-soluble fraction displayed lower EC_{50} value (59.86 μg/mL).

C. Reducing Power Activity

The antioxidant activity has been reported to be concomitant with the reducing power [13]. As shown in Table 5, the viscera extract exhibited a significantly (P < 0.05) better reducing ability than the other crude extracts. Among the four different fractions, the ethyl acetate-soluble fraction appeared to possess the highest reducing activity, followed by n-Butanol fraction, the same order as the antioxidant activities in both assay systems.

Table5. Reducing power of various crude extracts and fractions

Sample	AscA ± SD
Body	45.2 ± 0.91d
Viscera	121.6 ± 0.72e
Tunica	20.2 ± 0.46b
Petroleum ether fraction (PER)	32.8 ± 0.65c
Ethyl acetate fraction (ETH)	152.0 ± 1.12f
n-Butanol fraction (BUT)	50.7 ± 1.01d
Aqueous residue (AQU)	12.5 ± 0.26a

Reducing power is expressed as ascorbic acid equivalents (AscAE; mg/g dry weight of AscAE). Each value is presented as mean ± SD (n=3). Means within each column with different letters (a-f) differ significantly (P < 0.05).

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REFERENCES

[1] M. H. Gordon, "Dietary antioxidants in disease prevention," Nat. Prod. Red. vol. 13, pp. 265–273, 1996.

[2] C. A. Hamilton, W. H. Miller, S. A. Benna, M. J. Brosnan, R. D. Drummond, M. W. Mcbride and A. F. Dominiczak, "Strategies to reduce oxidative stress in cardiovascular disease," Clin. Sci. vol. 106, pp. 219–234, 2004.

[3] N. Ito, M. Hirose, S. Fukushima, H. Tsuda, T. Shirai and M. Tatematsu, "Studies on antioxidants: Their carcinogenic and modifying effects on chemical carcinogenesis," Food Chem. Toxicol. vol. 24, pp. 1071–1082, 1986.

[4] L. W. Wattenberg, "Protective effects of 2(3)-tert-butyl-4-hydroxyanisole on chemical carcinogenesis," Food Chem. Toxicol. vol. 24, pp. 1099–1102, 1986.

[5] T. Satoshi, W. H. Tyler, R. Ira, H. G. William, T. H. Mark and G. N. Dale, "Marine natural products as novel antioxidant prototypes," J. Nat. Prod. vol. 66, pp. 605–608, 2003.

[6] Y. Zhong, M. A. Khan and F. Shahidi, "Compositional characteristics and antioxidant properties of fresh and processed sea cucumber (*Cucumaria frondosa*)," J. Agric. Food Chem. vol. 55, pp. 1188–1192, 2007.

[7] J. Mamelona, É. Pelletier, K. Girard-Lalancette, J. Legault, S. Karboune and S. Kermasha, "Quantification of phenolic contents and antioxidant capacity of Atlantic sea cucumber, *Cucumaria frondosa*," Food Chem. vol. 104, pp. 1040–1047, 2007.

[8] Z. B. Morais, A. M. Pintão, I. M. Costa, M. T. Calejo, N. M. Bandarra and P. Abreu, "Composition and In Vitro Antioxidant Effects of Jellyfish *Catostylus tagi* from Sado estuary (SW Portugal)," J. Aquat. Food Prod. Technol. vol. 18, pp. 90–107, 2009.

[9] W. Binsan, S. Benjakul, W. Visessanguan, S. Roytrakul, M. Tanaka and H. Kishimura, "Antioxidative activity of Mungoong, an extract paste, from the cephalothorax of white shrimp (*Litopenaeus vannamei*)," Food Chem. vol. 106, pp. 185–193, 2008.

[10] G. K. Jayaprakasha, R. P. Singh and K. K. Sakariah, "Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models in vitro," Food Chem. vol. 73, pp. 285–290, 2001.

[11] G. C. Yen and P. D. Duh, "Antioxidative properties of methanolic extracts from Peanut Hulls," J. Am. Oil Chem. Soc. vol. 70, pp. 383–386, 1993.

[12] K. S. Sim, A. M. Sri Nurestri and A. W. Norhanom, “Phenolic content and antioxidant activity of crude and fractionated extracts of *Pereskia bleo* (Kunth) DC. (Cactaceae),” Afr. J. Pharm Pharmacol. vol. 4, pp. 193–201, 2010.

[13] M. Tanaka, C. W. Kuie, Y. Nagashima and T. Taguchi, “Application of antioxidative maillard reaction products from histidine and glucose to sardine products,” Nippon Suisan Gakkaishi. vol. 54, pp. 1409–1414, 1988.