DPPH AND HYDROXYL RADICAL SCAVENGING ACTIVITY EFFECT OF WILD AND FARMING ABALONE (*HALIOTIS DISCUS HANNAI*)

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ABSTRACT

The 1- diphenyl 2-picrylhyorazyl (DPPH) and hydroxyl radical scavenging activities from ethanol extracts of wild and farming abalones, *Haliotis discus hannai* were evaluated. DPPH scavenging activity was analyzed according to the method of Brand-Williams et al. Hydroxyl radical (OH) scavenging activity was measured by the Fenton reaction. DPPH scavenging activity of shell muscle of wild abalone evaluated at 1.0 mg/ml was 48.8% and that of gut was 56.1% at same concentration, and that of gonad was 63.7%. Form abalone were also observed that inhibition percentage values go on increasing with enhancements in concentration of research abalone extracts in the assay mixture. The all values of DPPH scavenging activity of wild abalones were higher than those of farming abalones. However, the all did not show a statistically significant difference ($p < 0.05$). OH scavenging activity of shell muscle of wild abalone was evaluated at 1.0 mg/ml was 47.2%, that of gut was 43.7% at same concentration, and gonad was 56.2%. The all values of OH scavenging activity of farming abalone were lower than those of wild abalone. However, the all groups did not showed a statistically significant difference ($p < 0.05$). A significant linear correlation (Correlation co-efficient r = 0.956, 95% confidence interval 0.114 - 0.129. Co-efficient of determination (r^2) = 0.914, $p < 0.01$) was established between DPPH and corresponding OH radical activity of extracts of abalone tissues.

Keywords: 1, 1- diphenyl 2-picrylhyorazyl (DPPH), *Haliotis discus hannai*, Hydroxyl radical (OH).

INTRODUCTION

Antioxidants are widely used in food preservation and healthcare practices, as they are capable of eliminating reactive oxygen species (ROS), counteracting the damaging effects of oxidation, and prolonging the preservation time of foods (Wang et al., 2014). There is great number of methods for determination of antioxidant capacity of foods and beverages based on different principles. DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol (Huang et al., 2005). This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to colorless ethanol solution. DPPH is very popular for the study of natural antioxidants (Villano et al., 2007). The use of the DPPH assay provides an easy and rapid way to evaluate antioxidants by spectrophotometry (Huang et al., 2005), so it can be useful to assess various products at a time.

The requirement of hydrogen peroxide in the Fenton reaction led to the misleading concept of oxidative stress that ignores the fact that hydroxyl radical (OH), known to be the most biologically active free radical, is formed in vivo under hypoxic conditions (Michiels, 2004).

Moreover, this free radical can be generated in vitro under the reducing condition in the presence of ascorbic acid and iron ions (Lipinski, 2011).

The Pacific abalone, *Haliotis discus hannai* Ino, which is mainly distributed in East Asia (Marchant et al., 2009), is the most commercially important species of gastropods in aqua-culture (Chen et al., 2005). The principal abalone farming regions are China, Taiwan, Japan, and Korea. Abalone is also farmed in Australia, Canada, Chile, France, Iceland, Ireland, Mexico, Namibia, New Zealand, South Africa, Spain, Thailand, and the United States (Campbell, 2000). At the 9th International Abalone Symposium, held in Korea in October 2015, representatives of various countries were invited to present data on abalone production in their region. The most significant change has been in China where the majority of the world's abalone are now produced (Cook, 2016). Farm production in most countries, over this period, has either been stable or grown very slowly, production in China and Korea has increased very rapidly. Over the past decade or so, Korea has become an important supplier of abalone to the world market. Before 2000, only small quantities of abalone were farmed, and the production method mainly used suspended baskets in land-based farms. Like China, production in Korea has evolved to a more efficient methodology, utilizing off-shore cage farms (Park & Kim, 2013). Today those China, Japan, and the Republic of Korea are the largest consumers of abalone as food. In the Republic of Korea, abalones are harvested or cultivated for food. The purpose of the present study is to evaluate wild and farming abalone extracts as sources of antioxidants for DPPH and OH radical to examine whether the extractions of abalone are not losing significant DPPH and OH activity during farming abalone.

METHODOLOGY

Sample extract

The wild abalones were obtained from a sea lady in the sea off at Tongyeong in Korea. Farming abalones were bought from three different supermarkets around Jindo Island in Korea. The sample was ground using a high-speed blender (HC-BL5000, Korea). Blanched samples were blended, and the juices were squeezed out, prior to analysis.

They were squeezed out with the muslin cloth and was put in 500 mL beaker. The samples were blended with 80% ethanol, and then an aliquot of the mixture (100 µL, 200 mg sample / ml 80% ethanol) was further mixed with 100 mM Tris-HCl buffer (400 µL, pH 7.4).The mixture was further stirred with a magnetic bar at 65°C for 12 hours. The sample was treated with ultrasound at room temperature for a given duration. The ultrasound extraction was carried out using an ultrasonic bath (5510, Branson, USA). The mixture was shaken vigorously for one hour at room temperature and left in the dark at room temperature for 20 min. Extracted sample was filtered. The sample was evaporated to remove solvent under reduced pressure and controlled temperature by using rotary vacuum evaporator (N-1001S-W, Eyela, Tokyo, Japan). To get dry powder, samples placed in a low temperature vacuum chamber.

DPPH free radical

The great diversity of methods and modifications is evident from its different names. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical. The antioxidant activity of the seaweed extracts was measured based on the scavenging activity of DPPH free radical according to the method described by Brand-Williams et al. (1995) with slight modifications. DPPH free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol (Cornish & Garbary, 2010). 1 ml of 0.1 mM DPPH solution in

ethanol was mixed with 1 ml of the previous algae extracts of various concentrations (0.1, 0.5, and 1.0 mg/ml). DPPH was added to the solutions prepared with sample extracts and standard antioxidant substances and stirred. A solution of DPPH was prepared by dissolving 5 mg DPPH in 2 ml of ethanol, and the solution was kept in the dark at 4°C. A stock solution of the compounds was prepared at 1 mg/ml in DMSO. The stock solution was diluted to varying concentrations in 96-well microplates. Then, 5 µL of ethanol DPPH solution (final concentration 300 µM) was added to each well. The plate was shaken to ensure thorough mixing before being wrapped with aluminum foil and placed into the dark. The radical scavenging reaction was carried out at 37 ºC in dark for 30 min. The optical density (OD) of the solution was read using the Microplate Reader (VersaMax, Califonia, USA) at the wavelength 515 nm. Corresponding blank sample was prepared and L-Ascorbic acid (1.0 µg/ml) was used as reference standard (positive control). Inhibition of free radical scavenging activity was calculated using the following equation.

Inhibition (%) = 100 × (absorbance of the control - absorbance of the sample)/ absorbance of the control. The ability of the extracts to scavenge at 50% of the DPPH, EC50 was determined from the graph plotted in GraphPad Prism software. All experiments were carried out in triplicate.

Hydroxyl radical assay

Hydroxyl radical (OH) scavenging activity was measured by the Fenton reaction. The deoxyribose method for determining the scavenging effect of the abalone extracts against hydroxyl radicals was performed according to a described procedure (Payá et al. 1992). Reaction mixtures contained, in a final volume of 1 ml, ascorbic acid (50 µM), $FeCl_3$ (20 µM), EDTA (100 µM), H_2O_2 (1.42 mM), deoxyribose (2.8 mM) and abalone extracts (0.1, 0.5, and 1.0 mg/ml). All components were dissolved in KH_2PO_4-KOH buffer 10 mM, pH 7.4. After incubation at 37 ºC for 1 h, 1 ml of 2.8% trichloroacetic acid (w/v) and 1 ml of 1% thiobarbituric acid (TBA) (w/v) were added and the mixture was heated in a water bath at 100 ºC for 15 min. The absorbance of the resulting solution was measured at 530 nm with Microplate Reader). This assay was also performed without ascorbic acid or EDTA, in order to check for pro-oxidant or metal chelation activities (Valentão et al.2003). The percent inhibition was calculated as the decolourization percentage of the test sample using the following formula:

Inhibition (%) = (IA-As)/IA×100

Where IA is the absorbance of the 100% initial and As is the absorbance of the sample. IA and As were the values which were subtracted the average absorbance of the blank wells.

EC_{50} is defined as the concentration of inhibitor necessary for 50% inhibition of the enzyme reaction of a maximum scavenging capacity. To determine the EC_{50} value of the active component, the technique using 96-well microplates was employed (Zubia et al., 2009). Regression analysis by a dose response curve was plotted to determine the EC_{50} values.

Statistical Analyses

The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The results were expressed as the mean ±SD. One-way analysis of variance was applied to determine differences in means. A P value <0.05 denoted statistical significance. Correlation co-efficient (R) to determine the relationship between two or more variables among Radical Scavenging activity tests were calculated using the SPSS software (Release 21.0).

RESULTS

Table 1 was shown the antioxidant activities for DPPH radical of the abalone. It was observed that inhibition percentage values go on increasing with enhancements in concentration of research plant extracts in the assay mixture. DPPH scavenging activity of shell muscle of wild abalone evaluated at 0.1 mg/ml was 4.3% and that of gut was 7.0% at same concentration, and that of gonad was 7.6%. The rates of antioxidant activities of the ethanol extracts for abalone were dependent on concentrations. DPPH scavenging activity of shell muscle of wild abalone evaluated at 1.0 mg/ml was 48.8% and that of gut was 56.1% at same concentration, and that of gonad was 63.7%. Form abalone were also observed that inhibition percentage values go on increasing with enhancements in concentration of research abalone extracts in the assay mixture. The high antioxidant activity for DPPH found on gonad extracts. The all values of DPPH scavenging activity of wild abalone were higher than those of Farming abalone. However, the all did not show a statistically significant difference ($p < 0.05$).

Hydroxyl radical (OH) scavenging activity was measured by the Fenton reaction. The results of the OH test of the alcoholic and ethanol extracts of abalone in comparison with the standard (H_2O_2) at 560 nm were shown in Table 2. The rates of antioxidant activities of the ethanol extracts for abalone were dependent on concentrations. Various concentrations of wild tissue extracts were higher than those of Farming abalone extracts. OH scavenging activity of shell muscle of wild abalone was evaluated at 0.1 mg/ml was 2.9%, that of gut was 5.8% at same concentration, and gonad was 5.5%. OH scavenging activity of shell muscle of wild abalone was evaluated at 1.0 mg/ml was 47.2%, that of gut was 43.7% at same concentration, and gonad was 56.2%. The all values of OH scavenging activity of farming abalone were lower than those of wild abalone. However, the all groups did not show a statistically significant difference ($p < 0.05$).

Figure 1 was shown the comparative data of DPPH contents, DPPH radical scavenging activity and OH as determined by the EC_{50} values of the different tissues. An EC_{50} value is the concentration of the sample required to scavenge 50% of the free radicals present in the system. EC_{50} value was inversely related to the antioxidant activity of crude extracts. The total DPPH contents activity of shell muscle (EC_{50} = 121.7 µg/ml for wild abalone and 130.6 µg/ml for Farming abalone) was at the same levels as that of L-Ascorbic acid. The values of EC_{50} for wild and Farming gut were 82.7 µg/ml and 88.9 µg/ml, respectively. Those of wild and Farming gonad were 72.7 µg/ml and 75.5 µg/ml, respectively. The OH activity of shell muscle (EC_{50} = 130.8 µg/ml for wild abalone and 134.9 µg/ml for Farming abalone) was at the same levels as that of H_2O_2 and EC_{50} of gut of wild abalone was 88.7 µg/ml, and that of gonad of wild abalone was 77.3 μg/ml.

A significant linear correlation (Correlation co-efficient r = 0.956, 95% confidence interval 0.114 - 0.129. Co-efficient of determination (r^2) = 0.914, p < 0.01) was established between DPPH and corresponding OH radical activity of extracts of abalone tissues (Fig. 3).

Table 1. The assay of DPPH by *Haliotis discus hannai* at different concentrations

Type	Concentration (mg/ml)	Shell muscle	Gut	Gonad
Wild	0.1	4.26±2.23	6.97±1.36	7.55±0.77
	0.5	14.62±0.98	22.41±1.26	28.34±4.41
	1.0	48.82±1.05	56.09±0.79	63.69±3.03
Farming	0.1	2.83±0.47	5.96±1.30	5.88±1.53
	0.5	13.63±1.16	21.10±0.96	25.97±3.35
	1.0	48.86±1.33	54.36±2.40	61.85±3.20
t-test		0.041	0.066	0.084

Data represent the mean ± SD from three replicates.

Table 2. The hydroxyl radical (OH) by *Haliotis discus hannai* at different concentrations

Type	Concentration (mg/ml)	Shell muscle	Gut	Gonad
Wild	0.1	2.89±0.51	5.81±1.11	5.51±0.46
	0.5	13.10±1.15	21.27±1.03	27.64±3.20
	1.0	47.24±3.21	43.73±2.02	56.21±1.33
Farming	0.1	2.22±1.02	5.18±1.06	4.38±3.68
	0.5	12.19±2.07	20.15±1.03	26.09±3.56
	1.0	46.92±1.89	41.17±3.65	55.22±2.23
t-test		0.033	0.097	0.059

Data represent the mean ± SD from three replicates.

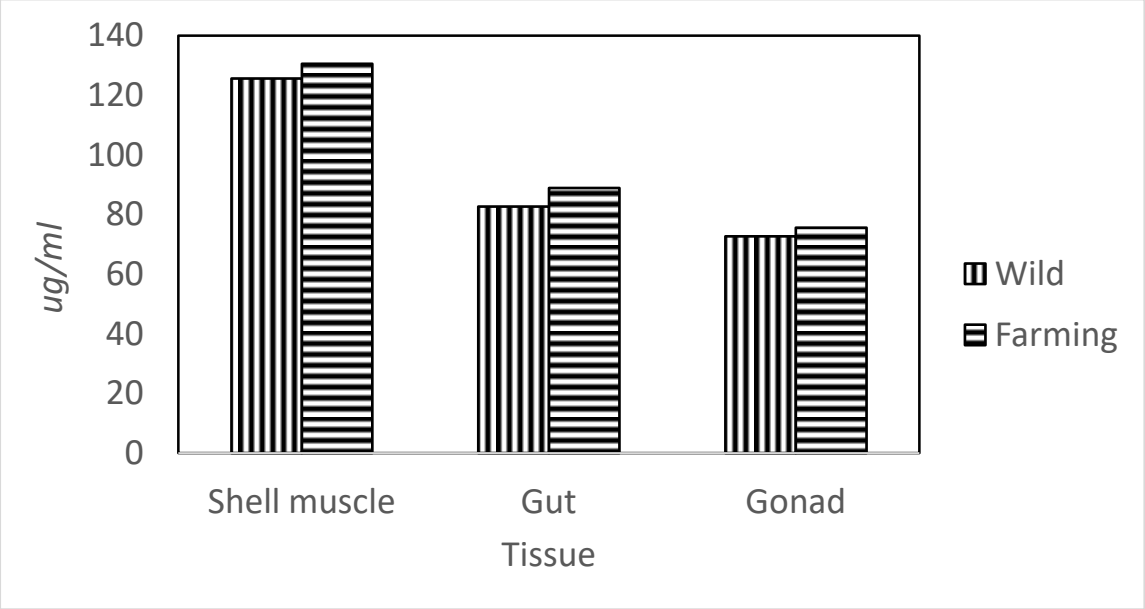

Fig. 1. Inhibitory effects {EC_{50} (mg/ml)} of DPPH by abalone on 1.0 M.

Fig. 2. Inhibitory effects {EC_{50} (mg/ml)} of OH by abalone on 1.0 M.

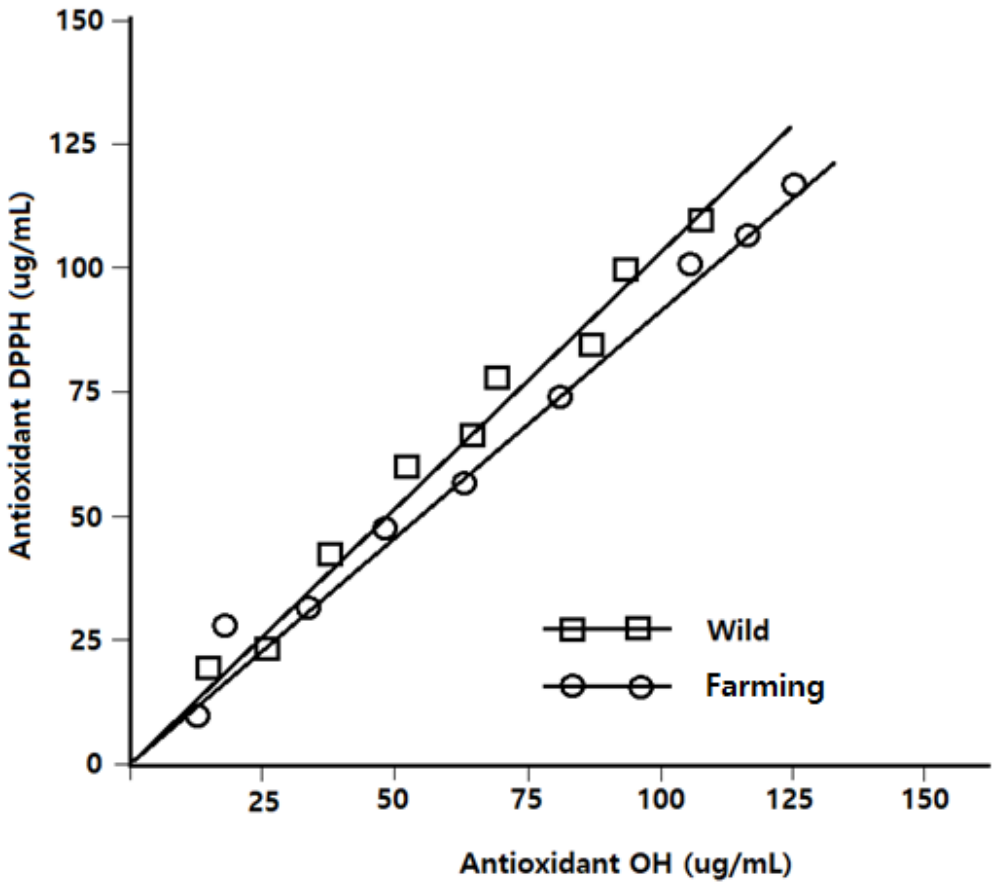

Fig. 3. Linear correlation between the amount of DPPH and OH. Correlation co-efficient of wild r = 0.956, Farming r = 0.547, 95% confidence interval 0.114 – 0.129.

DISCUSSION

Wang et al. (2014) demonstrated that the soluble matrix of abalone shell exhibited ferric reducing capacity and scavenging capacity of superoxide radicals ($O^{2-}\cdot$) similar to that of the pearl, while showing little or no inhibition of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl (OH)-free radicals (Liang et al., 2010). In the 0.01-2.0 mg/mL concentration range, the $O^{2-}\cdot$ scavenging ability of abalone shell polysaccharide increased from 1.88 to 80.4%, and that of abalone shell compound increased from 0.63 to 71.5% (Wang et al., 2014).
In the DPPH-scavenging assay, the Tris-HCl extract showed a 58.60±0.88% radical-scavenging activity, which was followed closely by the ethanol extract that had a 55.40±0.62% (Li et al., 2012). This result was similar to our results.

The activity in terms of scavenging reactive oxygen species (ROS) was likewise obtained with the use of 85% aq. MeOH (Lim, 2014). The antioxidant function of abalone was partially confirmed by these results. However, the ethanol extract did not have a high antioxidant capacity.

Regarding the fact that all cultivars were grown under identical conditions and in the same locality, it is possible to conclude that one can clearly see the cultivar variability, which is quite typical of fruit (Szajdek & Borowska, 2008). Farming abalone as well as wild abalone have lived to grow in similar saline regions. Reductions in antioxidant OH activity were similar for both analyzed wild and farming abalones. Liner correlation of antioxidant DPPH activity were strong positively correlated with OH antioxidants. The results of the farming abalones under investigation and their mutual comparison have not shown significant differences, and in this way, the work is a contribution to a further selection of the most suitable cultivars, which could become a part of nutrition participating in strengthening antioxidant effects of human organism.

CONCLUSIONS

The antioxidant function of abalone was partially confirmed by these results. The all values of DPPH and OH scavenging activity of wild abalones were higher than those of farming abalones. However, the all did not show a statistically significant difference ($p < 0.05$).

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