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Antioxidant Activity in Hepatopancreas of the Shrimp (*Pleoticus muelleri*) by Electron Paramagnetic Spin Resonance Spectrometry

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Free radical scavenging properties of hepatopancreas extracts of *Pleoticus muelleri* were evaluated by electron paramagnetic spin resonance spectrometry methods (EPR) against the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The present study was carried out to characterize different physiological stages of the shrimp under environmental and nutritional stress, evaluating the effect on growth, survival, and functional morphology of the hepatopancreas. Feeding trials were carried out on juveniles (1 g initial weight) held in aquaria. Each diet, with different concentrations of vitamins A and E, was tested in triplicate groups during 25 days. The control groups were fed with fresh squid mantle and with a vitamin-free diet. For all of the diets, the extracts exhibited strong DPPH radical scavenging activity, suggesting that the tissue is a powerful natural antioxidant. Individuals fed with different concentrations of vitamin E showed the strongest effect on the DPPH radicals, reducing the DPPH radicals to 50%, after an incubation period of 3 min. In contrast, the extracts of control animals, fed with squid mantle, had the weakest antioxidant activity (4%). These data indicated that the presence of vitamin E in the diet can provide immediate protection against free radicals.

KEYWORDS: Free radical scavenging; DPPH; electron paramagnetic spin resonance; antioxidant activity; hepatopancreas; *Pleoticus muelleri*

INTRODUCTION

Organisms have protective systems against free radical reactions, for example, endogenous antioxidants and oxidative enzymes. These protective systems may well function in association with each other. Oxidative stress causes tissue damage, and so we could follow the final results of free radical reactions. Lipid autoxidation, as well as enzymatic oxidation, during storage and processing is the major reaction in fats, oils, and fat-containing foods responsible for the deterioration in food quality (*1*).

One initiating radical may cause the oxidation of hundreds or even thousands of substrate molecules. Vitamin E (α-tocopherol) and other protective substances behave as chain-breaking antioxidants (*2*). Vitamin E lowers the rate of lipid autoxidation and has the effect of reducing the formation of hydroperoxides. It transfers hydrogen to peroxyl radicals very efficiently, thus allowing little opportunity for the peroxyl intermediates to revert. The antioxidants form a phenoxyl, stabilized by resonance, which may rapidly intercept a second peroxyl, yielding a nonradical product (*3*).

Electron paramagnetic spin resonance (EPR) spectrometry is considered to be the least ambiguous method for the detection of free radicals. The radical scavenging ability is determined by the disappearance of 1,1-diphenyl-2-picrylhydrazyl (DPPH), a stable free radical, with a distinctive EPR signal. A standard DPPH solution, protected from air contact, may be prepared and stored with a loss of free radical activity not exceeding 2−4% per week. DPPH reaction with antioxidants can be followed by loss of the EPR signal or loss of absorbance at 540 nm (*4*).

The Argentine red shrimp *Pleoticus muelleri* has a high commercial value and is distributed from 20 °S latitude in Espíritu Santo, Brazil, to 50 °S in Santa Cruz, Argentina (*5*). Most of the 78,866 ton catch along the coastal waters of Argentina in 2001 was exported (*6*). This shrimp has aroused great interest due to its potential use in culture in temperate areas, so it is important to determine the optimal culture conditions in order to have healthy individuals.

The aim of this study was to determine the free radical scavenging properties in the hepatopancreas of the Argentine red shrimp *P. muelleri* under experimental conditions and to characterize different physiological stages of the shrimp under environmental and nutritional stress, evaluating the effect on

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Table 1. Percent Composition of Experimental Diets

ingredients (g/100 g of dry diet)	diets D1	diets D2–D7	diets D8
casein, vitamin-free		37.5	37.5
cellulose		10	12
cholesterol	0.5	2	2
fish meal (65% crude protein)	27		
fish oil	2		
fish soluble	2		
free fatty acids		7	7
gelatin		12	12
lecithin	0.5	1	1
manioc starch	20	22	22
meat and bone meal (61% crude protein)	23		
mineral premix[a]		0.3	0.3
protein squid mantle (85.5% crude protein)		2.5	2.5
sodium alginate		3.7	3.7
soybean meal (42% crude protein)	17		
vitamin premix[b]	0.5	2	
wheat	7.5		
proximal composition			
moisture	7.3	7.1	7
total protein	33.4	44.5	43.2
total lipids	5.5	13.2	12.7
ash	5.5	4.2	4.5

[a] Mineral premix: calcium, 1000 mg; magnesium, 500 mg; potassium, 99 mg; zinc, 30 mg; 10 mg; iron, 10 mg; copper, 2 mg; iodine, 150 μg; selenium, 200 μg; chromium, 200 μg; molybdenum, 500 μg (Twin Laboratories, Inc.). [b] Vitamin premix (mg/kg): cholecalciferol, 35; thiamin, 163; rivoflavin, 156; pyridoxine, 213; calcium pantothenate, 250; biotin, 250; niacin, 500; folic acid, 25; B_{12} HCl, 20; ascorbic acid Rovimix STAY C, 781; menadione, 34; inositol, 300; choline chloride, 200 (Roche).

growth, survival, and functional morphology of the hepatopancreas.

MATERIALS AND METHODS

Experimental Animals. Feeding trials were carried out with 168 early juveniles (1.0 ± 0.05 g initial weight) held in 150 L glass aquaria with an under-gravel filter and a sand and crushed shell bed. The juveniles were reared from hatchery-raised postlarvae (wild broodstock from Mar del Plata, Argentina) at Nagera Station, of the Marine Science Department, Mar del Plata National University, Argentina.

The treatments consisted of nine diets that were developed from different food resources. One diet (D1), based on fish meal, meat and bone meal, and soybean meal, which produced good results in previous experiments (*7*), and seven semipurified diets (D2−D8) that contained casein and squid mantle protein were prepared (**Table 1**). D1 was tested at two salinity conditions, $D1_{33}$ (33‰) and $D1_{27}$ (27‰). Diets D2, D3, and D4 were supplemented with 140, 160, and 180 mg of vitamin A/kg. Diets D5, D6, and D7 were supplemented with 1250, 1500, and 1750 mg of vitamin E/kg, respectively. One control group was fed with fresh squid mantle (*Illex argentinus*) (C) and the other with a vitamin-free semipurified diet (D8). Diets D2−D8 and C were tested at normal salinity conditions (33‰). The animals were fed ad libitum once a day. Feeding rate was adjusted daily in each tank in order to maintain feed waste at a minimum. Diets were tested in three replicate groups of seven shrimps randomly chosen, during 30 days. Individual shrimp weights were determined at the beginning of the experiment and after 30 days. At the end of the experiment, animals were anesthetized on ice and were pooled by treatment for the analysis of hepatopancreas total lipid and EPR analysis. Hepatopancreas and muscle were stored at −30 °C.

Growth performance and survival were measured in terms of final individual weight, percentage of weight gain [[(final mean weight − initial mean weight)/initial mean weight] × 100], and percentage survival.

Hepatopancreas and muscle total lipid were analyzed from dry tissue with Soxhlet extraction with chloroform/methanol (2:1, v/v) for 6 h. The assays were done in triplicate (*8*).

Table 2. X-band Magnetic Resonance Spectra of DPPH

general instrumental settings	
microwave power (mW)	16000
attenuation (dB)	9
modulator frequency (kHz)	100
modulation amplitude (G)	2.5
temperature (K)	293
time constant or response time (s)	0.2
microwave frequency (GHz)	9.34
receiver gain	1.25×10^5
scan rate (s)	200

To determine differences among diets, data were analyzed with analysis of variance (ANOVA). A χ^2 test was used to assess survival. Statistical significance was accepted at the $P < 0.05$ level (*9*).

At the conclusion of the experiment, the individuals in intermolt stage were selected for histological studies. Hepatopancreases were fixed in Davidson's fluid (distilled water, ethanol, formaldehyde, acetic acid) for 24 h and embedded in paraffin, and tissue sections were cut to ∼5 μm and stained with hematoxylin and eosin (*10*).

Chemicals and Reagents. DPPH was obtained from Aldrich. The solvents were from Erba and Merck, and they were carefully purified following literature methods in order to have them free of alkenes and carbonyl compounds. They were also conveniently dried.

EPR Sample Preparation. A weighed portion (0.020 g) of the hepatopancreas tissue (pool of seven animals) was mixed with 0.2 mL of chloroform/methanol (2:1, v/v) solvent and kept under argon atmosphere. DPPH solution was also prepared in chloroform/methanol (2:1, v/v) solvent.

Samples for EPR measurements were prepared in quartz tubes with an internal diameter of 4 mm, to achieve an adequate tuning. Each reaction mixture contained 100 μL of the hepatopancreas solution and 100 μL of DPPH 1.8×10^{-4} M solution. All glassware and syringes were dried, and they were flushed with argon immediately before use. Control or blank preparations, containing 100 μL of solvent and 100 μL of DPPH 1.8×10^{-4} M, remained unaltered after a long period of time. DPPH proved to be a very stable free radical.

An experiment with different types of tissues was also carried out, with hepatopancreas and muscle tissue. A mass of 0.030 g of the particular tissue was mixed with 1 mL of DPPH solution at 37 °C. Aliquots of 200 μL were analyzed at fixed time intervals of 5, 12, 20, 80, and 140 min.

Instrumentation. A Bruker ER 200D spectrometer with a rectangular TE_{102} resonance cavity was used for recording X-band EPR spectra from samples contained in quartz tubes. A variable-temperature unit, type ER 4111 VT, was used, and so the measurements were carried out at constant temperature. The DPPH signal is sensitive to temperature changes. Electronic spectra of the DPPH solutions were recorded in a Shimadzu UVPC-2101. The standard plotting program, Origin, was used to fit all sets of points whenever necessary.

EPR Measurements. The potential antioxidant activity of tissue extracts was investigated on the basis of the scavenging activity of the stable DPPH free radical. The solutions were investigated throughout a scan range of 200 G. The spectra were computer-acquired. Typical experimental conditions used for acquiring EPR spectra are outlined in **Table 2**. First-derivative absorption curves were recorded. For quantitative measurements of radical concentrations we used the method of comparative determination, and the corresponding signal height was computed. A standard solution of diphenylpicrylhydrazyl in carbon tetrachloride was prepared, $\epsilon\lambda$, 520 nm = 12000 M^{-1} cm^{-1}, to control the concentration of the $HCCl_3$/methanol solutions (*4*).

All spectra were recorded immediately after shaking and followed at 293 K for ∼80 min. EPR spectra of samples and standards were made consecutively. The total experimental error due to the instrumental, sample, and spectrum processing factors was reduced to 5% (*11*).

All of the parameters were carefully kept the same for all samples, the sample volume, the EPR tube shape, and the instrumental setting,

Table 3. Weight Gain and Survival of Juveniles Fed Different Diets

	mean wt[a] (g)			
	initial	final	%Δ*w*[b]	% S[c]
D1 (33‰)	1.0 ± 0.18	1.5 ± 0.19	57	100 a
D1 (27‰)	1.0 ± 0.01	1.7 ± 0.17	63	86 a
D2	1.1 ± 0.38	1.7 ± 0.69	57	86 a
D3	1.0 ± 0.32	1.7 ± 0.67	73	67 b
D4	1.0 ± 0.33	1.7 ± 0.65	69	95 a
D5	1.1 ± 0.36	1.6 ± 0.51	50	90 a
D6	1.1 ± 0.38	1.7 ± 0.69	57	86 a
D7	1.0 ± 0.34	1.6 ± 0.62	65	90 a
D8	1.0 ± 0.05	1.4 ± 0.11	45	62 b
C	1.0 ± 0.06	1.5 ± 0.31	47	62 b

[a] Values are means ± standard error of triplicates. [b] %Δ*w* = percentage in mean weight. [c] % S = survival. Percentages with similar letters are not significantly different ($P < 0.05$).

to avoid nonreproducible results due to field drift. The measurements were repeated at least three times to minimize random errors.

RESULTS AND DISCUSSION

Weight gain and survival are presented in **Table 3**. After 30 days of the experiment, the percent of weight gain of the shrimps under different treatments ranged from 45 to 73% with no significant differences (ANOVA $P > 0.05$). Survival in all treatments was high, ranging between 62 and 100%. However, mean survival was significantly lower with control diet (fed squid mantle) (62%), vitamin-free diet (62%), and diet supplemented with 160 mg of vitamin A/kg of diet (67%).

In animals fed with $D1_{33}$ the hepatopancreas presents the four typical cells, E, F, R, and B, showing morphological features similar to those observed in wild shrimps (*12*). The hepatopancreas is the major organ in decapod crustaceans and has many biological functions, such as digestion, absorption, and storage of nutrients and metals (*13*). The digestion process is cyclical, and the specific role of the different cellular types has been determined through morphological, ultrastructural, histochemical, and immunohistochemical studies (*14*−*17*). It is important to recognize the structure of the hepatopancreas because of the role it plays in the metabolism and the rapid histological changes it suffers in response to different physiological demands and environmental variations (*17*, *18*). Malnourishment signs are reduction in cell size, condensation of nuclear chromatin, disruption of the brush border, and some ultrastructural changes (*19*). Hepatopancreas histology of shrimps fed with $D1_{27}$ shows some pathology. The epithelial cells showed signs of shrinkage, and the lumina were enlarged in the affected tubules. Cellular dysplasia and necrosis of B-cells in the proximal zone were observed, and the basal lamina presented infoldings in some of the tubules.

Variations in the structure of the epithelial cells of the hepatopancreas induced by suboptimal diets have been reported for other crustaceans (*20*−*23*). Histological analysis of the hepatopancreas of shrimps fed diet without vitamins (D8) showed cells with unclear nuclear and cellular contours. Different cell types can be not recognized, and there were not observed brush borders.

Shrimps fed with different levels of vitamin A (D2 and D3) and vitamin E (D5 and D6) exhibited some histopathological changes in the hepatopancreas, as well as cellular hyperplasia and hypertrophy, disorganized tissue, and shrinkage of cells. Individuals fed with 180 mg of vitamin A/kg (diet D4) and 1750 mg of vitamin E/kg (diet D7) did not reveal any pathology in the organ, showing a normal structure in all cases.

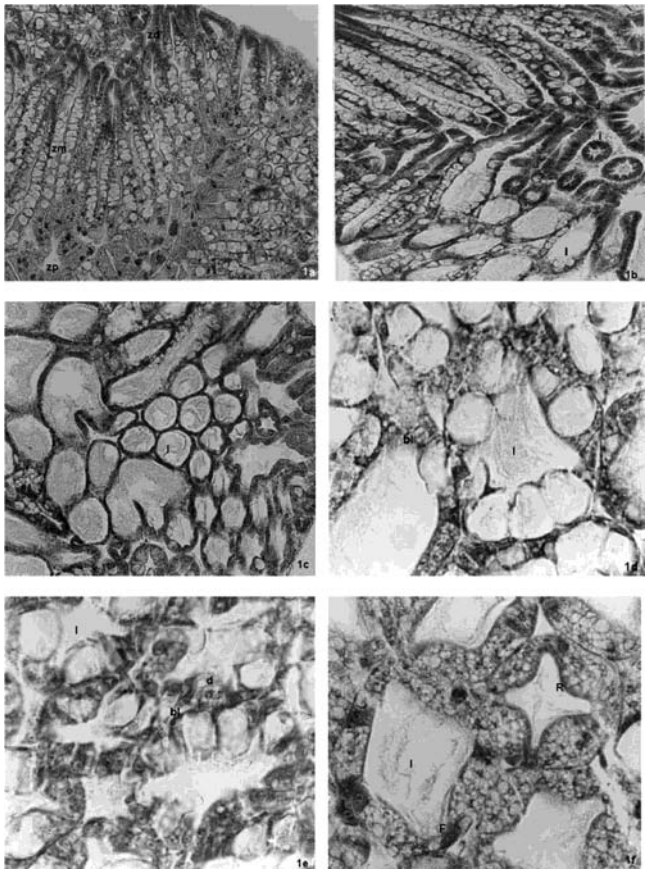

Figure 1. Microphotographs of *P. muelleri* hepatopancreas under different experimental conditions: (**a**) diet $D1_{33}$, longitudinal section through tubules showing the arrangement of the normal organ (×10); (**b**) diet $D1_{27}$, longitudinal section through tubules [note the large lumen and the ample intertubular space (×25)]; (**c**) diet D8, transverse section through tubules revealing severe cytological alterations [note complete loss or destruction of epithelial cells and scarce intertubular space (×25)]; (**d**) diet D2, transverse section through tubules, showing damage of the basal lamina and cellular retraction (×100); (**e**) diet D5, detail of the tubule showing desquamated epithelium and folding of the basal lamina (×100); (**f**) diet D7, transverse section through tubule showing the cellular types similar to those wild shrimps (×100). bl, basal lamina; d, desquamated cells; F, F cell; i, intertubular space; l, lumen; R, R cell; zd, distal zone; zm, medial zone; zp, proximal zone.

Figure 2 shows a typical sequence of EPR spectra measuring, in vitro, free radical scavenging activities of hepatopancreas tissue extracts of *P. muelleri*. The intensity of the signal, measured in arbitrary units, decays with time and represents the radical−tissue reaction.

In **Figure 3** we may observe the kinetics of the DPPH reaction. All of the samples exhibit powerful DPPH radical scavenging activity, which suggests the tissue is a natural antioxidant. Shrimps that have been fed control diets (D8 and C) cannot achieve a good weight and have a low survival; however, these fractions also exhibit DPPH scavenging. We, thus, infer that the shrimps have developed efficient protective substances, although they still needed more because their growth was impaired. The shrimps fed on vitamin E diets have the safest environment, so we may conclude that they have generated greater abundance of molecular antioxidants and that they have also good growth and survival.

Perhaps there is not a good correlation between the histological and EPR results, but it should be noted that antioxidant depletion does not necessarily mean that oxidative damage has

Table 4. Total Lipids and Percent Remaining DPPH at Different Incubation Times of Hepatopancreas Tissue

	D1 (33‰)	D1 (27‰)	D2	D3	D4	D5	D6	D7	D8	C
% total lipids (dry matter)	8.41	8.24	2.19	2.70	4.44	0.39	2.19	5.17	3.60	3.15
% DPPH 3′	70	91	42	74	96	42	42	56	51	96
% DPPH 50′	38	47	19	38	38	19	19	38	34	50

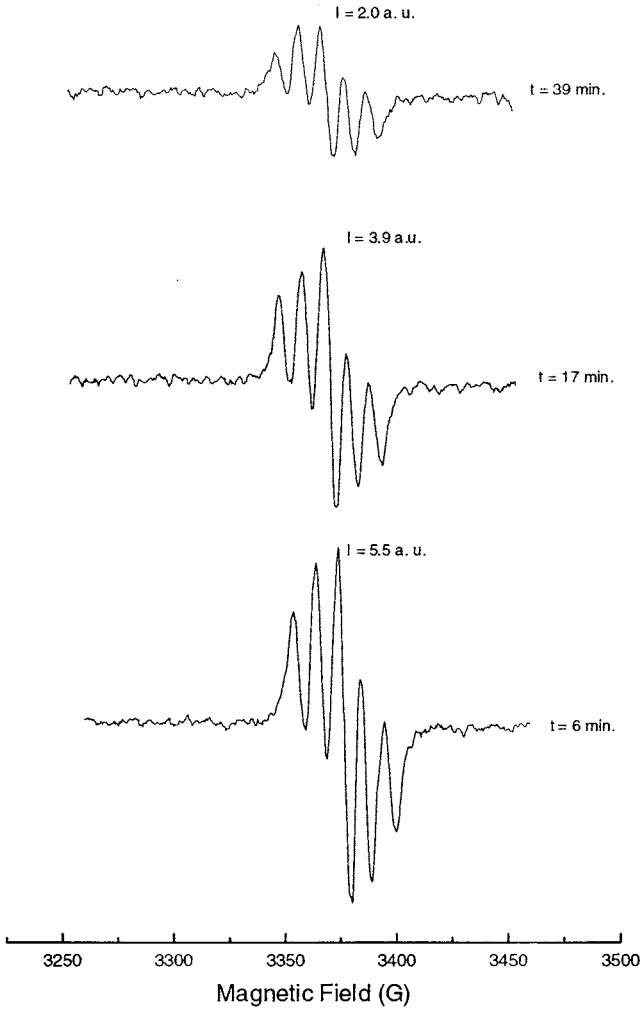

Figure 2. EPR spectra of DPPH free radical at 293 K, in $CHCl_3$/methanol solvent suspension of hepatopancreas tissue showing typical scavenging activity. DPPH signal disappears after some time.

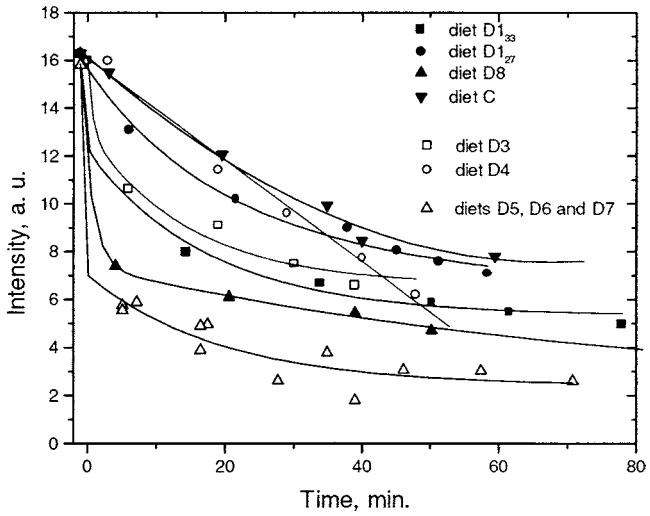

Figure 3. Free radical scavenging activity of hepatopancreas tissue preparations for a pool of seven animals. Shrimps were cultivated under different dietary conditions. DPPH radical concentration was 90 μM in all reaction mixtures. Tissue concentration was 0.05 g/mL. The signal intensity, in arbitrary units, represents the remaining DPPH. A control or blank preparation remained unaltered on time.

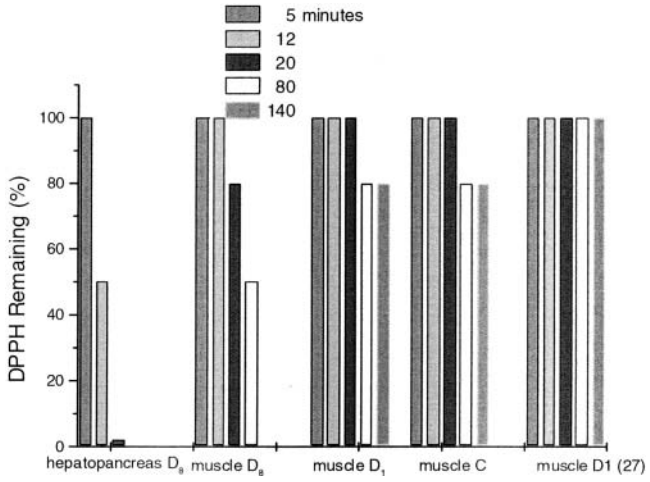

Figure 4. DPPH scavenging effect followed up to 140 min. Different tissues were tested at $T = 37$ °C by mixing 0.030 g of hepatopancreas or muscle with 1 mL of DPPH solution (110 μM).

taken place; it might simply mean that the defense mechanisms have performed their normal function.

In **Table 4** we can appreciate that the lipid content in dry hepatopancreas tissue is considerably high. In the hepatopancreas of shrimps fed with diets D2−D4 (supplemented with vitamin A) a lipid content increase took place. Similar results occur with diets D5−D7 (supplemented with vitamin E). The oxidant activity does not parallel the lipid content, so we may conclude that some antioxidant molecules or antioxidant factors do not belong to the lipid fraction.

We used muscle as a control tissue. In both hepatopancreas and muscle we analyzed the lipid content and the DPPH scavenging effect. The lipid content was in the range of 1.3−1.8%, measured over dry tissue. **Figure 4** shows a poor scavenging effect in almost all muscle extracts.

CONCLUSIONS

Loss of individual antioxidants and/or generation of oxidation products from them can be measured as an index of oxidative stress. Our results pointed out that the tissue investigated had a very efficient response to all of the stress situations imposed. The supply of vitamin E showed the better results. The results clearly indicate that the presence of vitamins in the diet promotes the protection of tissues.

Natural antioxidants can be used in the food industry, and there is evidence that these substances may exert their antioxidant effects within the human body. Many biochemical and clinical studies suggest that natural and synthetic antioxidant compounds are helpful in treating diseases mediated by oxidative stresses. Our study demonstrates that a chloroform/methanol extract from shrimp hepatopancreas has excellent antioxidant activity. Therefore, it is worthwhile to investigate the composi-

tion of the extract and look for the compounds responsible for its antioxidant activity.

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