

A Temperature Resistant Extracts Prepared from the *Perinereis aibuhitensis* and Its Antioxidative Characterization

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Received: March 21, 2019 Accepted: April 15, 2019 Published: April 16, 2019

doi:10.5296/ast.v7i2.14673 URL: <https://doi.org/10.5296/ast.v7i2.14673>

Abstract

The *Perinereis aibuhitensis* (*P. aibuhitensis*), a traditional Chinese medicine, has a function of delaying senility-according to “Compendium of Materia Medica”. It is considered a useful animal model in antioxidant activity tests. The *P. aibuhitensis* has already been utilized as biochemical responses to specific pollutants and the food supply for various birds. In the

present work, the extracts from *P. aibuhitensis* were prepared by the traditional decocting method and the biocompatibility was characterized by hemolysis and MTT tests. The materials showed no hemolytic for the hemolysis rate (lower than 0.2 %) while exhibited moderate cytotoxic effects on HUVECs (Human Umbilical Vein Endothelial Cells). In extracellular antioxidant activity against O_2^- and OH , the extracts from *P. aibuhitensis* could eliminate O_2^- and OH efficiently. In cellular ROS level tests, the ROS level was reduced effectively after treated with *P. aibuhitensis* extracts. The extracts had a potential prospect as a biological antioxidant to reduce redundant reactive oxygen species damage and great significance for humans health.

Keywords: *P. aibuhitensis*, biocompatibility, antioxidant activity, biological antioxidant

1. Introduction

The *P. aibuhitensis*, a marine invertebrate, is a conspicuous elements of benthos in intertidal mudflats and estuaries with a worldwide distribution (Zhang et al., 2008). They form an important part of the food supply for various birds and bottom-dwelling fishes. *P. aibuhitensis* has already been utilized in ancient China. Besides, the *P. aibuhitensis* was used as a traditional Chinese medicine to delay senility according to “Compendium of Materia Medica”, which the special characteristic of *P. aibuhitensis* might relate with the scavenge of ROS in the point of modern medicine.

Much attention has been focused on the *P. aibuhitensis* in recent years because several bioactive components have been identified from *P. aibuhitensis* including antimicrobial peptide (Pan, 2004), glycerophospholipids (Hirobe et al., 2015) and protease (Li et al., 2006), etc. Owing to *P. aibuhitensis* good adaptability to stressful environmental conditions, some researchers have regarded them as biochemical responses to specific pollutants as well as potentially ecological keystone species for monitoring programs (Perez, Blasco, & Sole, 2004).

It is well known that free radicals oxidative stress can damage cellular lipids, proteins, or DNA and inhibit their normal function (Valko et al., 2007). Furthermore, free radicals have been proved to be implicated in the pathogenesis of a variety of human diseases such as atherosclerosis, diabetes mellitus, hypertension and inflammation (Lowenfeld et al., 2017). Among free radicals, reactive oxygen species such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and superoxide anion (O_2^-) are usually generated during cellular metabolism (Yuan, Chen, Zhou, Liu, & Yang, 2010). Hydroxyl radical is a neutral form from hydroxide ion and has a high reactivity, making it a very dangerous radical to the human cells (Du et al., 2017). Superoxide anion, a primary species of reactive oxygen species (ROS) function as a defense against viral or bacterial attack, also lead to damage of proteins, DNA, and lipid structures in human body (Tian, Mao, Okajima, & Ohsaka, 2005; Wang et al., 2013). Hence, for protecting the organisms' health, removing the redundant reactive oxygen species are of great significance in a variety of in vitro and in vivo models.

It has reported that several environmental contaminants including heavy metals can induce oxidative stress in marine animals by generating reactive oxygen species (Yuan et al., 2010).

Moreover, nowadays researcher were focused on identifying natural antioxidants and a lot of papers have been published on this topic (A., 2018; Butterfield, 2015; Ott et al., 2017; Qiao et al., 2018). Tian et al have found that the antioxidant system of *P. aibuhitensis* has defensive responses to Pb^{2+} exposure (Tian, Liu, Wang, Zhou, & Tang, 2014). Hence, *P. aibuhitensis* is a good model animal for the study of the antioxidant activity via bioassays in laboratory.

In the current study, the extracts from *P. aibuhitensis* were prepared by the traditional decocting method and the biocompatibility such as blood compatibility and cytotoxicity were evaluated by hemolysis rate and MTT assay, respectively. To reflect antioxidant activity of extracts completely, the scavenging rate of O_2^- and OH was measured in different temperature and concentration. Then, cellular ROS levels were evaluated after the treatments with different concentration of *P. aibuhitensis* extracts. The results indicated that the O_2^- and OH could be eliminated by *P. aibuhitensis* extracts. The antioxidant potential of *P. aibuhitensis* extracts showed a good prospect as a biological antioxidant.

2. Materials and Methods

2.1 Materials

Adult individuals of *P. aibuhitensis* were purchased from Marine Biology Institute of Shandong Province. Tris-HCl (pH 8.2), DL-Dithiothreitol (DTT), and Pyrogallol were obtained from Qingdao Yunshan Biotechnology Co., Ltd. Phosphate buffer solution (PB), 1,10-Phenanthroline, $FeSO_4$ and H_2O_2 were purchased from Huasheng Chemical. Other reagents were all analytical grade.

2.2 Preparation of *P. Aibuhitensis* Extracts

Fresh individuals of *P. aibuhitensis* were rinsed with tap water and distilled water several times to remove impurities. Then the clamworm was immersed into the distilled water for 12 h. Subsequently, the clamworm was added into a beaker with fresh distilled water and boiled for 3 h. Fresh distilled water was supplemented during the boiling process to avoid burning dry. The obtained extracts were filtered and freeze-dried which were used in the following experiments.

2.3 Hemolysis Rate of *P. Aibuhitensis* Extracts

The hemolysis rate of four different concentrations of extracts was evaluated. In brief, the hemodilution was prepared at 37 °C for 1 h. 20 μ l blood was added into the extract solution (1 ml) and treated for 1 h at 37 °C. Then the mixture was centrifuged 5 minutes at 2000 rpm/min to obtain the supernatant. The negative control groups were administered with normal physiological saline. The absorbance was measured at 316 nm. The ratio of hemolysis rate (HR %) was calculated which is defined as:

$$HR \% = (D_{sample} - D_{nc}) / (D_{pc} - D_{nc}) \times 100\%$$

Where D_{sample} was the absorbance of sample group; D_{nc} represented the absorbance of the

normal saline groups; D_{pc} was the absorbance of deionized water group.

2.4 Cytotoxicity Assays of Extracts from *P. Aibuhitensis*

In vitro cell viability in the presence of extracts from *P. aibuhitensis* was investigated by the MTT assay using human umbilical vein endothelial cells (HUVECs). HUVECs were seeded into a 96-well plate at a density of 1×10^4 cells per well and incubated for 24 h at 37 °C, CO₂ 5 % with culture medium. The medium was removed and replaced by 100 µl extracts solutions at 0.0125, 0.025, 0.05, and 0.1 mg/ml and incubated for 24 h and 48 h. Culture medium was used as blank control. At the end of the incubating, MTT (10 µl) was added and the plates were incubated for an additional 4 h. Then the supernatants were removed, and 100 µl DMSO was added to dissolve the blue-violet crystals for 10 min. The absorbance was measured at 490 nm. The relative growth rate (RGR) was expressed by the following equation:

$$\text{RGR (\%)} = D_t / D_{nc} \times 100 \%$$

Where D_t and D_{nc} were the absorbances of the tested sample and the negative control.

2.5 Antioxidant Activity

2.5.1 Determination of Antioxidant Activity Using Superoxide Anion (O₂⁻) Radical Scavenging Method

The antioxidant activities were determined using O₂⁻ as a model free radical. In the experiment, different concentrations of *P. aibuhitensis* extracts were used to evaluate its antioxidation. Briefly, the extracts were added into solution contained 5 ml 0.1 mol/ml Tris-HCl, followed by adding 40 µl 25 mmol/l pyrogallol to react in a shaker at 25 °C for 3 min accurately. At last, 50 µl 50 mg/ml DTT was added into the mixed solution to stop the reaction and the solution was remained 10 min at room temperature. The absorbance was measured at 316 nm. The O₂⁻ scavenging activity was expressed by the following equation:

$$\text{O}_2^- \text{ scavenging activity (\%)} = (A_0 - A_1 + A_2) / A_0 \times 100 \%$$

Where A_0 was the absorbance of the autoxidation of pyrogallol; A_1 represented the absorbance of the pyrogallol which had been in contact with sample (extract from *P. aibuhitensis*); A_2 was the absorption of sample.

2.5.2 Determination of Antioxidant Activity Using ·OH Radical Scavenging Method

The antioxidant activities were determined using ·OH as another model free radical. In brief, 1 ml of extraction from *P. aibuhitensis* was used and 0.15 mol/l phosphate buffer saline (pH 7.4) was prepared as a stock standard. Samples were incubated at 37 °C for 1 h, after that,

1,10-Phenanthroline monohydrate was added. Followed by adding FeSO₄ and H₂O₂ and keeping reacting 10 min at 37°C. The absorbance was measured at 536 nm. The ·OH scavenging activity was expressed by the following equation:

$$\text{OH scavenging activity (\%)} = (A_1 - A_0 - A_2) / (A_3 - A_2) \times 100 \%$$

Where A_1 represented the absorbance of 1,10-Phenanthroline monohydrate which had been in contact with sample; A_2 was the absorbance of 1,10-Phenanthroline monohydrate, FeSO₄ and H₂O₂; A_3 was the absorbance of 1,10-Phenanthroline monohydrate with FeSO₄; A_0 was the absorption of sample.

2.6 Determination of Cellular ROS Levels

Cellular ROS levels of fibroblasts cell line (L929) treated with extracts from *P. aibuhitensis* were investigated with reactive oxygen species assay kit. L929 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % FBS and penicillin-streptomycin at 37 °C and 5 % CO₂. Then, L929 cells were seeded in wells of 96 well plate at a density of 6×10⁴ cells/well (Huang, Zhang, Yang, Zhang, & Xu, 2013; Yan et al., 2016). Subsequently, the medium was replaced with fresh medium with a gradient concentration of *P. aibuhitensis* extracts (ranging from 2.5 mg/ml to 10 mg/ml). The cells were incubated with carboxy-2',7'-dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe for 20 min. The cells were dealt with Rosup as positive control. The fluorescence of each group was measured at 488 nm (excitation) and 525 nm (emission) wavelengths on confocal laser scanning microscope.

2.7 Statistical Analysis

The assays were performed at least in triplicate on separate occasions. A statistical analysis was performed using one-way ANOVA for data. $p < 0.05$ was considered as significant.

3. Results

3.1 Hemolysis rate of *P. aibuhitensis* Extracts

The *P. aibuhitensis* extracts were successfully prepared and the hemolysis rate was evaluated. As 100 % and 0 % values we used normal saline and deionized water, respectively. The extracts with the concentration range from 0.1 g/ml to 0.4 g/ml caused 0.29 % up to 1.72 % hemolysis (Fig. 1). According to the relative of the hemolysis rate and hemolysis level, the material showed no hemolytic that the hemolysis rate was lower than 0.2 %. The data showed that the extracts had excellent blood compatibility as biological antioxidant.

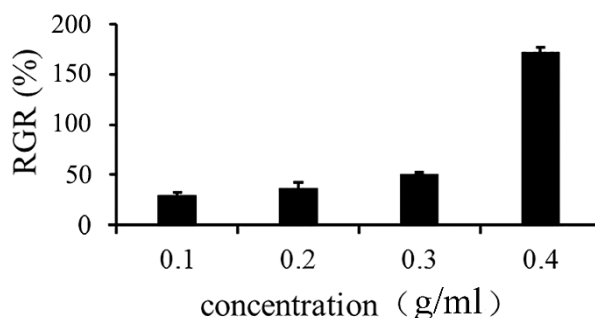


Fig. 1. Hemolysis rate of the extracts with different concentrations

3.2 Cytotoxicity Assays of Extracts from *P. aibuhitensis*

To determine effects of the extracts on biological antioxidant, the MTT test was used. Cells were immersed in the extract solution with the concentration range of 0.0125–0.1 mg/ml and treated for up to 48 h (Fig. 2). The data showed that the extract solution was non-toxic to HUVECs with the concentration range from 0.0125 mg/ml to 0.05 mg/ml and all the relative growth rates were nearly 100 %, which only exhibited moderate cytotoxic effects at the concentration of 0.1 mg/ml. The result demonstrated that the extracts showed perfect cellular compatibility and a good candidate as biological antioxidant.

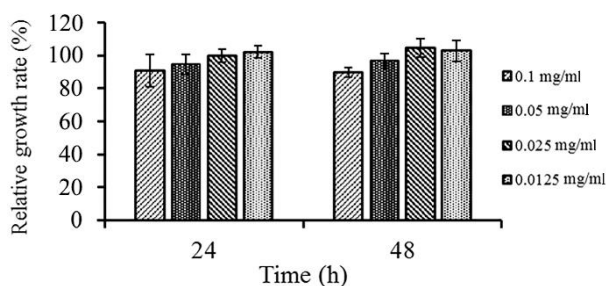


Fig. 2. Cell viability measured by MTT assay for the extracts from *P. aibuhitensis* with different concentrations after 24 h and 48 h incubation

3.3 Antioxidant Activity

3.3.1 O₂⁻ Scavenging Activity of the Extracts from *P. aibuhitensis*

Superoxide anion (O₂⁻) could be generated during the process of pyrogallol auto-oxidation. With this method, the extracts from *P. aibuhitensis* were studied as scavengers of O₂⁻ generated by autoxidation of pyrogallol. The scavenging activity of the extract (40 mg/ml) was tested at different time ranging from 5 to 30 min Fig. 3 showed that the O₂⁻ scavenging rate changed slightly over time (near 65 %), which indicated that the extracts had accomplished the action of scavenging within 5 min.

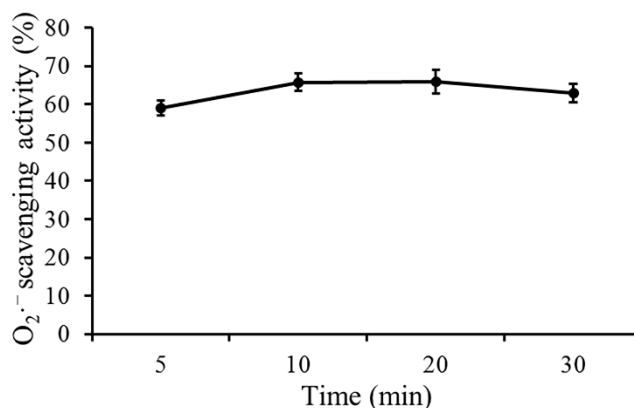


Fig. 3. Antioxidant activity of the extracts measured by pyrogallol auto-oxidation method

To reflect antioxidant activity completely, the extracts concentration and temperature were also considered as main facts to evaluate the antioxidant activity of extracts. The scavenging rates at 37 °C were slightly higher than that of 25 °C (Fig. 4). It indicated that temperature had no apparent influence on the antioxidant activity of extracts. As O₂⁻ is an undesirable compound especially in food industry which may be detrimental to health, the development of biological antioxidant become urgent. According to the results, the extracts from *P. aibuhitensis* could eliminate O₂⁻ efficiently, then it had a wide application with different surrounding conditions as biological antioxidant.

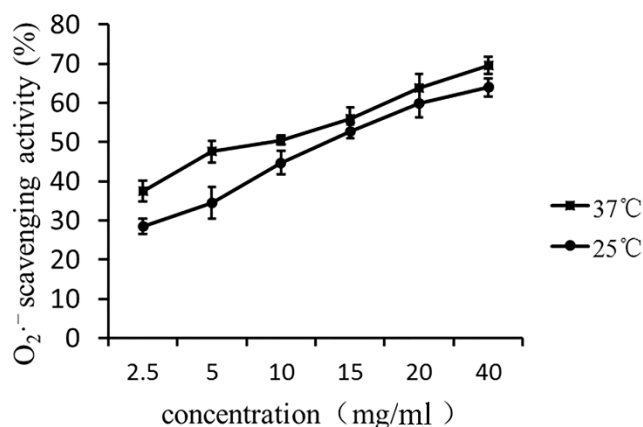


Fig. 4. Antioxidant activity of the extracts with gradient concentration at room temperatures and 37°C

3.3.2 OH Radical Scavenging Activity of the Extracts from *P. aibuhitensis*

Hydroxyl Radical (OH radical) is easily generated in damaged tissue and has powerful oxidization capacity. The redundant hydroxyl radical should be removed because of its ability of destroying cell membranes and DNA. In this study, the OH radical scavenging activity of the extracts from *P. aibuhitensis* was evaluated (Fig. 5). With the concentration increased, OH radical scavenging activity of the extracts improved rapidly and achieved approximately 60 % at the concentration of 40 mg/ml. In addition, compared with O₂⁻

scavenging activity, the low concentration extracts showed lower scavenging effect on OH radical (< 40mg/ml).

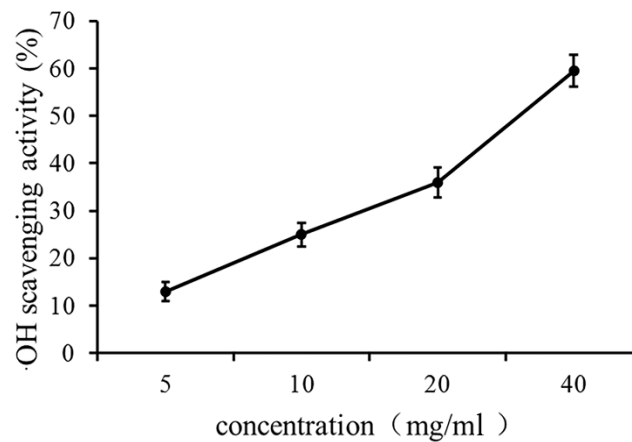


Fig. 5. OH radical scavenging activity of extracts with different concentrations

3.4 Cellular ROS Level

In this study, the ROS in L929 cells was removed by the treatment of the extracts after 20 min incubation, which presented that the green fluorescent in experimental groups' cells became significantly weaker than the control. Moreover, the fluorescent decreased with the increase of concentrations of *P. aibuhitensis* extracts (from 2.5 to 10 mg/ml) (Fig. 6 (a), (b) and (c)). The data showed that the extract of *P. aibuhitensis* could diffuse into cells easily and cause a chemical reaction with ROS, which reduced cellular ROS level effectively.

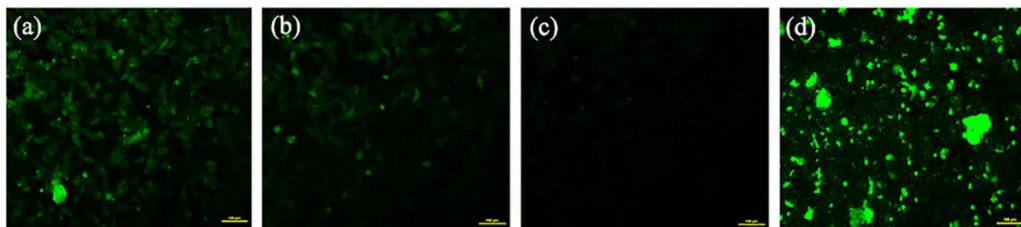


Fig. 6. The fluorescence photograph of cells treated with *P. aibuhitensis* extracts. (a), (b) and (c) represent the cell with different concentration of *P. aibuhitensis* extract ranging from 2.5 to 10 mg/ml, respectively. D means the blank control

4. Discussion

The *P. aibuhitensis* was used as a traditional Chinese medicine in the forms of individuals, which had the function in delaying senility. In the study, the *P. aibuhitensis* extracts were successfully prepared with traditional decocting method and the characteristics of extracts were evaluated and made a preliminary research with the active ingredient firstly.

As biological antioxidant, the material should be safe and nontoxicity (Morales et al., 2014). The hemolysis rate and cellular compatibility were evaluated to study the biocompatibility of *P. aibuhitensis* extracts. The break rate of red blood cells was used to quantify the hemolysis

properties of the extracts from *P. aibuhitensis*. The extracts displayed no hemolytic effects up to 0.4 mg/ml, which indicated that the extracts had no detectable disturbance of red blood cells membranes. In addition, the extracts from *P. aibuhitensis* were obtained by the traditional decocting method which had the characteristics of safety and non-toxic to HUVECs. We could conclude that the extracts had excellent biocompatibility as biological antioxidant.

In humans, oxidative damage is usually not involved in the initiation of chronic disease although it can be a promoter of disease (MacDonald-Wicks, Wood, & Garg, 2006). To remove redundant reactive oxygen species are of great significance for human health. The extracts from *P. aibuhitensis* could eliminate O_2^- and OH efficiently in this study. The scavenging rate or antioxidant activity was enhanced with increasing concentration of the extracts. It was also proved that the extracts had a wide application with different-conditions (different time and temperature). The DCFH oxidation method was used for the quantitation of oxidative burst activity at a single-cell level. The non-fluorescent substrate, 2,7-dichlorofluorescein-diacetate (DCFH-DA), diffused through the plasma membrane and was trapped within the cell upon hydrolysis to DCFH. Intracellular oxidation turned DCFH into 2,7-dichlorofluorescein (DCF) with green fluorescent which could be clearly detected (Aranda et al., 2013; Christensen, Korsholm, Andersen, & Agger, 2011). The extracts from *P. aibuhitensis* could reduce cellular ROS level effectively and the results was consistent with that of in vitro model against O_2^- and OH . In the experiment, it was confirmed that the extracts from *P. aibuhitensis* had antioxidant activity against O_2^- and OH . During the process of decocting, the majority of protein would inactivate and degrade to short peptide with small molecular weight. In this way, the short peptide might be the main factors to scavenge the free radicals in the extracts (data not shown). And more experiment should be done to determine the active ingredients of extracts.

5. Conclusions

The extracts from *P. aibuhitensis* were prepared by the traditional decocting method and it showed perfect cellular compatibility and benign antioxidant activity against O_2^- and OH . The cellular ROS level reduced effectively after the treatment with *P. aibuhitensis* extraction. Moreover, the fluorescent decreased with the increase of concentrations of *P. aibuhitensis* extracts. Future experiments will aim to determine the active ingredients of extracts. In summary, *P. aibuhitensis* extracts had a potential prospect as a biological antioxidant.

Acknowledgements

This work was supported by The Key Research and Investigate Plan of Shandong Province (2016YYSP014), and the Taishan Scholar Program, China.

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