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Evaluation of antibacterial and antioxidant activity of extracts of eelgrass *Zostera marina* Linnaeus

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***Zostera marina* L. is one of marine flowering plants, which plays an important role in coastal ecosystems by generating oxygen and organic substance and providing a habitat for other organisms. However, its residues on seashore have caused serious environmental problems in some coasts worldwide. In this paper, crude extract of *Z. marina* L. was prepared using methanol-water (1:1, v/v) and further separated into petroleum ether fraction, ethyl acetate fraction, *n*-butanol fraction and water fraction. The antibacterial activity and antioxidant activity of different fractions from *Z. marina* L. were evaluated in order to provide information for utilizing the marine plant. Antibacterial bioassay showed that *n*-butanol fraction was effective only for *Staphylocccus aureus*, petroleum ether fraction was effective for *S. aureus* and *Bacillus anthracis*, ethyl acetate fraction was effective for *S. aureus, B. anthracis, Diphtheroid bacilli* and *Staphylococcus epidermidis*, while water fraction had no effect on all the tested strains. Antioxidant assay indicated that all of the four fractions showed α-diphenyl-β-picrylhydrazyl (DPPH) radical-scavenging activity and Fe^{3+} reducing activity, and ethyl acetate fraction exhibited the maximum activity.**

Key words: *Zostera marina* L., extracts, antibacterial activity, antioxidant activity.

INTRODUCTION

Antibiotic resistance has become a global concern (Westh et al., 2004), and there has been an increasing incidence of multiple resistances in human pathogenic microorganisms in recent years, largely due to indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. Therefore, it is essential to search new antimicrobial substances from various sources like plants. Many researches have shown that higher plants represent a potential source of novel antibiotic prototypes (Afolayan, 2003; Clark et al., 1993; Srinivasan et al., 2001). Some traditional herbs have already been proved to be effective against antibiotic-resistant bacteria (Kone et al., 2004), and further research is necessary to identify the antibacterial compounds (Romero et al., 2005). The pharmacological studies of antibacterial compounds will lead to synthesis of a more potent drug with reduced toxicity (Ebana et al., 1991; Manna and Abalaka,

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2000). Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, oxygen-centered free radicals, which are continuously, produced *in vivo*, result in cell death and tissue damage. Oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell and Gutteridge, 1984). Although almost all organisms possess antioxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to prevent the damage entirely (Simic, 1988). However, antioxidant supplements, or foods containing antioxidants, may be used to help the human body reduce oxidative damage. Antioxidants are very important not only for the prevention of food oxidation but also for the defense of living cells against oxidative damage (Kim et al., 2003). Antioxidants reduce harmful reactive free radicals and reactive oxygen species (ROS) in cells (Chanwitheesuk et al., 2005), thereby preventing cancer and heart disease (Yan et al., 1998; Qi et al., 2005). Several synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) are extensively used because of their excellent efficacy and low cost. However, these artificial chemicals have major side-effects including toxicity and DNA damage problems (Choi et al., 1993; Sasaki et al., 2002). Therefore, the identification and isolation of new natural antioxidants from aquatic and terrestrial plants are also essential (Nishida et al., 1996).

Eelgrass (*Zostera marina* L.) is a flowering angiosperm belonging to Potamogetonaceae, which is an important species in coastal ecosystems because it contributes to nutrient cycling and sediment stabilizer, and provides food stuffs and habitat for many marine organisms such as invertebrates and fishes (Harrison, 1982; Moore and Short, 2006). Since halophytes growing in stressful environments make phenolic compounds to suppress the growth of yeast and mold (Bandaranayake, 2002), *Z. marina* L. might produce many secondary metabolites for protecting itself against microorganisms, epiphytes and predation (Harrison and Chan, 1980; Buchsbaum et al., 1990; Vergeer and Develi, 1997). Previous studies indicated that water-soluble extracts of eelgrass leaves could inhibit the growth of micro-algae and bacteria, and grazing by amphipods on dead leaves (Harrison, 1982; Lu and Foo, 1999). These observations indicate that eelgrass is a good resource for screening natural antibiotics.

Kawasaki et al. (1998) analyzed the compounds in the essential oil from eelgrass shoots and found that the major constituents were phytol, hexadecanamide, octadecanamide, pentadecane, heptadecane, nonadecane, (8*Z*,11*Z*)-heptadecadienal, (8*Z*)-heptadecenal, (9*Z*,12*Z*,15*Z*)-octadecatrienal and (9*Z*,12*Z*)-octadecadienal. Zosterin, a bioactive pectin from the eelgrass *Z. asiatica* Miki decreases the toxicity of antitumor drugs and purges heavy metals from human organisms (Loenko et al., 1977). Achamlale et al. (2009) isolated and quantified rosmarinic acid (RosA) in *Z. noltii* Hornem. and *Z. marina* L., and RosA from *Z. marina* L. showed good nematocidal activity and antibacterial activity against pine wood nematode and its associated four bacterial strains (Wang et al., 2012). Antioxidant activity for the polysaccharide zosterin isolated from *Z. marina* L. has been examined by the scavenging of free radical induced peroxide oxidation in mice, and results showed that the low etherified pectin normalizes the level of malonic dialdehyde and the activities of glutathione reductase and glutathione peroxidase in the liver (Khasina et al., 2003).

Eelgrass shows great potential in exploring anti-bacterial and antioxidant products, but there have been few studies about inhibition effects of extract of *Z. marina* L. on human pathogenic bacteria. In the present study, we prepared four extracts of *Z. marina* L. and evaluated their antioxidant and antimicrobial activities in order to lay a foundation for further utilizing this promising marine plant.

MATERIALS AND METHODS

Eelgrass collection and treatment

Living eelgrass (*Z. marina* L.) was collected at low tide from the sub-tidal beds at Qingdao, China. The eelgrass leaves were washed carefully in fresh water to remove sediment and dried naturally at room temperature, and then crushed in smaller pieces by a disintegrator. Prior to experiment, samples were stored in a refrigerator at 4°C.

Preparation of extracts of *Z. marina* L.

Crushed eelgrass powder 300 g was mixed with 12000 ml methanol:water (1:1, v/v), extracted for 4 h at 40°C and filtrated with filter paper. After the methanol in the extract was removed by vacuum evaporation, the remaining residue was dissolved in 200 ml distilled water followed by subsequent extraction with 200 ml petroleum ether, 200 ml ethyl acetate, and 200 ml *n*-butanol, respectively. Each fraction was also evaporated to dryness, and the residue of each fraction was dissolved in distilled water and stored at 4°C for further use. Each extraction was performed three times.

Assay of antimicrobial activity

The various fractions of *Z. marina* L. were used for assessing their antibacterial activity. The antimicrobial assay was performed by agar well diffusion method (Perez et al., 1990). Six bacteria were used in this study, the four Gram positive bacteria were *Staphylocccus aureus, Staphylococcus epidermidis, Bacillus anthracis* and *Diphtheroid bacilli*, while the two Gram negative bacteria used were *Escherichia coli* and *Typhoid bacillus.*

The nutrient broth medium plate was inoculated with the 100 µl bacterial suspension (1 x 10^8 cfu/ml), and then, wells was punched in the plates with a sterilized borer (6 mm in diameter). After 30 µl sample was introduced into each well, the plates were incubated at 37°C for 24 h in an incubator. 0.1 mg/ml Oxolinic acid was used as positive control and sterilized water was used as negative control. Antibacterial activity was assayed by measuring the diameter of the

Table 1. Inhibitory effect of *n*-butanol fraction on *Staphylocccus aureus.*

Bacteria	Concentration (mg/ml)	Diameter of inhibitory zone (mm)[a]	MIC (mg/ml)
Staphylocccus aureus	20.0	13.67 ± 1.70	20
	25.0	13.67 ± 0.47	
	30.0	14.33 ± 0.47	
	35.0	15.67 ± 0.47	
	40.0	16.67 ± 0.47	

a: Inhibition zones are the mean including cup borer (6 mm) diameter.

inhibition zone. Each experiment was performed three times and the mean values are presented.

The minimum inhibitory concentration (MIC) for each fraction was determined by agar dilution method. Each fraction of *Z. marina* L. was mixed with nutrient agar to final concentrations ranged from 0.156 to 20 mg/ml to prepare the nutrient broth medium plates. The plates were inoculated with the 100 µl bacterial suspension (1 x 10^8 cfu) respectively and incubated at 37°C for 24 h in an incubator. Oxolinic acid (0.1 mg/ml) was used as positive control and sterilized water was used as negative control. The lowest concentration of each fraction which could inhibit completely the growth of bacteria was defined as MIC.

α-Diphenyl-β-picrylhydrazyl radical-scavenging activity

To test radical scavenging activity, reactions containing α-diphenyl-β-picrylhydrazyl (DPPH) were carried out according to the method of Wangensteen et al. (2004). Each fraction of *Z. marina* L. was diluted with distilled water to various concentrations and mixed with equal volume of DPPH solution, which was prepared at a concentration of 0.2 mmol/L in ethanol. After standing for 30 min at room temperature, absorbance of each reaction system was measured by a UV-VIS spectrophotometer at 517 nm (Persee, TU-1810, China). For control, ethanol was mixed with equal volume of DPPH solution and incubated at room temperature for 30 min. All of the determinations were performed three times. Radical scavenging capacity was calculated as $(A_0 - A_i)/A_0 \times 100\%$, where A_0 is the absorbance of the control and A_i is the absorbance of treatment group. Ascorbic acid was used as the positive control, and the concentration range was 0.04 - 0.20 mg/ml.

Reducing power

Reducing activity of various fractions from *Z. marina* L. was determined according to the methods of Yen and Chen (1995). Briefly, 2.5 ml extract in phosphate buffer (0.2 mol/L, pH 6.6) were added to 2.5 ml potassium ferricyanide (10 g/L) and the mixture was incubated at 50°C for 30 min. After 2.5 ml trichloroacetic acid (100 g/L) was added to the mixture, the solution was centrifuged for 10 min at 3,000 rpm. The upper layer (2.5 ml) was mixed with 2.5 ml distilled water and 0.5 ml 0.1% ferric trichloride solution, and then absorbance of the resultant solution was recorded at 700 nm. The experiment was replicated for three times. Ascorbic acid was used as the positive control, and the concentration range was 0.02 - 0.10 mg/ml.

RESULTS

Antimicrobial activity

Different fractions isolated from *Z. marina* L. were assayed for their antibacterial activities. The results indicated that eelgrass extracts could only inhibit the growth of Gram positive bacterial strains, and had no effect on Gram negative bacterial strains such as *E. coli* and *T. bacillus.*

The antibacterial activities on the four Gram positive strains of various fractions of eelgrass also differed greatly. The *n*-butanol fraction was only effective for *S. aureus* (Table 1) with minimum inhibitory concentration (MIC) of 20 mg/ml, petroleum ether fraction was effective for *S. aureus* and *B. anthracis* (Table 2) with MIC of 0.156 and 1.25 mg/ml, respectively. Ethyl acetate fraction showed antibacterial activity against *S. aureus, B. anthracis, D. bacilli* and *S. epidermidis* (Table 3), with MIC of 0.156, 5, 5 and 10 mg/ml, respectively. Water fraction had no effect on all the tested bacterial strains. The negative control, sterilized water had no effect on all the tested strains, while the positive control, oxolinic acid was effective for all the tested strains at the concentration of 0.1 mg/ml.

Antioxidant activity

The various fractions of eelgrass crude extracts were investigated for their reducing power and DPPH radical scavenging activity. The ethyl acetate fraction showed the strongest antioxidant activity, followed by the *n*-butanol and water fraction (Tables 4 and 5). In the DPPH test, ethyl acetate fraction had the highest radical scavenging activity with EC_{50} of 0.278 mg/ml, and the inhibition rate reached 77.97% at the concentration of 0.5 mg/ml.

The *n*-butanol fraction and water fraction also exhibited DPPH radical scavenging activity to some extents, with EC_{50} of 3.110 and 4.849 mg/ml, respectively (Table 4). There was a significant difference among the EC_{50} of ethyl acetate fraction, n-butanol fraction, water fraction and ascorbic acid, and radical scavenging activity of the three fractions of eelgrass were less than that of ascorbic acid.

In the determination of reducing power, the reduction of Fe^{3+} to Fe^{2+} by various fractions of *Z. marina* L. was measured. Results showed that all of the fractions could reduce Fe^{3+} with different efficiency, and the reducing power of all the fractions increased with increase of

Table 2. Inhibitory effects of petroleum ether fraction on *Staphylocccus aureus* and *Bacillus anthracis.*

Bacteria	Concentrations (mg/ml)	Diameter of inhibitory zone (mm)[a]	MIC (mg/ml)
Staphylocccus aureus	0.2	10.67 ± 0.47	0.156
	0.4	11.33 ± 0.47	
	0.8	14.67 ± 0.94	
	1.2	17.33 ± 1.25	
	1.6	23.67 ± 1.25	
Bacillus anthracis	0.2	ND	1.250
	0.4	ND	
	0.8	ND	
	1.2	9.33 ± 0.47	
	1.6	13.00 ± 0.82	

a: Inhibition zones are the mean including cup borer (6 mm) diameter. ND: not detected.

Table 3. Inhibitory effects of ethyl acetate fraction on four sensitive bacterial strains.

Bacteria	Concentration (mg/ml)	Diameter of inhibitory zone (mm)[a]	MIC (mg/ml)
Staphylocccus aureus	0.2	10.33 ± 0.47	0.156
	0.4	11.33 ± 0.47	
	0.6	13.67 ± 0.47	
	0.8	16.67 ± 0.47	
	1.0	19.00 ± 0.82	
Bacillus anthracis	4.0	12.67 ± 0.47	1.250
	8.0	18.67 ± 0.94	
	12.0	20.33 ± 0.47	
	16.0	22.33 ± 0.47	
	20.0	23.00 ± 0.82	
Diphthemid bacillus	4.0	13.33 ± 1.25	5.000
	8.0	17.67 ± 0.47	
	12.0	21.33 ± 0.47	
	16.0	21.67 ± 0.47	
	20.0	21.67 ± 0.47	
Staphylococcus epidermidis	4.0	ND	10.000
	8.0	8.00 ± 0.82	
	12.0	10.67 ± 0.47	
	16.0	12.33 ± 0.47	
	20.0	13.33 ± 0.47	

a: Inhibition zones are the mean including cup borer (6 mm) diameter. ND: not detected.

concentration. The reducing power of the ethyl acetate fraction exhibited highest activity than that of the other fractions (Table 5), and the reducing power was 0.199 at the concentration of 0.1 mg/ml, which was similar to that for ascorbic acid at the concentration of 0.04 mg/ml. However, reducing power was significantly lower compared with that of ascorbic acid.

DISCUSSION

Intact eelgrass leaves decay very slowly (Wang et al., 2012), which indicated they have special chemical constitutes preventing microorganisms from utilizing them, and they are widely selected and used as roof materials in some seaside villages in Northern China

Table 4. Dose-dependent DPPH inhibition activity (%, n = 3) of different fractions.

Fraction	Concentration (mg/ml)	DPPH inhibition activity (%)	Regression equation
Petroleum ether fraction	0.20	4.439 ± 0.001	y=0.1291x+0.0096 R^2=0.9517 EC_{50}=3.799
	0.40	6.951 ± 0.001	
	0.60	7.454 ± 0.001	
	0.80	12.479 ± 0.001	
	1.00	13.149 ± 0.001	
Ethyl acetate fraction	0.10	26.957 ± 0.002	y=1.5362x+0.0744 R^2=0.9644 EC_{50}=0.278
	0.20	40.870 ± 0.000	
	0.30	59.130 ± 0.000	
	0.40	70.145 ± 0.000	
	0.50	77.971 ± 0.000	
n-Butanol fraction	1.00	22.995 ± 0.001	y=0.1415x+0.0600 R^2=0.9701 EC_{50}=3.110
	2.00	36.715 ± 0.001	
	3.00	53.527 ± 0.001	
	4.00	63.768 ± 0.000	
	5.00	71.208 ± 0.001	
Water fraction	2.00	23.722 ± 0.001	y=0.0885x+0.0710 R^2=0.9491 EC_{50}=4.849
	4.00	48.160 ± 0.000	
	6.00	70.450 ± 0.001	
	8.00	80.061 ± 0.005	
	10.00	85.583 ± 0.000	
Ascorbic acid	0.04	24.932 ± 0.001	y=3.8411x+0.0622 R^2=0.9709 EC_{50}=0.114
	0.08	38.571 ± 0.000	
	0.12	57.655 ± 0.001	
	0.16	69.767 ± 0.001	
	0.20	76.832 ± 0.001	

(Yang, 2012). Plants have developed chemical defenses against invasion of microorganisms. Vergeer et al. (1995) reported that the production of phenolic compounds in *Z. marina* L. increased when infected by *Labyrinthula zosterae*, indicating that the production of phenolic compounds is an antimicrobial response. In marine plants, bioactive chemicals such as phenolic compounds play important roles for plant survival and growth, and are now being used to explore new drugs and health foods for human (Baker and Joseph, 1984; Smit, 2004; Katalinic et al., 2006).

In the present study, extracts of *Z. marina* L., especially the ethyl acetate fraction, showed good antibacterial activity against Gram positive bacteria, but had no effect on Gram negative bacteria. The antibacterial activity of ethyl acetate fraction against *S. aureus* is higher than that of ethanol extract of *Ecballium elaterium* (Adwan et al., 2011), but the activity against *S. epidermidis* is little lower than that of water extract of *Aquilaria crassna* (Kamonwannasit et al., 2013). *B. anthracis* is much more sensitive to ethyl acetate fraction of *Z. marina* L. than that of stem bark crude extract of *Antidesma venosum* (Mwangomo et al., 2012). This result was consistent to the observation that Gram positive bacteria are more susceptible towards plants extracts as compared to Gram negative bacteria (Lin et al., 1999; Parekh and Chanda, 2006). These differences may be attributed to fact that the cell wall in Gram positive bacteria is of a single layer, whereas the Gram negative cell wall is multilayered structure (Yao and Moellering, 1991). Alternatively, the passage of the active compound through the Gram negative cell wall may be inhibited.

It is well known that ROS induces oxidative damage to biomolecules like nucleic acids, lipids, proteins and carbohydrates, which causes cancer and other disease in human bodies (Duan et al., 2006). Plant products such as flavonoids, cumarins, phenolic acids and terpenoids are reported to have DPPH scavenging activity (Puertas-

Table 5. Dose-response of reducing power (mean ± SE, n = 3) of different fractions.

Fraction	Concentrations(mg/ml)	OD at 700 nm	Regression equation
Petroleum ether fraction	0.20	0.005 ± 0.000	y=0.0187x+0.0007 R^2=0.9951
	0.40	0.009 ± 0.000	
	0.60	0.012 ± 0.001	
	0.80	0.015 ± 0.000	
	1.00	0.019 ± 0.001	
Ethyl acetate fraction	0.10	0.199 ± 0.000	y=1.8486x+0.0162 R^2=0.9974
	0.20	0.417 ± 0.000	
	0.30	0.577 ± 0.001	
	0.40	0.739 ± 0.000	
	0.50	0.938 ± 0.000	
n-Butanol fraction	1.00	0.183 ± 0.001	y=0.1466x+0.0191 R^2=0.9969
	2.00	0.325 ± 0.000	
	3.00	0.464 ± 0.000	
	4.00	0.587 ± 0.000	
	5.00	0.756 ± 0.000	
Water fraction	1.00	0.178 ± 0.000	y=0.1554x+0.0066 R^2=0.9953
	2.00	0.304 ± 0.000	
	3.00	0.493 ± 0.001	
	4.00	0.598 ± 0.000	
	5.00	0.798 ± 0.000	
Ascorbic acid	0.02	0.131 ± 0.000	y=5.1257x+0.0100 R^2=0.9949
	0.04	0.198 ± 0.000	
	0.06	0.329 ± 0.000	
	0.08	0.425 ± 0.001	
	0.10	0.515 ± 0.000	

Mejía et al., 2002). Jiménez-Escrig et al. (2001) found that DPPH radical scavenging activities are positively correlated with the total polyphenol contents in aqueous and organic solvent extracts of brown and red algae. At the same concentration, the DPPH quenching activity of non-polar solvents (chloroform, ethyl acetate and acetone) extracts was stronger than that of polar solvents (water, ethanol, methanol) extracts of Rhodomelaceae seaweeds (Yan et al., 1998; Yuan et al., 2005). Four solvents were used to prepare the extracts of eelgrass in this study and the resultant fractions were investigated for their antioxidant activity. The ethyl acetate fraction of *Z. marina* L. showed the best DPPH radical scavenging activity and reducing power compared with other solvent fractions.

In conclusion, *Z. marina* L. is an aquatic plant found in wide grasslands that contributes to the coastal ecosystems. However, the residues reaching the coastlines cause an environmental problem with high costs for their disposal. In this study, we found that the ethyl acetate fraction of *Z. marina* L. possessed both antibacterial and antioxidant activities, and this study will lay a solid foundation for utilizing this versatile plant in the future.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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