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Antimicrobial activity of extracts from giant knotweed *Polygonum sachalinense* against animal pathogenic bacteria

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Abstract

The antimicrobial activities of extracts from the leaves and rhizomes of giant knotweed *Polygonum sachalinense* against animal pathogenic bacteria were investigated. The methanol extracts from both the dried leaves and dried rhizomes of the plant had the strongest effective inhibitory activity against *Photobacterium damselae* subsp. *piscicida* (*Pasteurella piscicida*) among 16 species (27 strains) of fish and veterinary pathogenic bacteria. Subsequently the methanol extracts from the leaves and rhizomes were serially partitioned between organic solvents and water. Five fractions extracted with hexane, chloroform, ethyl acetate, 1-butanol and, residual water were consequently obtained. Of these fractions, the ethyl acetate- and 1-butanol-separated fractions from both the leaves and rhizomes had greater inhibitory activity against *P. damselae* subsp. *piscicida*. The ethyl acetate-soluble fraction in methanol extracts from the rhizome was confirmed to show the similar antimicrobial activity against 12 strains of *P. damselae* subsp. *piscicida*, including some antibiotic-resistant strains.

Key words: Antimicrobial activity, *Photobacterium damselae* subsp. *piscicida, Polygonum sachalinense*, Rhizome, Leaf

Introduction

It is undoubtedly essential to maintain the health and sound growth of livestock, poultry, and cultured fish which play prominent roles in our dietary life. Many pharmaceuticals or chemicals including antibiotics have been developed for that purpose. Recently, novel antimicrobial substances from natural sources have been desired as substitutes for antibiotics, to avoid appearance of antibiotic-resistant bacteria and remaining of antibiotics. The botanical products like extracts or essential oils from herbs and spices have been remarked as potential antimicrobial agents or biopreservatives (Cowan, 1999; Draughon, 2004).

Giant knotweed (Sakhalin knotweed), *Polygonum sachalinense* F. Schmidt ex Maxim. (Polygonaceae), is a perennial plant widely distributed in northern Japan and has spread to North American and European countries. This plant is strongly rhizomatous and the taller one can grow up to 3 m tall. Based on its vigorous growth, this plant could provide a potential source of biomass, though it might also create ecological problems. Giant knotweed, as well as its closely related species, Japanese knotweed *Polygonum cuspidatum* Siebold & Zucc., has been treated as an edible plant for years. Their younger buds and stems have been cooked as a green vegetable. The rhizomes of both Japanese and giant knotweeds have been used in traditional medicine as a hydragogue, aperient, emmenagogue, and balm (Okuda, 1986).

The physiological activities and constituents of *P. cuspidatum* and *P. sachalinense* have been subjected to considerable study (Nonomura et al., 1963; Kudo et al., 1966; Kimura et al., 1983; Yeh et al., 1988; Inoue et al., 1992; Jayasuriya et al., 1992; Vastano et al., 2000; Xiao et al., 2002). Saito et al. (1997) showed that the extracts from both the leaves and rhizomes of *P. sachalinense* have antimicrobial activities against several food-borne bacteria. Konstantinidou-Doltsinis and Schmitt (1998) also reported that this plant's extracts have a fungicidal activity against fungi which cause powdery mildew in cucumbers. The present paper describes the inhibitory activities of the extracts from giant knotweed against certain fish and livestock pathogens.

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Materials and Methods

Preparation of extracts from leaves and rhizomes of giant knotweed

Leaves and rhizomes of giant knotweed *P. sachalinense* were collected in Hakodate, Hokkaido, Japan. They were washed in tap water and air-dried at room temperature. The leaves and rhizomes were then broken into pieces and individually immersed in six-fold volumes of methanol for 10 d. The solvent was removed using a rotary evaporator. Furthermore, a part of the methanol extracts obtained were suspended with water and extracted in turn with an equal volume of hexane, chloroform, ethyl acetate, and 1-butanol. The phase-separated fractions were evaporated *in vacuo*.

Bacterial strains

Sources of bacterial strains used for antimicrobial assay are represented in Tables 1 and 2. Test strains are pathological isolates from diseased fish or livestock, stock cultures in Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan, cultures from American Type Culture Collection, Rockville, MD, USA, and cultures for animal hygiene supplied through Japanese Association of Veterinary Biologics, Tokyo, Japan. *Staphylococcus aureus* 209P and *Escherichia coli* NIHJ were also used as standard strains for minimum inhibitory concentration (MIC) measurement specified by the Japanese Society of Chemotherapy (1981).

For fish pathogenic bacteria, the strains were maintained on Brain Heart Infusion (BHI) agar (Eiken Chemical, Co., Ltd., Tokyo, Japan) containing 2.0% NaCl at 25°C. These strains were freshly cultured in Mueller-Hinton broth (Difco Laboratories, Detroit, MI, USA) containing 2.0% NaCl at 25°C for 18 h. The veterinary-related bacteria were maintained on BHI agar and freshly cultured in Mueller-Hinton broth at 37°C for 18 h. These cultures were diluted to 10^6 cells/ml with the same culture media before antimicrobial assay.

Antimicrobial assay

Antimicrobial assay was conducted by the method based on the National Committee for Clinical Laboratory Standards (NCCLS, 2002). MIC of the extracts was determined by agar dilution method. A loopful of the pre-cultured microbial cells with the culture broth was spotted on Mueller-Hinton agar containing various concentrations of the extracts, and the plates were incubated at 25 or 37°C for 48 h. In the case of fish pathogenic bacteria, media included 2.0% NaCl. Antimicrobial activity was determined according to the inhibition of colonization.

The antimicrobial activity of the extracts was also measured by disk diffusion method on the agar plates.

Table 1. Minimum inhibitory concentration (μg/ml) of methanol extracts from *Polygonum sachalinense* against fish pathogenic bacteria

Bacterial species	Strain	Leaf extract	Rhizome extract	Source
Photobacterium damselae subsp. *piscicida*	DPp-1	50	50	Kidney, yellowtail
	DPp-2	50	100	Kidney, yellowtail
	DPp-3	100	100	Kidney, yellowtail
Vibrio anguillarum	DVa-1	>1,000	>1,000	Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan
	DVa-2	>1,000	>1,000	Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan
Vibrio parahaemolyticus	DVp-1	>1,000	>1,000	Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan
Aeromonas salmonicida subsp. *salmonicida*	ATCC14174	>1,000	>1,000	American Type Culture Collection, Rockville, MD, USA
	DAs-2	250	250	Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan
Aeromonas hydrophila	ATCC7966	1,000	>1,000	American Type Culture Collection, Rockville, MD, USA
	DAh-2	>1,000	>1,000	Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan
Edwardsiella tarda	FPC-22	>1,000	1,000	The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan
Enterococcus seriolicida	DEs-1	>1,000	1,000	Kidney, yellowtail
	DEs-2	>1,000	1,000	Kidney, yellowtail
	DEs-3	>1,000	1,000	Kidney, yellowtail

Table 2. Minimum inhibitory concentration (μg/ml) of methanol extracts from *Polygonum sachalinense* against veterinary-related bacteria

Bacterial species	Strain	Leaf extract	Rhizome extract	Source
Escherichia coli	NIHJ	>1,000	>1,000	Standard strain specified by Japanese Society of Chemotherapy, Tokyo, Japan
	S6W	>1,000	>1,000	Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan
Salmonella Enteritidis	DSe-1	>1,000	>1,000	Feces, human
Salmonella Dublin	ST-8	>1,000	>1,000	Feces, cattle
Salmonella Typhimurium	SIC-8401	>1,000	>1,000	The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan
Bordetella bronchiseptica	S1	>1,000	>1,000	Snivel, swine
	SM2-4	>1,000	>1,000	Snivel, swine
Pasteurella multocida	TI-18	>1,000	>1,000	Lights, cattle
Mannheimia haemolytica	N-791	1,000	500	Nisseiken, Co. Ltd., Tokyo, Japan
Staphylococcus aureus	209P	>1,000	1,000	Standard strain specified by Japanese Society of Chemotherapy, Tokyo, Japan
	TI-1	>1,000	1,000	Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan
Streptococcus sp.	1032-B	>1,000	>1,000	Milk, cattle
	76-LB-B	>1,000	1,000	Milk, cattle

The bacterial test strains were pre-cultured at appropriate conditions and mixed with a Mueller-Hinton agar to make a plate. Then, paper disks (8 mm in diameter, thick-type, Toyo Roshi Kaisha, Tokyo, Japan) bearing 1 mg of the extracts were placed on the agar media inoculated with the test bacteria, and the plates were incubated for 48 h. The diameter of the inhibition zone was measured as a positive indication of the antibacterial activity.

To compare antimicrobial activity, ampicillin, florfenicol and thiamphenicol (Sigma Chemical Co., St. Louis, MO, USA) were used.

Results

Inhibitory activity of methanol extracts from giant knotweed against fish and veterinary pathogenic bacteria

The MIC values of methanol extracts from giant knotweed against various species of pathogenic fish and veterinary-related bacteria are shown in Tables 1 and 2, respectively.

The methanol extracts of both leaves and rhizomes were specifically inhibitive against three strains of *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*) with 50-100 μg/ml of MIC. The extracts also showed inhibitory activity against a strain of *Aeromonas salmonicida*, although much less than against *P. damselae* subsp. *piscicida*. Veterinary-related *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*) N-791 was somewhat inhibited by the rhizome extract. The other test strains were not influenced by the methanol extracts from both leaves and rhizomes.

Inhibitory activity of solvent-separated fractions from the methanol extracts against Photobacterium damselae subsp. piscicida

The methanol extracts from leaves and rhizomes of the knotweed were serially separated to five fractions with various polarities of solvents. The antibacterial activities of these fractions from the plant against a fish pathogenic strain *P. damselae* subsp. *piscicida* DPp-1 evaluated by the disk diffusion method are shown in Table 3.

The fractions extracted with ethyl acetate and 1-butanol for both methanol extracts of the leaves and rhizomes had relatively greater inhibitory activities against the test strain. As well, the fractions separated with hexane, chloroform, and water from leaves, and chloroform and water fractions from rhizomes showed somewhat antibacterial activities. Of all the fractions separated from the methanol extracts, the ethyl acetate-soluble fraction from rhizomes showed the highest activity.

The yields of methanol extracts and their separated fractions with various solvents from leaves and rhizomes are also shown in Table 3. From one kg of dried leaves and rhizomes were obtained 204.04 and 72.35 g of methanol extracts, respectively. The ethyl acetate fractions extracted from the methanol extracts of leaves and rhizomes were 14.79 and 5.30 g, respectively.

Table 3. Inhibitory activities against *Photobacterium damselae* subsp. *piscicida* DPp-1 and yields of solvent-separated fractions from leaf and rhizome extracts of *Polygonum sachalinense*

Fraction	Inhibitory diameter (mm)[a]		Yield (g/kg dried matter)	
	Leaf extract	Rhizome extract	Leaf extract	Rhizome extract
Methanol	20.0	31.5	204.04	72.35
Hexane	17.1	—[b]	25.40	0.20
Chloroform	20.0	11.0	10.22	2.51
Ethyl acetate	30.0	39.4	14.79	5.30
1-Butanol	29.5	35.0	38.38	18.86
Water	19.8	18.8	92.21	42.94

[a] Inhibitory activities are represented as diameter (mm) in clear zones around the disks bearing 1 mg of each fraction.
[b] Inhibitory zone was not detected.

Table 4. Minimum inhibitory concentration (μg/ml) of the ethyl acetate-soluble fraction from *Polygonum sachalinense* rhizome and antibiotics against various strains of *Photobacterium damselae* subsp. *piscicida*

Bacterial strain[a]	Test fraction	Ampicillin	Florfenicol	Thiamphenicol
DPp-1	25	<0.05	0.2	>100
DPp-2	50	<0.05	0.2	6.25
DPp-3	25	<0.05	25	>100
DPp-4	25	50	0.2	>100
DPp-5	25	<0.05	12.5	>100
DPp-151	50	<0.05	12.5	>100
DPp-171	25	<0.05	25	>100
DPp-174	25	50	0.2	>100
DPp-175	25	<0.05	0.2	>100
DPp-209	50	50	0.2	>100
DPp-219	25	<0.05	6.25	>100
DPp-225	25	50	0.2	>100

[a] All bacterial strains were isolates from kidney of diseased yellowtail.

Inhibitory activity of the ethyl acetate-soluble fraction against various strains of Photobacterium damselae subsp. piscicida

Antibacterial activity of the ethyl acetate-soluble fraction in methanol extracts from the rhizome was evaluated by the serial dilution assay on agar plates against 12 strains of *P. damselae* subsp. *piscicida*, comparing to that of three antibiotics (Table 4). Some test strains had resistant properties against ampicillin, florfenicol, and/or thiamphenicol. The fraction showed the similar inhibitory activities with 25-50 μg/ml of MIC against all strains including antibiotic-resistant strains.

Discussion

The extracts from the leaves and rhizomes of giant knotweed showed an efficacy on *P. damselae* subsp. *piscicida* among many fish pathogenic bacteria. *P. damselae* subsp. *piscicida* is a gram-negative and halophilic coccobacillus. This bacterium is a pathogen for pasteurellosis, pseudotuberculosis, notably in yellowtail and sea bream, which is one of most prominent diseases in aquaculture in many countries. The infected fish suffers small white spots on the kidney, spleen and branchiae and generally dies within weeks (Zorrilla et al., 1999; Romalde, 2002).

In the aquaculture industry, frequent outbreak of fish diseases has constituted an important problem, causing significant economic losses. Pharmaceuticals including antibiotics have been used appropriately in diseased fish, observing the relevant regulations to avoid the appear-

ance of resistance; nevertheless, antibiotic-resistant bacteria have often appeared. *P. damselae* subsp. *piscicida* has been shown to gain resistance to many antibiotics (Aoki, 1992).

Ampicillin, florfenicol and thiamphenicol are known to be effective antibiotics for preventing pasteurellosis by *P. damselae* subsp. *piscicida*. So we used these antibiotics to compare inhibitory activity of the fraction. As pointed out above, actually, some of the test strains used in this study showed resistant properties against these antibiotics.

Vaccination is an effective method for preventing microbial infection. The vaccines against fish diseases have been developed. In several European countries, the vaccine has been successfully employed for preventing pasteurellosis of fish. However, the vaccine for pasteurellosis of yellowtail has not been practically available in Japan (Nakanishi, 1998).

Mannheimia haemolytica also showed certain sensitivity to the methanol extract of *P. sachalinense*. *M. haemolytica* is a weakly hemolytic, gram-negative coccobacillus. This is an etiological agent of bovine pneumonic pasteurellosis, also called bovine respiratory disease, that is economically significant to both the beef and the dairy cattle industry in North America and Western Europe (Jeyaseelan et al., 2002).

Both *P. damselae* subsp. *piscicida* and *M. haemolytica* are members of the family Pasteurellaceae and etiological agents of pasteurellosis in animals. They might possess similarities in their cell surface structures, which are related to virulence factors, including the capsule, outer membrane proteins, and adhesion properties (Magariños et al., 1992; Magariños et al., 1996a; Magariños et al., 1996b).

It is suggested that the extracts from giant knotweed *P. sachalinense* might have a potential as antimicrobial substances against pathogens for pasteurellosis. Further researches are required to elucidate the mode of antimicrobial action of the plant extracts, and to determine the main constituents responsible for the antimicrobial action of the extracts.

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