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In vitro Antibacterial Activity of Herbal Medicines and Combinations of Herbal Medicines and Antibiotics against *Edwardsiella tarda*

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Abstract

The antibacterial activity of five medicinal herbs, alone and together with other herbs or one of six antibiotics, against *Edwardsiella tarda* was determined by World Health Organization-International Collaborative Study (WHO-ICS) agar dilution protocol. Minimal inhibitory concentrations (MIC) of the herbs were significantly lower (≤4 mg/ml) in treatments using the combinations *Rhizoma coptidis*+*Radix scutellariae*, *Galla chinensis*+*Radix et Rhizoma rhei*, *Galla chinensis*+*Radix scutellariae*, and *Radix et Rhizoma rhei*+*Radix scutellariae* than in treatments using *Rhizoma coptidis*, *Radix et Rhizoma rhei*, or *Flos lonicerae* (≥32 mg/ml). Almost all the combination treatments were more effective than treatment by a single herb. Combinations containing herbal medicines and regular antibiotics resulted in varying results: antibacterial effects increased with some combinations and decreased with others. *Galla chinensis* is suitable for use with most antibiotics, while streptomycin sulfate is suitable for use with many herbal medicines.

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Introduction

Edwardsiella tarda, a gram-negative bacterium of the family Enterobacteriaceae, is the causative agent of one of the most serious bacterial diseases of freshwater and marine fish in farmed and wild populations throughout the world. Outbreaks of the disease, called edwardsiellosis, have been recorded in an array of commercially important fish, including crimson sea bream (Kusuda and Kawai, 1998), eels (Wakabayashi and Egusa, 1973), channel catfish (Meyer and Bullock, 1973), tilapia (Kubota et al., 1981), Chinook salmon (Amandi et al., 1982), flounder (Nakatsugawa, 1983), and carp (Sae-Oui et al., 1984). The bacterium also causes disease in reptiles, birds (Sae-Oui et al., 1984; Van Damme and Vandepitte, 1984), and mammals (White et al., 1973; Van Damme and Vandepitte, 1984), including humans (Ma et al., 1998). In fish, typical symptoms of edwardsiellosis are septicaemia with extensive skin lesions and swelling, plus hemorrhage and necrosis of the liver, spleen, and other tissues. Eventually, the infection spreads throughout the internal organs and muscles, with suppurative abscesses being the main characteristic.

Many antibiotics have been effective against *E. tarda*, including gentamicin sulfate, tetracycline hydrochloride, and streptomycin sulfate (Kashiwaga et al., 1980; Chen et al., 1984). However, due to acquired resistance by pathogens, most of these have become ineffective and their widespread usage has given rise to many mutant resistant strains (Aoki and Kitao, 1981; Choi and Park, 1995). Hence, there is an urgent need to find new drugs that can combat resistant strains.

Herbal medicines have a broad range of anti-bacterium and anti-virus effects, have relatively low toxic side effects, and are inexpensive. Traditional Indian herbal medicines have an effect on *Aeromonas hydrophila* (Bhuvaneswari and Balasundaram, 2006). Resistance is not easily generated in the pathogen when compounds of herbal medicines are applied (Li et al., 2007). Herbal medicines can improve the immunity of host organisms (Wen and Liu, 2004) and may have associative effects between the pathogen and the host. However, high medicine concentrations in the blood of the host are difficult to maintain using herbal medicines, and they require large doses. Hence, application of herbal medicines combined with antibiotics is proposed to treat illnesses caused by bacteria with high resistance.

In this study, the anti-bacterium effects of five medicinal herbs in combination with six antibiotics was measured by growing *E. tarda* with the goals of finding new prescriptions against *Edwardsiella* and providing a theoretical foundation for combining herbal medicines and antibiotics.

Materials and Methods

Bacterial strain. The ATCC 15947 strain of *Edwardsiella tarda* used in this study was obtained from infected marine Malaba grouper, *Epinephelus malabaricus* (Haifa Titbit Enterprise Development Co. Ltd., Tianjin) and identified by the College of Biotechnology, Tianjin University of Science and Technology. The bacteria were maintained in glycerol broth at -80°C until use.

The bacteria were inoculated into Mueller Hinton (M-H) medium, placed in a shaker at 28°C, and cultivated for 24 h. The concentration of the bacteria solution was determined and the solution was diluted with M-H culture medium to reach a concentration of 4.0×10^6 cfu/ml.

Medicinal herbs and antibiotics. The following medicinal herbs (Anshun Pharmaceuticals, Tianjin) were used: *Rhizoma coptidis* (coptis root)*, Galla chinensis* (leaf gall)*, Radix et Rhizoma rhei* (rhubarb)*, Radix scutellariae* (Baikal skullcap root)*,* and *Flos lonicerae* (honeysuckle flower). To prepare the herbal medicines, 50 g of the herb was placed in a flask. To prepare combinations of two herbs, 25 g of each herb was used, while in combinations of three herbs, 16.7 g of each

was used. The herbs were soaked in water (3-8 times the amount of the herb), except for *Radix et Rhizoma rhei* which was soaked in ammonia (0.3%), for 2-4 h, heated to boiling, filtered through four layers of gauze, decocted twice for 20-30 min, and mixed with water (50 ml) to obtain a concentration of 1 g herb/ml solution. The herbal medicines were stored at 4°C and used within one week after sterilization at 121°C for 20 min.

The following antibiotics were used: kanamycin sulfate, tetracycline hydrochloride, rifampicin, streptomycin sulfate, gentamycin sulfate, and cefotaxim sodium (AMRESCO, USA). The antibiotics were diluted with physiological saline to obtain a concentration of 1000 μg/ml.

Susceptibility test. Minimum inhibitory concentrations (MIC) of the herbs and antibiotics were determined using the World Health Organization-International Collaborative Study agar dilution protocol (Ericsson and Sherris, 1971; Washington and Sutter, 1980) in 10 x 10 cm petri plates prepared as follows. Sterilized M-H agar was melted and 16 ml of the agar was extracted under sterile conditions and placed in a conical flask. The experimental herb, antibiotic, or combination of herb(s)/antibiotic (4 ml) was also placed into the flask. The solution was shaken and poured onto the experimental plates.

Suspensions of log phase strains were adjusted so that 10^5 strains were inoculated in 0.002 ml volumes onto the surface of the plates using a Steers' replicator (Steers and Foltz, 1959). The plates were incubated at 31°C for 18 h after which the MIC was determined. For all drugs, the MIC was defined as the lowest concentration of drug resulting in complete inhibition of visible growth.

All treatments, including positive and negative controls, were tested in five replicates.

Testing of individual herbs. The five medicinal herbs listed above were tested at nine dilutions: 1, 2, 4, 8, 16, 32, 64, 128, and 256 mg/ml. For a positive control, plates contained bacteria but no herbs. For a negative control, plates contained 1 mg/ml solution but no bacteria. The total number of plates in this test was 235.

Testing of combinations of herbs. Nine combinations of two or three herbs were tested at the dilutions mentioned above. Control groups were as above. The total number of plates in this test was 415.

The six antibiotics listed above were tested at nine dilutions: 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64 μg/ml. For a positive control, plates contained bacteria but no antibiotics. For a negative control, plates contained 0.25 μg/ml antibiotic but no bacteria. The total number of plates in this test was 280.

Testing of herbs together with antibiotics. Forty-one combinations of herbs and antibiotics were tested at nine dilutions: 0.5/0.125, 1/0.25, 2/0.5, 4/1, 8/2, 16/4, 32/8, 64/16, and 128/32 (mg herb/μg antibiotic per ml solution). For a positive control, plates contained bacteria but no herb or antiobiotics. For a negative control, plates contained 0.5 mg herb plus 0.125 μg antibiotic per ml, but no bacteria. The total number of plates in this test was 1855.

Results

Testing of individual herbs. Except for *Flos lonicerae*, all MIC_{50} values were ≤32 mg/ml and all MIC_{90} values were ≤8 mg/ml (Table 1).

Testing of combinations of herbs. Four of the herbal combinations demonstrated evident antibacterial effects with MIC_{50} values of ≤2 mg/ml and MIC_{90} values ≤4 mg/ml (Table 2). Two of the combinations prevented even minimal antibacterial ability, indicated by an MIC_{90} value under 1 mg/ml. These two could be considered new herbal medicines for treating *Edwardsiella* among fish.

Table 1. Growth of the bacteria *Edwardsiella tarda* in solutions containing medicinal herbs (mg/ml).

	Medicinal herb				
	Rhizoma coptidis	*Galla chinensis*	*Radix et Rhizoma rhei*	*Radix scutellariae*	*Flos lonicerae*
Concentration (range)	4-64*	1-32	4-64	1-32	64-256
MIC_{50}	8	2	8	4	128
MIC_{90}	32	16	32	16	256

* Bacteria colonies in concentrations below 4 mg/ml did not differ from those of the positive control. No bacteria grew in concentrations above 64 mg/ml.

Table 2. Growth of the bacteria *Edwardsiella tarda* in solutions containing two or three medicinal herbs (mg/ml).

	*Combination of medicinal herbs**								
	RC+ RS	*GC+ RRR*	*GC+ RS*	*RC+ RRR +RS*	*RRR+ FL*	*RC+ RRR*	*RRR+ RS*	*RC+ GS*	*RS+ FL*
Concentration (range)	*1-32*	*1-32*	*1-32*	*1-32*	*1-32*	*1-32*	*1-32*	*1-32*	*1-32*
MIC_{50}	2	<1	<1	4	2	1	<1	2	1
MIC_{90}	4	<1	<1	8	8	8	2	32	16

* RC = *Rhizoma coptidis*, RS = *Radix scutellariae*, GC = *Galla chinensis*, RRR = *Radix et Rhizoma rhei*, FL = *Flos lonicerae*

Testing of antibiotics. All six antibiotics had relatively good antibacterial ability as all MIC_{50} values were ≤2 μg/ml and all MIC_{90} values were ≤4 μg/ml (Table 3). Gentamycin sulfate was the best as both MIC_{50} and MIC_{90} were under 0.25 μg/ml.

Testing of herbs together with antibiotics. When combined with any of the six antibiotics, the herb *Galla chinensis* produced the best results; the MIC90 value of *Galla chinensis* dropped from 16 mg/ml to less than 1 mg/ml (Table 4). In combination with *Galla chinensis,* the MIC90 value for five of the antibiotics also dropped: from 4 μg/ml to less than 0.25 μg/ml for streptomycin sulfate, from 2 μg/ml to less than 0.25 μg/ml for cefotaxim sodium and rifampicin, and from 1 μg/ml to less than 0.25 μg/ml for kanamycin sulfate and tetracycline hydrochloride. The antibacterial effect of streptomycin sulfate was improved when combined with seven combinations of herbs: the MIC90 value dropped from 4 μg/ml to less than 0.25 μg/ml when combined with *Galla chinensis*, *Radix et Rhizoma rhei* and *Galla chinensis,* or *Radix scutellariae* and *Galla chinensis*, to less than 0.5 μg/ml when combined with *Rhizoma coptidis* or *Rhizoma coptidis* and *Radix et Rhizoma rhei* and

Table 3. Growth of the bacteria *Edwardsiella tarda* in solutions containing an antibiotic (μg/ml).

	Antibiotic					
	Kanamycin sulfate	*Tetracycline hydrochloride*	*Rifampicin*	*Streptomycin sulfate*	*Gentamycin sulfate*	*Cefotaxim sodium*
Concentration (range)	0.25-2	0.25-2	0.5-4	0.5-8	0.25-2	0.5-4
MIC_{50}	0.5	0.5	1	2	<0.25	1
MIC_{90}	1	1	2	4	<0.25	2

Radix scutellariae, and to less than 2 mg/ml when combined with *Radix et Rhizoma rhei* or *Rhizoma coptidis* and *Radix scutellariae*.

In addition, protection improved with the following combinations: *Rhizoma coptidis* and rifampicin*, Rhizoma coptidis* and cefotaxim sodium, *Rhizoma coptidis* and *Radix scutellariae* and kanamycin sulfate.

Other combinations did not produce obvious changes or results worsened. The worst combination was *Radix et Rhizoma rhei* and *Rhizoma coptidis* and *Radix scutellariae*.

Discussion

The five herbs used in this study are capable of suppressing or killing pathogens such as bacteria and viruses. *Galla chinensis* has relatively good antibacterial effects against *Edwardsiella tarda* (Jiang and Zheng, 2005). In this experiment, MIC_{90} values of *Galla chinensis* and *Radix scutellariae* were higher than reported by Zhu and Shi (2007), perhaps due to the difference in strains. *Galla chinensis* can improve nonspecific immunity of the fish body and has good effects in terms of preventing and treating blood poisoning (Jiang, 2006). Among the nine combinations of herbs, all demonstrated increased antibacterial effects except *Rhizoma coptidis* and *Galla chinensis,* and *Radix scutellariae* and *Flos lonicerae.* The dissolution rate (effective ingredient/total weight of the herb) almost doubled for the combination of *Radix et Rhizoma rhei* and *Radix scutellariae* which contains the compounds anthraquinone and flavone (Lin et al., 1989; Guan et al., 2000). For the combination of *Galla chinensis* and *Radix scutellariae*, the dissolution rate of its ingredient, berberine, was higher when decocting the herbs together than decocting each herb separately (Li, 1998). The mechanism of associative effects of the combination of *Flos lonicerae* and *Radix et Rhizoma rhei* probably resulted from a change in cell membrane potential (Kang, 2003). Acidity of *Flos lonicerae* can change the permeability of materials inside and outside of membranes and allow materials like anthraquinone to enter the bacteria and take effect in the corresponding site (Kang, 2003). The combination of *Radix et Rhizoma rhei* and *Galla chinensis* has good suppressing effect against streptococcus (Xiong et al., 2005).

The antibiotics used in this experiment have similar antibacterial effects against *E. tarda* as reported by Stock (2001). The antibacterial effect increased for certain combinations of herbal medicines and antibiotics, and decreased for others. *Galla chinensis* can be used

Table 4. Growth of the bacteria *Edwardsiella tarda* in solutions containing different concentrations* of one, two, or three medicinal herbs and one antibiotic.

Medicinal herb(s)	*Antibiotic*					
	Kanamycin sulfate	*Tetracycline hydrochloride*	*Rifampicin*	*Streptomycin sulfate*	*Gentamycin sulfate*	*Cefotaxim sodium*
Rhizoma coptidis						
Concentration range	1-8/0.25-2	1-8/0.25-2	1-8/0.25-2	1-8/0.25-2	1-4/0.25-1	1-8/0.25-2
MIC_{50}	1/0.25	<1/<0.25	1/0.25	<1/<0.25	<1/<0.25	<1/<0.25
MIC_{90}	2/0.5	2/0.5	2/0.5	2/0.5	2/0.5	2/0.5
Galla chinensis						
Concentration range	1-4/0.25-1	1-4/0.25-1	1-4/0.25-1	1-4/0.25-1	1-4/0.25-1	1-4/0.25-1
MIC_{50}	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25
MIC_{90}	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25
Radix et Rhizoma rhei						
Concentration range	1-8/0.25-2	1-8/0.25-2	2-16/0.5-4	1-8/0.25-2	1-4/0.25-1	1-8/0.25-2
MIC_{50}	2/0.5	2/0.5	4/1	2/0.5	1/0.25	2/0.5
MIC_{90}	4/1	4/1	8/2	4/1	2/0.5	4/1
Rhizoma coptidis+Radix scutellariae						
Concentration range	1-4/0.25-1	2-16/0.5-4	1-8/0.25-2	1-8/0.25-2	1-4/0.25-1	1-8/0.25-2
MIC_{50}	<1/<0.25	4/1	2/0.5	2/0.5	1/0.25	2/0.5
MIC_{90}	<1/<0.25	8/2	4/1	4/1	2/0.5	4/1
Galla chinensis+Radix et Rhizoma rhei						
Concentration range	1-4/0.25-1	1-4/0.25-1	1-4/0.25-1	1-4/0.25-1	1-4/0.25-1	1-4/0.25-1
MIC_{50}	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25
MIC_{90}	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25
Galla chinensis+Radix scutellariae						
Concentration range	1-4/0.25-1	1-4/0.25-1	1-4/0.25-1	1-4/0.25-1	1-4/0.25-1	1-4/0.25-1
MIC_{50}	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25
MIC_{90}	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25	<1/<0.25
Rhizoma coptidis+Radix et Rhizoma rhei+Radix scutellariae						
Concentration range	2-16/0.5-4	2-16/0.5-4	1-8/0.25-2	1-4/0.25-1	1-8/0.25-2	1-8/0.25-2
MIC_{50}	4/1	2/0.5	2/0.5	<1/<0.25	2/0.5	2/0.5
MIC_{90}	8/2	8/2	4/1	2/0.5	4/1	4/1

* Concentrations are shown as mg of herbal medicine/µg of antiobiotic per ml solution.

with most antibiotics while streptomycin sulfate can be used in combination with most medicinal herbs.

In this experiment, direct antibacterial effects of herbal medicines on *in vitro* growth of *E. tarda* were measured. Antibacterial effects obtained by adjusting immunity inside the fish body were not examined. For example, ginseng treatment produces T helper 1, 2, or other cell effects *in vivo* and reduces bacterial loads and lung pathology in chronic *Pseudomonas aeruinosa*, but has no antibacterial effect *in vitro* (Stock and Wiedemann, 2001). To effectively use herbal medicines to treat contagious diseases, further discussion and research are needed that considers the molecular structure of medical and clinical experimental results.

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