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Original Research Articles

In vitro antibacterial activity of 40 Chinese herbal medicines against three aquatic pathogenic bacteria

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Chinese herbal medicines could be an alternative treatment for antibiotics against fish bacterial disease. The present research was carried out to investigate the antimicrobial potential of 40 herbs against *Edwardsiella tarda*, *Streptococcus iniae* and *Klebsiella pneumoniae*. Firstly, the anti-bacterial activity of forty herbal aqueous extracts (HAEs) was determined using the plate perforation method on brain heart infusion (BHI) agar. Then, effective HAEs were selected to detect the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against the pathogenic bacteria through the micro broth dilution method. Lastly, the inhibitory effects of HAEs combination were also assessed. The results showed that *Caesalpinia sappan*, *Fructus mume*, *Rhus chinensis* and *Punica granatum* could effectively inhibit all strains of the test bacteria, with the inhibitory circles in 15.00–17.00 mm diameter, MIC and MBC ranging from 31.25 to 62.50 mg/mL and 62.50 to 125.00 mg/mL, respectively. Among the compound of HAEs, the combination of *Fructus mume* + *Rhus chinensis* exhibited the most potent antibacterial efficacy against *E. tarda* and *S. iniae*, producing inhibitory circles of 19.5±0.41 mm and 19.5±0.41 mm, respectively. The MIC and MBC of this combination against *E. tarda* were 15.625 and 31.25 mg/mL, whereas, the MIC and MBC of this combination against *S. iniae* were 7.8125 and 15.625 mg/mL. The compound of *C. sappan* + *F. mume* showed the most potent antibacterial efficacy against *K. pneumoniae*, with inhibition zone diameter, MIC and MBC were 24.50±0.41 mm, 7.8125 mg/mL and 15.625 mg/mL, respectively. Overall, the results indicate that *C. sappan*, *F. mume*, *R. chinensis* and *P. granatum* showing significant potential for inhibiting pathogens, and their combinations could be a potential alternative to treat the aquatic bacteria diseases.

INTRODUCTION

China has a long history of aquaculture, dating from 2000 years. To date, China is now the world's largest producer, contributing nearly 70% of global aquaculture production.[1] In recent years, the growing demand for aquatic products has driven an increasing scale and density of aquaculture. With the rapid development of intensive aquaculture, subsequent outbreaks of various bacterial diseases have seriously obstructed the development of the aquaculture industry, leading to giant economic losses.[2]

Bacterial disease is considered as one of the main challenges in the sustainable development of aquaculture, especially amongst high-density. *S iniae* is a Gram-positive beta-haemolytic facultative anaerobic bacterium primely isolated from dolphins.[3] In recent years, *S iniae* infection has been observed in a wide range of aquatic animals, such as American bullfrogs (*Rana catesbeiana*),[4] Nile tilapia (*Oreochromis niloticus*),[5] yellow catfish (*Tachysurus fulvidraco*)[6] and Adriatic sturgeon (*Acipenser naccarii*).[7] In in-

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fected aquatic animals, the most common clinical symptoms of *S. iniae* infection are panophthalmitis and meningitis.[8] Furthermore, studies have shown that *S iniae* is also a potential zoonotic pathogen that can cause soft tissue infections and septicemia in humans.[9] *E. tarda*, a gram-negative facultative anaerobic bacterium of the Enterobacteriaceae family,[10] is the causative agent of edwardsiellosis in fish.[11] This bacterium is widely distributed in natural ecosystems (lakes, streams, seawater, mud), and has a broad range of hosts.[12] Edwardsiellosis, a typical disease caused by *E. tarda*, characterized by symptoms of ascites, hernia, and exophthalmia, is often found in aquatic animals, some as turbot (*Scophthalmus maximus*),[13] barramundi (*Lates calcarifer*),[14] giant mottled eel (*Anguilla marmorata*),[15] gold fish (*Carassius auratus*),[16] and hybrid fish (*Salvelinus fontinalis*).[17] *K. pneumonia* is a Gram-negative, non-motile bacterium from the family Enterobacteriaceae. It is widely distributed in different environments[18] and is the most common cause of pneumonia infections in hospitals. Hosts infected with *K. pneumonia* is associated with various symptoms, including septicemia, urinary tract infections, soft tissue infections, and meningitis. In addition to infecting humans, there have been increasing reports of mortality of aquatic animals caused by *K. pneumonia* in recent years, such as black-spotted frogs (*Pelophylax nigromaculatus*),[19] Nile tilapia,[20] and Chinese mitten crab (*Eriocheir sinensis*).[21]

At present, antibiotic treatment is the main strategy for aquatic animal diseases. Although the application of antibiotics can effectively control aquaculture diseases, the abuse of antibiotics has boosted the emergence of drug-resistant bacteria. Moreover, traditional antibiotics could pollute the environment and leave residues in aquatic products, thereby threatening human health.[22] In this context, it is urgent to find a highly efficient, low-toxic, safe, and green drug that can effectively control diseases.

Recently, Chinese herbal medicine has emerged as an alternative therapy against aquatic disease, showing great potential for application. It showed that herbal bacterial inhibition research has become a prominent research field in aquaculture. Compared to conventional antibiotics, Chinese herbal medicine has many advantages, being safe without side effects, efficient, low residue, inexpensive, and environmentally friendly. Chinese herbals have anti-viral, anti-bacterial, anti-inflammatory activities in aquaculture due to the presence of different bioactive ingredients such as organic acids, terpenoids, alkaloids, phenolics, volatile oils and flavonoids.[23] In addition, Chinese herbal medicine is often used as a feed additive to improve growth performance and immune function in aquatic animals.[24]

In this study, we investigated the *in vitro* antimicrobial effects of forty herbal aqueous extracts (HAEs) and their combinations against three pathogenic bacteria strains, such as *S. iniae*, *E. tarda* and *K. pneumonia*, which are etiologies of different diseases in aquaculture. These results provide a valuable reference for the development of herbal medicines and the prevention and control of aquatic bacterial diseases.

MATERIALS AND METHODS

1.1. PREPARATION OF PATHOGENIC BACTERIA

Pathogenic bacteria used in the experiment were two types of Gram-negative rod (*E. tarda* and *K. pneumoniae*) and one type of Gram-positive coccus (*S. iniae*). They were isolated from the diseased aquatic animals and stored in our laboratory.[6,19,25] All the cultures were preserved in brain heart infusion (BHI, HopeBio, China) containing 30% sterile glycerol at -80 °C. Bacteria were cultured at 28°C in BHI medium for 24 h, and then harvested by centrifugation at 10000 *g* for 10 min. Thereafter, the bacteria were wash with sterile phosphate-buffered saline (PBS) and the bacteria suspensions were adjusted to 3.0×10^8 CFU/mL using a McFarland turbidity meter (LOOBO Qingdao, China).

1.2. PREPARATION OF HAES

Forty kinds of candidate herbals were purchased from Gutian Health Care Wholesale Market, Wuhan, China. The obtained herbals were taxonomically recognized and verified, and detailed information was listed in Table 1. Each herbal plant was grinded into powder and then used for preparation of candidate HAEs as described previously with minor modification.[26] Briefly, 30 g of each herbal powder sample was soaked in 30 mL sterile distilled water for 10 min, extracted at 100 °C for 30 min, and centrifuged at 10000 *g* for 30 min at 28 °C. While, two herbals were mixed at 1:1, and treated as afore mentioned to obtain combination of HAEs. The HAEs were filtered and sterilized through a 0.22 μm microfilter and then stored at 4 °C for later use.

1.3. ANTIBACTERIAL ACTIVITY ASSAY

The antibacterial activity of 40 HAEs against three pathogenic bacteria were determined using the plate perforation method with minor modification.[27] In brief, 100 μL of bacteria suspension was swabbed onto BHI agar plates, and then wells of 6 mm diameter were punched with sterile cork borer into the agar medium and filled with 100 μL of each previously prepared HAEs (1000 mg/mL), followed diffusion at 28 °C for 1 h. The plates were then incubated at 28 °C for 24 h. Finally, the diameter of the inhibition zones around each well was measured in millimeter (mm). Three replicates were carried out for each extract against each of the test bacterium and the average values were recorded. Five classifications were made based on the zone of inhibition: no sensitive (0-1 mm), slightly sensitive (1-10 mm), moderately sensitive (10-15 mm), highly sensitive (15-20 mm) and extremely highly sensitive (> 20 mm).[28]

1.4. BACTERIOSTATIC AND BACTERICIDAL ACTIVITY

The bacteriostatic or minimum inhibitory concentration (MIC) is generally regarded as the lowest concentration of a given HAEs that prevents growth of a bacteria after a specified incubation period. Based on the preliminary screening, HAEs showing highly and extremely highly sensitive inhibition against bacteria were examined and further tested to

Table 1. 40 kinds of experimental herbals

Drug name	Family	Test site	Drug name	Family	Test site
Caesalpinia sappan	Fabaceae	leaf	*Terminalia chebula*	Combretaceae	Ripe fruit
Senna obtusifolia	Fabaceae	seed	*Astragali radix*	Fabaceae	root
Eleutherococcus gracilistylus	Araliaceae Juss	rootstock	*Cynanchum otophyllum* Schneid	Asclepiadaceae	root
Mentha canadensis L	Lamiaceae	leaf	*Syringa oblata* Lindl	Oleaceae	leaf
Scutellaria baicalensis	Lamiaceae	root	*Forsythia suspensa*	Oleaceae	Ripe fruit
Scutellaria barbata	Lamiaceae	herb	*Fructus mume*	Rosaceae Juss	Ripe fruit
Perilla frutescens	Lamiaceae	seed	*Rubus idaeus*	Rosaceae Juss	Ripe fruit
Herba Lycopodii	Lycopodiaceae	herb	*Houttuynia cordata*	Saururaceae	stem
Xeloseptum Juglandis	Juglandaceae	Wooden diaphragm	*Rehmannia glutinosa*	Scrophulariaceae	stolon
Rhus chinensis	Anacardiaceae	Ripe fruit	*Viola phillipina*	Violaceae	Root
Eupatorium japonicum	Asteraceae	herb	*Punica granatum*	Punicaceae	pericarp
Centipedae Herba	Asteraceae	herb	*Cortex phellodendri*	Rutaceae	barks
Artemisia caruifolia Buch	Asteraceae	Stem	*Polygoni multiflori* Caulis	Polygonaceae	vine stem
Artemisia capillaris	Asteraceae	leaf	*Reynoutria japonica*	Polygonaceae	stolon
Artemisia argyi	Asteraceae	leaf	*Radix et rhizoma* rhei	Polygonaceae	root
Taraxacum mongolicum	Asteraceae	herb	*Radix paeoniae* rubra	Ranunculaceae	root
Dendranthema indicum	Asteraceae	capitulum	*Moutan cortex*	Ranunculaceae	rootstock
Cistanche deserticola Ma	Orobanchaceae	herb	*Rhizoma coptidis*	Ranunculaceae	root
Siraitia grosuenorii	Cucurbitaceae	root	*Clematis chinensis*	Ranunculaceae	root
Alumen	Aluminium compounds	-	*Isatis indigotica*	Brassicaceae Burnett	leaf

determine the MIC for each bacterial sample using twofold broth dilution method.[29] Briefly, the HAEs (1000 mg/mL) were resuspended in sterile BHI to produce serial twofold dilutions in the range of 1.95-1000 mg/mL. The final concentration of each HAEs was 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.91, 1.95, and 0.98 mg/mL. Then 1 mL of diluted HAEs were transferred to sterile culture tubes and mixed with 10 μL of suspended bacterial suspension (3.0×10^8 CFU/mL). After incubation at 28 °C for 24 h, a spectrophotometer set to 600 nm was used to measure turbidity as the lowest concentration of HAEs that prevented the bacterial isolates in the test tubes from growing. The MIC value was defined as the lowest drug concentration corresponding to the tube concentration at which no turbidity was detected. Two test tubes including the HAEs with no bacteria and bacteria with no HAEs were considered as negative control and positive control, respectively. The above experiment was set up with three parallel groups. Each test was performed in triplicates.

Minimum bactericidal concentration (MBC) was determined by sampling 100 μL solution from all test wells that did not show any apparent growth and then inoculating them on BHI agar at 28 °C for 24 h. The lowest concentration at which there was no apparent bacterial colony was considered as the MBC values of the HAEs. All samples were done in triplicate.

1.5. ANTIBACTERIAL, BACTERIOSTATIC AND BACTERICIDAL ACTIVITY OF HAES COMBINATIONS

Based on the results of antibacterial activity assay, HAEs with inhibition zone diameters > 10 mm were selected and used for detection of combined effect of HAEs. Following the methodology described above, these compound formulations' inhibition zones, MIC and MBC against three pathogenic bacteria were systematically analyzed.

1.6. STATISTICAL ANALYSES

All data were expressed as mean ± standard deviation (SD), and data were processed and analyzed using SPSS software version 23.0 for Windows.

RESULTS

2.1. IN VITRO BACTERIOSTATIC EFFECTS OF 40 HAES AGAINST THREE PATHOGENIC BACTERIA

The HAEs of 40 herbals, as shown in Fig. 1, have different antibacterial activity utilizing the plate perforation method against three fish pathogenic bacteria. The results indicated that 10, 14 and 4 HAEs exhibited obvious inhibitory zones against *E. tarda*, *S. iniae* and *K. pneumoniae*, respectively. The diameters of inhibition circles of 40 HAEs against three pathogenic bacteria were shown in Table 2. For *E. tarda*, *Caesalpinia sappan* had the largest inhibition diameter (19.00 ± 4.00 mm), followed by *Fructus mume*, *Rhus chinensis* and *Punica granatum*, which had inhibition diameters of 17.00 ± 1.00 mm, 16.00 ± 3.00 mm, and 15.00 ± 1.00 mm, respectively. *E. tarda* was moderately susceptible to *Terminalia chebula*, *Rubus idaeus* and *Radix paeoniae* rubra, with inhibition diameters of 13.33 ± 4.33 mm, 11.67 ± 0.33 mm and 10.67 ± 4.33 mm, respectively. While *E. tarda* showed low sensitivity to *Moutan cortex*, *Tuber Fleeceflower* Stem and *Semen Juglandis*, and no sensitivity to the remaining HAEs, such as *Scutellaria baicalensis*, *Scutellaria barbata* and *Syringa oblata* Lindl. For *S. iniae*, *F. mume* and *R. chinensis* exhibited extremely antibacterial effects, with the inhibitory zones were 20.00 ± 0.33 mm and 20.00 ± 0.33 mm, respectively. Moreover, *S. iniae* was moderately susceptible to *R. rhizoma* rhei, *S. baicalensis* and *P. granatum*, with inhibition diameters of 13.50 ± 0.50 mm, 13.00 ± 0 mm and 12.67 ± 0.33 mm, respectively. The remaining HAEs showed low or no inhibition against *S. iniae*. Like *S. iniae*, *F. mume* also showed the strongest inhibitory effect against *K. pneumonia*, with an inhibition diameter of 17.00 ± 1.00 mm. Additionally, *C. sappan*, *R. chinensis* and *P. granatum* exhibited general antibacterial effects against *K. pneumoniae*, with inhibition diameters of 12.67 ± 1.33 mm, 11.00 ± 1.00 mm and 11.00 ± 1.00 mm, respectively. The remaining HAEs showed no inhibition effect.

2.2. THE MIC AND MBC OF 40 HAES AGAINST THREE PATHOGENIC BACTERIA

The HAEs with high and extremely high inhibitory effects were selected for further test of MIC and MBC against three pathogenic bacteria. The results obtained from the broth dilution assay demonstrated that *F. mume*, *R. chinensis* and *P. granatum* had bacteriostatic effects against all three pathogenic bacteria. The MIC of *F. mume* against the three strains ranged from 31.25 to 62.25 mg/mL, with the best antibacterial effect against *S. iniae*, having an MIC of 31.25 mg/mL. The MIC of *R. chinensis* against the three strains ranged from 62.5 to 250 mg/mL, with the best antibacterial effect against *S. iniae* and *E. tarda*, both with an MIC of 62.50 mg/mL. The MIC of *P. granatum* against the three strains ranged from 62.5 to 250 mg/mL, with the best antibacterial effect against *S. iniae*, having an MIC of 62.5 mg/mL. Moreover, *C. sappan* demonstrated the best antibacterial effect, with MIC for the *E. tarda* and *K. pneumonia* ranging from 31.25 to 125 mg/mL, and the best antibacterial effects against *K. pneumonia*, with an MIC of 31.25 mg/mL (Table 3).

The MBC results revealed that *F. mume*, *R. chinensis* and *P. granatum* had bactericidal effects at ⩾ 500 mg/mL concentrations. *F. mume* had the best bactericidal activity compared with the other two herbs, which have the lowest MBC ranging from 62.5 to 125 mg/mL. The MBC of *R. chinensis* and *P. granatum* against the three strains ranged from 62.50–500 and 125–500 mg/mL, respectively. Additionally, the MBCs of *C. sappan* against the *E. tarda* and *K. pneumonia* were 62.50 and 125 mg/mL, respectively (Table 3).

2.3. IN VITRO BACTERIOSTATIC EFFECTS OF HAES COMPOUND AGAINST THREE PATHOGENIC BACTERIA

The combination of *C. sappan* and *F. mume* exhibited the strongest inhibition against *K. pneumoniae* (24.50 ± 0.41 mm). Whereas, *F. mume* paired with *R. chinensis* demonstrated broad-spectrum efficacy, showing highest inhibitory circle against both *S. iniae* (24.50 ± 0.41 mm) and *E. tarda* (19.5 ± 0.41 mm). *P. granatum* combined with *R. chinensis* showed synergistic effects against all three pathogens, with inhibition zones of 21.17 ± 0.23 mm for *S. iniae* and *K. pneumoniae*. *S. iniae* displayed heightened sensitivity to formulations containing *R. rhizoma* rhei, such as *F. mume* + *R. rhizoma* rhei (23.17 ± 0.23 mm). *K. pneumoniae* was notably inhibited by tannin-rich combinations, including *C. sappan* + *R. chinensis* (21.83 ± 0.82 mm). Combinations involving *R. idaeus* or *M. cortex* exhibited minimal efficacy. For instance, *R. idaeus* + *R. paeoniae rubra* yielded weak inhibition against *E. tarda* (9.50 ± 0 mm). Several combinations showed no detectable inhibition, including *C. sappan* + *Terminalia chebula* against *S. iniae* and *K. pneumoniae*. (Table 4).

2.4. MIC AND MBC OF HAES COMPOUND AGAINST THREE PATHOGENIC BACTERIA

All results from MIC and MBC agree with the previous diameters of inhibition zones. As shown in Table 5, the combination of *F. mume* + *R. chinensis* exhibits the best antibacterial effect against *E. tarda*, with the MIC and MBC values being 15.625 mg/mL and 32.5 mg/mL, respectively. For *S. iniae*, the MIC and MBC of *F. mume* + *R. chinensis* were 7.8125 mg/mL and 15.625 mg/mL. In addition, the MIC and MBC of *F. mume* + *R. chinensis* against *K. pneumoniae* are both 31.25 mg/mL. Different from the above two pathogenic bacteria, the combination of *C. sappan* + *F. mume* shows the best antibacterial effect against *K. pneumoniae*, with the MIC and MBC values being 7.8125 mg/mL and 15.625 mg/mL, respectively.

Based on the results of the inhibitory zone, MIC and MBC tests involving the combined application of traditional herbal medicines, we found that the *F. mume* + *R. chinensis* group exhibited the best antibacterial effect against *E. tarda* and *S. iniae*. Additionally, *C. sappan* + *F. mume* showed the strongest antibacterial effect against *K. pneumoniae*. For both *S. iniae* and *K. pneumoniae*, the compound treatment was significantly more effective than a single application.

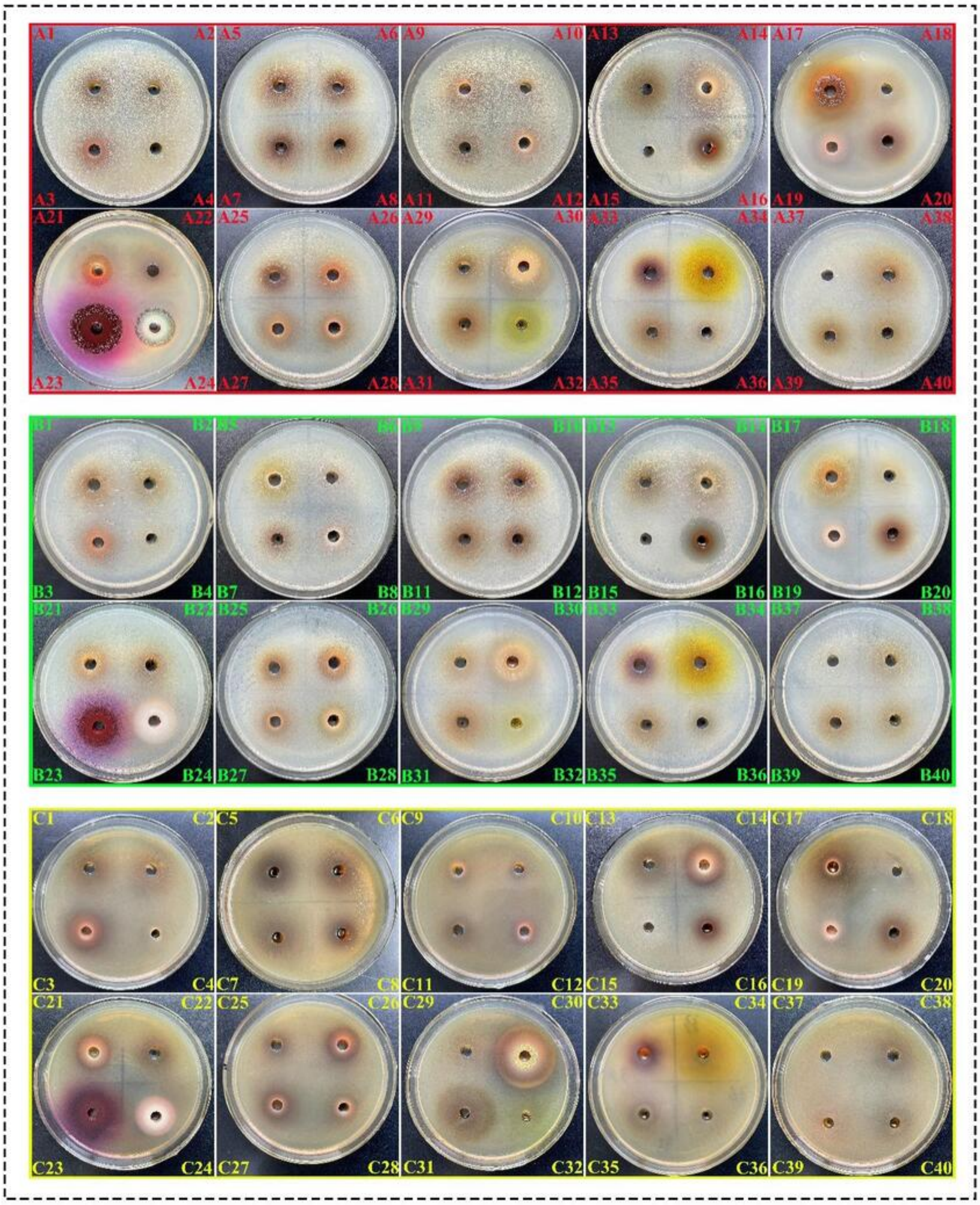

Figure 1. The inhibition zone (mm) of extracts of *Eupatorium japonicum* Thunb (1), *Artemisia caruifolia* Buch (2), *Xeloseptum Juglandis* (3), *Herba Lycopodii* (4), *Cistanche deserticola* Ma (5), *Houttuynia cordate* (6), *Eleutherococcus gracilistylus* (7), *Artemisia capillaris* (8), *Reynoutria japonica* (9), *Cynanchum otophyllum* Schneid (10), *Rehmannia glutinosa* (11), *Moutan cortex* (12), *Artemisia argyi* Levl.et Vant (13), *Terminalia chebula* (14), *Astragali radix* (15), *Fructus mume* (16), *Scutellaria baicalensis* (17), *Taraxacum mongolicum* (18), *Polygonimultiflori Caulis* (19), *Siraitia grosuenorii* (20), *Rubus idaeus* (21), *Mentha canadensis* L (22), *Caesalpinia sappan* (23), *Rhus chinensis* (24), *Senna obtusifolia* (25), *Radix paeoniae* rubra (26), *Syringa oblata* Lindl (27), *Radix et rhizoma* rhei (28), Alumen (29), *Punica granatum* (30), *Scutellaria barbata* (31), *Cortex phellodendri* (32), *Viola phillipina* (33), *Rhizoma coptidis* (34), *Centipedae Herba* (35), *Clematis chinensis* (36), *Perilla frutescens* (37), *Forsythia suspensa* (38), *Isatis indigotica* (39) and *Dendranthema indicum* (40) against *Edwardsiella tarda* (A), *Klebsiella pneumoniae* (B) and *Streptococcus iniae* (C).

DISCUSSION

Contemporary methods for controlling outbreaks of bacterial diseases in intensive aquaculture systems primarily rely on antibiotics, despite the evidence of their negative effects on the environment and human health.[30] The increasing national awareness of antibiotics' potential negative impact on health has spurred the search for alternative, more natural antibacterial medicines that can improve aquatic product safety and quality. Herbs are rich sources of antimicrobial substances for drug development, since its outstanding features of being natural, inexpensive, easy

Table 2. Bacterial inhibition zone of 40 HAEs

Chinese herbal medicine	Antibacterial zone/mm (Antibacterial level)		
	Edwardsiella tarda	*Streptococcus iniae*	*Klebsiella pneumoniae*
Caesalpinia sappan	19.00±4.00(+++)	-	12.67±1.33(++)
Fructus mume	17.00±1.00(+++)	20.00±0.33(+++)	17.00±1.00(+++)
Rhus chinensis	16.00±3.00(+++)	15.00±0.33(+++)	11.00±1.00(++)
Punica granatum	15.00±1.00(+++)	12.67±0.33(++)	11.00±1.00(++)
Terminalia chebula	13.33±4.33(++)	9.33±0.33 (+)	-
Rubus idaeus	11.67±0.33(++)	8.00±0(+)	-
Radix et rhizoma rhei	-	13.50±0.50(++)	-
Scutellaria baicalensis	-	13.00±0(++)	-
Radix paeoniae rubra	10.67±4.33(++)	9.00± 0(+)	-
Moutan cortex	9.33±0.33(+)	8.33± 0.33(+)	-
Reynoutria japonica	-	8.33± 0.33(+)	-
Tuber Fleeceflower Stem	8.00± 0(+)	9.00± 0(+)	-
Semen Juglandis	9.00± 0(+)	-	-
Scutellaria barbata	-	9.00± 0(+)	-
Radix Paeoniae Alba	-	8.33± 0.33(+)	-
Syringa oblata Lindl	-	8.00± 0(+)	-
Eupatorium japonicum Thunb	-	-	-
Centipedae Herba	-	-	-
Herba Artemisiae Annuae	-	-	-
Artemisia capillaris	-	-	-
Artemisia argyi Levl.et Vant	-	-	-
Taraxacum mongolicum	-	-	-
Dendranthema indicum	-	-	-
Cistanche deserticola Ma	-	-	-
Schisandrae Chinensis Fructus	-	-	-
Alumen	-	-	-
Mentha haplocalyx Briq	-	-	-
Eleutherococcus gracilistylus	-	-	-
Perillafrutescens	-	-	-
Herba Lycopodii	-	-	-
Forsythia suspensa	-	-	-
Houttuynia cordata	-	-	-
Rehmannia glutinosa	-	-	-
Viola phillipina	-	-	-
Astragali radix	-	-	-
Catsia tora Linn	-	-	-
Rhizoma coptidis	-	-	-
Clematis chinensis	-	-	-
Radix isatidis	-	-	-
Rehmannia glutinosa	-	-	-

preparation, biocompatible and few side effects for aquatic animals.[31] Herbs have the ability to damage cell walls, inhibit nucleic acid and protein synthesis and increase intracellular osmotic pressure.[32] Some herbals, such as the *Rhizoma coptidis*,[33] *Ramulus Cinnamomi*,[34] *Allium sativum*,[35] *Portulaca oleracea* L,[36] *Eryngium campestre*,[37] *Eichhornia crassipes*,[38] *Zingiber officinale*,[39,40] exhibit strong antibacterial effects against *Aeromonas* spp, *Staphylococcus aureus*, *Vibrio* spp and other common pathogens in medicine and aquaculture. In the present research, we determined the *in vitro* antimicrobial activity of 40 HAEs against three bacteria. Based on a systematic evaluation of the collected data, four HAEs exhibited optimal inhibitory effects against three

Table 3. MIC and MBC of HAEs against pathogenic bacteria

Chinese herbal medicine	*Edwardsiella tarda*		*Streptococcus iniae*		*Klebsiella pneumoniae*	
	MIC	MBC	MIC	MBC	MIC	MBC
Caesalpinia sappan	31.25	62.5	-	-	125	250
Fructus mume	62.5	62.5	31.25	62.5	62.5	125
Rhus chinensis	62.5	62.5	62.5	62.5	250	500
Punica granatum	62.5	125	125	250	250	500
Terminalia chebula	125	125	-	-	-	-
Rubus idaeus	125	250	-	-	-	-
Radix paeoniae rubra	250	250	-	-	-	-
Radix et rhizoma rhei	-	-	125	125	-	-
Scutellaria baicalensis	-	-	125	125	-	-

bacterial pathogens, especially *C. sappan* against *E. tarda* as well as *F. mume* against *K. pneumoniae* and *S. iniae*.

F. mume, a traditional Chinese herbal medicine, has been used for chronic cough therapy in China for over a millennium.[41] *F. mume* and its components, including organic acids, polysaccharides, flavonoids, amygdalin, terpenes and sterols, have been demonstrated to have potential antibacterial activity *in vitro* and *in vivo*.[42] Previous studies have found that *F. mume* exhibits bactericidal activity against many pathogens, such as *Streptococcus* mutans, *Salmonella* spp, *Staphylococcus aureus*, *Escherichia coli*, and *Helicobacter pylori*.[43,44] According to Chen,[43] the composition of *F. mume* can significantly reduce bacterial biofilm activity, thereby inhibiting bacterial growth. Furthermore, the citric acid content in *F. mume* induces an acidic milieu (pH < 3.0), thus creating a bacteriostatic environment that suppresses microbial proliferation. In the present study, all three pathogens were successfully inhibited by *F. mume* with the circle of inhibition diameter ⩾ 17.00 mm and MIC of 62.5 mg/mL.

C. sappan, belongs to the Leguminosae family, is widely distributed in China and Southeast Asia.[45] Phytopharmacological analysis revealed that *C. sappan* consists of multiple bioactive components, including xanthones, coumarins, chalcones, flavonoids, isoflavonoids, and brasilin.[46] Especially, brasilin, the main active component of *C. sappan*, has potent inhibition of many antibiotic-resistant bacteria due to the compound's ability to block the synthesis of DNA and protein.[47] In this research, *C. sappan* extract good inhibitory effect on *K. pneumoniae* and *E. tarda*, but showed no effect against *S. iniae*. The difference was probably related to components of bacterial wall.

P. granatum, belonging to the pomegranate family, is the dried peel of the pomegranate. *P. granatum* accounts for approximately 50% of the total fruit weight and is an important source of pharmacologically active constituents.[48] Previous studies have demonstrated that *P. granatum* has antibacterial functions due to its multiple pharmacologically active ingredients, such as polyphenols, polysaccharides, terpenes and lignans.[49] Zazharskyi et al.[50] found that the ethanol extract of *P. granatum* have strong bactericidal activity on *K. pneumonia*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Escherichia coli*, *Corynebacterium xerosis*, *Proteus vulgaris*.[32] Moreover, the study carried out by Pandit and Vyas[51] reported that *P. granatum* showed significant activity against *Streptococcus* spp (20.16 ± 0.76), similar to our findings.

R. chinensis, is a sac-shaped proliferative tissue structure induced by the parasitic activity of aphids on the leaves or petioles of Chinese sumac.[52] According to phytochemical composition analysis, the main chemical components of *R. chinensis* include flavonoids, triterpenoids, geranylgeranyl derivatives, lignans, and phenolic acid glycosides (Wang, Sheng-Tian et al. 2024). Gallnut water extract was shown to have a good inhibitory effect on *V. parahaemolyticus*.[32] In this study, we found that *R. chinensis* exhibited antibacterial activity against three aquatic pathogenic bacteria, confirming that *R. chinensis* has great promise for antibacterial applications.

Various studies have demonstrated that the combined effect of Chinese herbal prescriptions may be greater than the sum of the individual effects.[32] Therefore, it's of great significance to understand the compatibility and synergistic effects of herbal combinations, and then develop novel drug combinations.[32] Based on our *in vitro* susceptibility test, *C. sappan*, *F. mume*, *R. chinensis*, *P. granatum* and their combinations with other herbs appear to be excellent candidates for the treatment of infections caused by *K. pneumoniae* and *E. tarda*. While, *F. mume*, *R. chinensis*, *P. granatum* and their combinations with other herbs appear to be excellent candidates for the treatment of *S. iniae*. Moreover, *F. mume* + *R. chinensis* exhibited the best inhibitory effect against *E. tarda* and *S. iniae*, while *C. sappan* + *F. mume* showed the strongest inhibitory effect against *K. pneumoniae* among all the HAEs combinations. However, assessing the efficacy and safety of HAEs are based on clinical practice rather than in a laboratory, it's necessary to establish the clinical utility of HAEs for the treatment of *K. pneumoniae*, *S. iniae* and *E. tarda* infections in aquatic animals.

CONCLUSION

In this study, the *in vitro* bacteriostatic effects of forty HAEs and their compounds against three common aquatic pathogens were investigated. The results showed that four

Table 4. Bacterial inhibition zone of herbal prescriptions

Compound herbal medicine	Antibacterial zone/mm (Antibacterial level)		
	E. tarda	*S. iniae*	*K. pneumoniae*
Caesalpinia sappan + Fructus mume	17.67±0.23(+++)	-	24.50±0.41(++++)
Caesalpinia sappan + Punica granatum	17.17±0.23(+++)	-	17.67±0.23(+++)
Caesalpinia sappan + Rhus chinensis	17.00±0(+++)	-	21.83±0.82(++++)
Caesalpinia sappan + Terminalia chebula	16.83±0.23(+++)	-	-
Caesalpinia sappan + Rubus idaeus	16.00±0(+++)	-	-
Caesalpinia sappan + Radix paeoniae rubra	15.17±0.23(+++)	-	-
Caesalpinia sappan + Moutan cortex	14.17±0.23(++)	-	-
Fructus mume + Punica granatum	14.5±0.41(++)	21.17±0.23(++++)	21.17±0.23(++++)
Fructus mume + Rhus chinensis	19.5±0.41(+++)	24.50±0.41(++++)	23.17±0.23(++++)
Fructus mume + Terminalia chebula	12.17±0.23(++)	-	-
Fructus mume + Rubus idaeus	12.00±0(++)	-	-
Fructus mume + Radix paeoniae rubra	10.33±0.23(++)	-	-
Fructus mume + Moutan cortex	8.67±0.47(+)	-	-
Fructus mume + Radix et rhizoma rhei	-	23.17±0.23(++++)	-
Fructus mume + Scutellaria baicalensis	-	22.5±0.41(++++)	-
Punica granatum + Rhus chinensis	16.17±0.23(+++)	21.17±0.23(++++)	21.17±0.23(++++)
Punica granatum + Terminalia chebula	11.33±0.23(++)	-	-
Punica granatum + Rubus idaeus	9.83±0.23(+)	-	-
Punica granatum + Radix paeoniae rubra	9.17±0.23(+)	-	-
Punica granatum + Moutan cortex	8.00±0(+)	-	-
Punica granatum + Radix et rhizoma rhei	-	16.83±0.23(+++)	
Punica granatum + Scutellaria baicalensis	-	18.83±0.82(+++)	
Rhus chinensis + Terminalia chebula	15.17±0.23(+++)	-	-
Rhus chinensis + Rubus idaeus	14.17±0.23(++)	-	-
Rhus chinensis + Radix paeoniae rubra	11.50±0(++)	-	-
Rhus chinensis + Moutan cortex	11.00±0(++)	-	-
Rhus chinensis + Radix et rhizoma rhei	-	21.83±0.82(++++)	-
Rhus chinensis + Scutellaria baicalensis	-	17.67±0.23(+++)	-
Terminalia chebula + Rubus idaeus	12.33±0.47(++)	-	-
Terminalia chebula + Radix paeoniae rubra	11.50±0(++)	-	-
Terminalia chebula + Moutan cortex	14.83±0.23(++)	-	-
Rubus idaeus + Radix paeoniae rubra	9.50±0(+)	-	-
Rubus idaeus + Moutan cortex	10.17±0.23(++)	-	-
Radix paeoniae rubra + *Moutan cortex*	9.16±0.23(+)	-	-
Radix et *rhizoma* rhei + *Scutellaria baicalensis*	-	12.83±0.23(++)	-

single HAEs (*F. mume*, *C. sappan*, *P. granatum*, *R. chinensis*) and their compounds (*F. mume* + *R. chinensis* and *C. sappan* + *F. mume*) have significant inhibitory effect and could be used for future treatment of aquatic diseases caused by *S. iniae*, *E. tarda* and *K. pneumoniae*. In our future research, the active components and antibacterial mechanisms of HAEs which have significant bacteriostatic and bactericidal activity will be investigated.

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Table 5. MIC and MBC of herbal prescriptions against pathogenic bacteria

Compound herbal medicine	*E. tarda*		*S. iniae*		*K. pneumoniae*	
	MIC	MBC	MIC	MBC	MIC	MBC
Caesalpinia sappan + *Fructus mume*	31.25	62.5	-	-	7.8125	15.625
Caesalpinia sappan + *Punica granatum*	31.25	62.5	-	-	62.5	62.5
Caesalpinia sappan + *Rhus chinensis*	31.25	62.5	-	-	31.25	62.5
Caesalpinia sappan + *Terminalia chebula*	31.25	62.5	-	-	-	-
Caesalpinia sappan + *Rubus idaeus*	62.5	62.5	-	-	-	-
Caesalpinia sappan + *Radix paeoniae* rubra	62.5	125	-	-	-	-
Caesalpinia sappan + *Moutan cortex*	62.5	125	-	-	-	-
Fructus mume + *Punica granatum*	62.5	125	31.25	62.5	31.25	62.5
Fructus mume + *Rhus chinensis*	15.625	31.25	7.8125	15.625	31.25	31.25
Fructus mume + *Terminalia chebula*	125	250	-	-	-	-
Fructus mume + *Rubus idaeus*	125	250	-	-	-	-
Fructus mume + *Radix paeoniae* rubra	250	250	-	-	-	-
Fructus mume + *Moutan cortex*	250	500	-	-	-	-
Fructus mume + *Radix et rhizoma* rhei	-	-	31.25	31.25	-	-
Fructus mume + *Scutellaria baicalensis*	-	-	15.625	31.25	-	-
Punica granatum + *Rhus chinensis*	62.5	62.5	31.25	62.5	31.25	62.5
Punica granatum + *Terminalia chebula*	125	250	-	-	-	-
Punica granatum + *Rubus idaeus*	250	250	-	-	-	-
Punica granatum + *Radix paeoniae* rubra	250	500	-	-	-	-
Punica granatum + *Moutan cortex*	500	500	-	-	-	-
Punica granatum + *Radix* et *rhizoma* rhei	-	-	62.5	125	-	-
Punica granatum + *Scutellaria baicalensis*	-	-	62.5	62.5	-	-
Rhus chinensis + *Terminalia chebula*	62.5	125	-	-	-	-
Rhus chinensis + *Rubus idaeus*	62.5	125	-	-	-	-
Rhus chinensis + *Radix paeoniae* rubra	125	250	-	-	-	-
Rhus chinensis + *Moutan cortex*	125	250	-	-	-	-
Rhus chinensis + *Radix et rhizoma* rhei	-	-	31.25	62.5	-	-
Rhus chinensis + *Scutellaria baicalensis*	-	-	62.5	62.5	-	-
Terminalia chebula + *Rubus idaeus*	125	250	-	-	-	-
Terminalia chebula + *Radix paeoniae* rubra	125	250	-	-	-	-
Terminalia chebula + *Moutan cortex*	62.5	125	-	-	-	-
Rubus idaeus + *Radix paeoniae* rubra	250	500	-	-	-	-
Rubus idaeus + *Moutan cortex*	250	500	-	-	-	-
Radix paeoniae rubra + *Moutan cortex*	500	500	-	-	-	-
Radix et rhizoma rhei + *Scutellaria baicalensis*	-	-	125	125	-	-

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