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Antimicrobial property of gallic acid from *Terminalia chebula* Retz. effects on two pathogenic *Aeromonas* species isolated from cultured eel

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Abstract

Aeromonas spp. are prevalent pathogens in farmed eel, which induce highly infectious diseases, such as hemorrhagic septicemia and gill-rot disease. Research on the application of ethanol and water extracts from traditional Chinese herbs in controlling the diseases has increased. However, the exact antibacterial components in majority of these herbs were not well-studied. Four fractions or components were isolated from *Terminalia chebula* Retz. ethanol extract in our previous study. In the present study, their antibacterial effects on *A. hydrophila* and *A. veronii* were assessed using microcalorimetry. The results showed that component III (a single chemical constituent, gallic acid) had the strongest antibacterial effect compared with three other fractions. The inhibitory effect was enhanced as the concentration of component III increased, and the minimum inhibitory concentrations of component III to *A. hydrophila* and *A. veronii* were found to be 0.56 mg/ml and 0.74 mg/ml respectively. This is the first study in which the antibacterial component of *T. chebula* extract on aquatic bacteria growth was screened and determined by microcalorimetry.

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Introduction

It is widely recognized that *Aeromonas* spp. infections produce septicaemia, and ulcerative and haemorrhagic diseases in fish, causing heavy mortalities in farmed and feral fishes throughout the world (Harikrishnan and Balasundaram, 2005; Beaz-Hidalgo and Figueras, 2013). It also has been confirmed that *Aeromonas* spp. are prevalent pathogens in farmed eel, including *A. hydrophila* and *A. veronii* (Guo 2006). Several methods are considered to prevent and treat the disease. Researches on the application of traditional Chinese herbs in controlling diseases induced by *A. hydrophila* by dip, feed or intraperitoneal treatment has increased. These medicinal plants produced a large number of antibacterial organic chemicals and/or increased the immune response and resistance to aquaculture pathogens, including *A. hydrophila* (Harikrishnan and Balasundaram, 2005; Rao et al., 2006; Cao et al., 2012; Bilen et al., 2016; Stratev et al., 2018).

The ripened and dried fruit of *Terminalia chebula* Retz. is extensively used in traditional Chinese medicine. To date, however, studies on Chinese herbs including *T. chebula* have mainly focused on the pharmacological activity of its ethanol and water extracts in aquaculture (Guo and Yang, 2013). It is essential to identify the antibacterial compound from *T. chebula* fruits against *A. hydrophila* and develop an optimal extraction method to obtain high yield of the compound. In the previous study, we isolated four fractions or components from an ethanol extract of *T. chebula* by high-speed countercurrent chromatography (HSCCC). Among them, only component III contained a large amount of single chemical constituent, gallic acid (with high purity of 96.40%), which was confirmed by electrospray ionization for mass spectrometry (ESI-MS) and high performance liquid chromatographic (HPLC) analysis. Through further purification, 17.2 mg gallic acid was obtained from a 200 mg ethanol extract of *T. chebula* (Li et al., 2014).

In the present study, we analyzed the antibacterial activity of these isolated fractions through high-sensitivity microcalorimetry with real-time dynamic monitoring. Since growing microorganisms produce heat, microcalorimetry can be useful for characterizing the effect of drugs on their growth by measuring the heat output before and after the addition of the drug (Koenigbauer, 1994). These techniques allowed the establishment of a highly efficient isolation method and rapid and accurate activity measurement, which provide a scientific basis and reference for screening the active components of traditional Chinese medicinal extracts.

Materials and Methods

Bacterial strain

A. hydrophila B11 and *A. veronii* B59 isolated from cultivated eels were obtained from the strain library of eel pathogens (Engineering Research Center of the Modern Technology for Eel Industry, Ministry of Education, China).

Preparation of T. chebula ethanol extract

T. chebula was crushed into a 200-mesh powder using an ultrafine frozen crusher. Next, 7.5 g of the powder was added to 150 ml of 60% ethanol at a powder/ liquid ratio of 1:20 w/v. Crude extraction was performed for 50 min in a 50°C ultrasonic bath. The residue was filtered, and remaining solution was placed in a multi-sample parallel evaporator for vacuum concentration in order to yield the extract, which was then stored at 4°C for further use.

Isolation and preparation of fractions from T. chebula ethanol extract

Before analytical HSCCC (TBE-20A, Tongtian), the upper and lower phases, used as the stationary and mobile phases respectively, were ultrasonically degassed for 20 min. The solvent system of n-hexane-ethyl acetate-methanol-water (1:5:1:5, v/v) using in analytical HSCCC was optimized, and then four fractions (I, II, III, and IV) were obtained in our previous study (Li et al., 2014). Subsequently, large batches of the individual fractions were isolated and prepared using preparative HSCCC (TBE-300B, Tongtian). The wavelength for UV detection was 254 nm, and the column temperature was 25°C. The solvent system was optimized and the effects of various parameters were tested at a

mobile phase flow rate of 2 ml/min, rotation rate of 850 rpm, and maximum injection amount of 300 mg. Under these conditions, the retention rate of the system was 63%, and the isolation efficiency was better than that of analytical HSCCC (Li et al., 2014). These fractions (I, II, III, and IV) were collected, frozen, and dried respectively for further use. Component III was identified as gallic acid, with the high-purity of 96.4%, by comparative HPLC analysis with standard products.

Microcalorimetry-based determination of the antibacterial effects of the isolated fractions

The inhibitory effects of the isolated fractions on *A. hydrophila* B11 and *A. veronii* B59 were examined by microcalorimetry (TAM III, Waters LC). The fractions (I, II, III, and IV) were dissolved respectively in 5% DMSO to prepare different concentrations of the medicated solution. Under aseptic conditions, 1 ml MH broth was added to each ampoule of the bacteria to obtain a bacterial concentration of 10^7 CFU/ml. Then different concentrations of medicated solutions were added to 50 μ l *A. hydrophila* B11 or *A. veronii* B59 suspension in ampoules respectively. The ampoules were sealed and then placed in the microcalorimeter. The solution was cultured at 28°C, and bacterial growth and metabolism curves (P_t - t) were recorded dynamically in real time.

Microcalorimetric calculation method

In the exponential growth phase of bacterial growth thermograms, two formulae— $P_t = P_0 \exp(kt)$ and $\ln P_t = \ln P_0 + kt$ (Kong et al., 2008a)—are valid, where P_0 and P_t are the thermal powers of the bacteria at the starting point and at time t of the exponential growth phase, respectively, while k is the growth rate constant. A series of P_t - t data was selected from the exponential growth phase to perform linear fitting for t using $\ln P_t$ based on the above formulas, whereby the value of k was calculated. The antibacterial effects of the medicinal extract were assessed by analyzing the linear relationship between the k and extract concentration, the maximum heat production power (P_m), and the peak time (T_m) from the characteristic parameters represented in the bacterial thermograms.

Results

Thermogram of A. hydrophila B11 and A. veronii B59 treated with fractions isolated from T. chebula

Curve 1 in **Figure 1 (a-d)** represented the thermogram of the growth and metabolism of untreated *A. hydrophila* B11 (blank sample) at 28°C. It was divided into five phases: delay phase, first exponential growth phase, second exponential growth phase, stabilization phase, and decay phase. The growth curves of *A. hydrophila* B11 were similar for 5% DMSO (Curve 2 in **Figure 1 (a-d)**). As the concentration of various medications increased, the delay phase became prolonged, the peak of the first exponential growth phase was delayed, and the second exponential growth phase disappeared and tended to decay, suggesting that the intensity of the antibacterial effects increased gradually. The growth of the pathogen was almost inhibited within 24 h at a component III concentration of 0.30 mg/ml (**Figure 1c**).

The thermogenic curves of *A. veronii* B59 growth at 28°C without any substance were shown in **Figure 2** (curve 1 of **a-d**). It was divided into three phases: delay phase, exponential growth phase, and decay phase. The growth curves of *A. veronii* B59 were similar for 5% DMSO (Curve 2 of **Figure 2 (a-d)**). These curves changed with the increase of concentration of various medications, showing that the T_m is suspended when the microbe growth was restrained. The growth of the pathogen was completely inhibited within 24 h at a component III concentration of 0.45 mg/ml (**Figure 2c**).

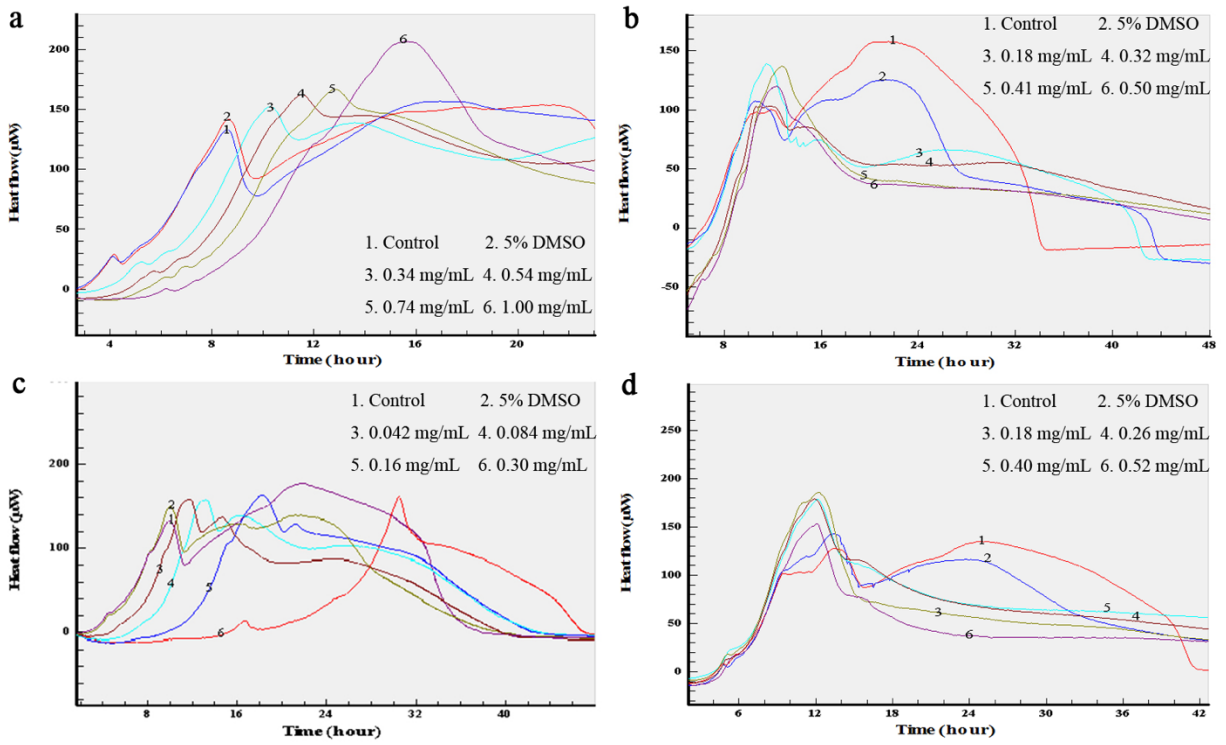


Figure 1 Power-time curves of *A. hydrophila* B11 in four fractions with different concentrations. **a** Fraction I. **b** Fraction II. **c** Fraction III. **d** Fraction IV

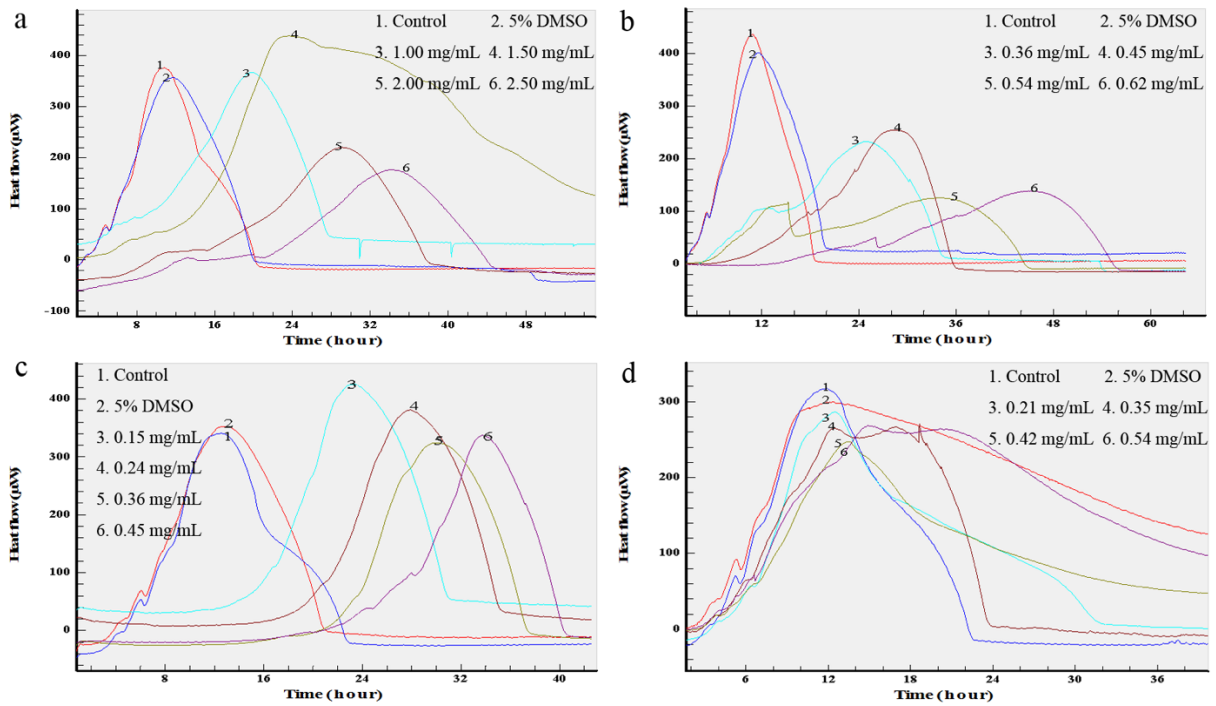


Figure 2 Power-time curves of *A. veronii* B59 in four fractions with different concentrations. **a** Fraction I. **b** Fraction II. **c** Fraction III. **d** Fraction IV

Minimum inhibitory concentrations of isolated T. chebula fractions on A. hydrophila B11 and A. veronii B59

Changes in the bacterial growth rate constant, k , was used to evaluate the antibacterial effects of fractions. The k value of *A. hydrophila* B11 and *A. veronii* B59 was calculated, and a linear relationship between k and the corresponding drug concentration c was fitted to calculate the minimum inhibitory concentrations (MICs) of the fractions (at $k = 0$) (**Table 1**). As shown in Table 1, the four fractions isolated from *T. chebula* (I, II, III, and IV) inhibited *A. hydrophila* B11 and *A. veronii* B59 growth in the order III > II > IV > I. Component III had a lower MIC of 0.56 mg/ml or 0.74 mg/ml compared to three other fractions.

Relationship between quantitative thermokinetic parameters and concentration of component III

The P_t - t values of the first exponential growth phase in the thermogram (Figure 1, 2) were fitted to calculate the k of *A. hydrophila* B11 and *A. veronii* B59 respectively. The relevant bio-thermokinetic parameters are listed in **Table 2**. As the concentration of component III increased, the k gradually decreased while the T_m increased compared to the control.

Table 1 The correlation between the growth rate constant of *A. hydrophila* B11, *A. veronii* B59 and concentrations of the fractions separated from *Terminalia chebula*

pathogen	fraction	formula	correlation coefficient (R)	MIC (mg/ml)
<i>A. hydrophila</i> B11	I	$k = -0.0044 c + 0.0077$	0.9873	1.75
	II	$k = -0.0258 c + 0.0219$	0.9947	0.85
	III	$k = -0.0122 c + 0.0068$	0.9967	0.56
	IV	$k = -0.0014 c + 0.0019$	0.9863	1.35
<i>A. veronii</i> B59	I	$k = -0.0014 c + 0.0056$	0.9527	4.00
	II	$k = -0.0060 c + 0.0055$	0.9945	0.92
	III	$k = -0.0090 c + 0.0067$	0.9837	0.74
	IV	$k = -0.0029 c + 0.0057$	0.9828	1.97

k , growth rate constant; c , concentrations of the fractions

Table 2 The thermokinetic parameters of *A. hydrophila* B11 and *A. veronii* B59 growth in different concentrations of component III separated from *Terminalia chebula*

pathogen	groups	c (mg/ml)	T_m /min	$P_m/\mu W$	$10^{-2}k/\text{min}^{-1}$
<i>A. hydrophila</i> B11	1	0	599	133	0.67
	2	0	602	149	0.69
	3	0.042	706	158	0.64
	4	0.084	791	157	0.56
	5	0.16	1097	162	0.49
	6	0.30	1829	161	0.32
<i>A. veronii</i> B59	1	0	752	341	0.70
	2	0	781	352	0.69
	3	0.15	1401	426	0.51
	4	0.24	1673	381	0.42
	5	0.36	1807	324	0.38
	6	0.45	2023	337	0.26

c, concentrations of the fractions; T_m , the peak time at maximum heat production power; P_m , maximum heat production power; k , growth rate constant

Discussion

In the present study, microcalorimetry was used to determine the inhibitory effects of *T. chebula* fractions on two *Aeromonas* species. The thermogenic curves of *A. hydrophila* growth affected by four fractions from *T. chebula* indicated that the component III (gallic acid) had the strongest antibacterial effect. As the concentration of component III increased, the k gradually decreased while the T_m increased. The longer the T_m are, the stronger antibacterial activity the drugs possess. The same result was found in the restraining effect of *Rheum palmatum* L. on *Bifidobacterium adolescentis* growth (Wang et al., 2010). The MIC of component III (gallic acid) was found to be 0.56 mg/ml and 0.74 mg/ml for *A. hydrophila* B11 and *A. veronii* B59 respectively. This is the first study in which the antibacterial component of *T. chebula* extract on aquatic bacteria growth was determined by microcalorimetry. The present study provided theoretical and material bases for further analysis of the pharmacological effects of *T. chebula* as well as its use in the prevention and treatment of diseases in aquatic animals.

A. hydrophila was extremely sensitive to the phenolic compounds from grape (*Vitis vinifera* L.) seed extracts and cranberry (*Viburnum opulus* L.) fruit extract, which containing over 500 mg and 130 mg gallic acid equivalent per gram extract respectively. The extracts had bactericidal activities against *A. hydrophila* at low concentrations of 1% and 5% respectively (Baydar et al., 2006; Sagdic et al., 2006). Lu et al., (2013a) used the agar diffusion method to determine the inhibitory effect of gallic acid (reference substance) on *A. hydrophila* and found the MIC to be 0.25 mg/ml. However, gallic acid purified from the flowers of *Rosa chinensis* Jacq. was proved to have not antibacterial activity against *A.*

hydrophila by disc diffusion assay (Li et al., 2007). The different MIC of gallic acid probably attribute to inconsistency of the methods or antibacterial resistance. Since the need of determining the biological activity studies of drugs on microbes in high sensitivity, application of microcalorimetry, a new method, was developed (Kong et al., 2008b; Maniewska et al., 2009). Some methods, such as paper disk and agar diffusion, can also be used to assay the antibacterial activity. But the indexes are simple, and the dynamic process of bacterium growth with the effect of medicines cannot be given out in those methods (Feng et al., 2011). Therefore, microcalorimetry has an advantage over those methods. And the MIC of component III (gallic acid) against *A. hydrophila* determined in the present study is convincing. In addition, previous research showed *Aeromonas* species was resistant to different antimicrobial agents in different area and country (Cai et al., 2012). With the increase in antibiotic resistant organisms due to extensively overusing antibiotics and chemotherapy in eel farm (Lin et al., 2016), we had reasons to infer that *A. hydrophila* B11 and *A. veronii* B59 showed a higher antibacterial resistance.

The antioxidative, antiapoptotic, cardioprotective, neuroprotective, and anticancer properties of gallic acid and its esters, the gallates have been widely reported in human and mammals (Khadem and Marles, 2010; Kosuru et al., 2018; Zhang et al., 2018). The antibacterial activity of plant extracts containing 5.27% gallic acid is due to the disruption of the cell membrane and the leakage of cytoplasmic contents (Lu et al., 2013b). It has also been found the inhibition effects of gallic acid on the biofilm formation of human pathogenic bacteria, such as *Escherichia coli*, *Streptococcus mutans* and *Pseudomonas aeruginosa* (Borgesa et al., 2012; Shao et al., 2015). Gallic acid showed a great potential in being developed as a naturally sourced, nontoxic, and novel inhibitor of bacteria (Shao et al., 2015). *T. chebula* has definite application prospects in the prevention and treatment of *A. hydrophila* and *A. veronii* infection among aquatic animals.

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