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Comparison study of antibacterial properties of curcumin from *Curcuma longa* and enrofloxacin against *Aeromonas hydrophila*

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Abstract

Antibacterial properties of curcumin from turmeric (*Curcuma longa*) and enrofloxacin against *Aeromonas hydrophila* were assayed. The minimum inhibitory concentration (MIC) values of curcumin and enrofloxacin against *A. hydrophila* were found to be 100ug/ml and 9.375ug/ml, respectively. To realize the mechanisms of action of curcumin against *A. hydrophila,* we researched the antibacterial activity and bacterial membrane permeability of *A. hydrophila* cells treated with curcumin or enrofloxacin. All results elucidated that curcumin increased membrane permeabilization and caused leakage of intracellular contents, while its role was not as good as enrofloxacin. Moreover, a synergistic effect was shown between curcumin and enrofloxacin. The present study suggests that curcumin extracted from turmeric has the potential to be used as an antimicrobial for the control of *A. hydrophila*.

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Introduction

In recent years, the proportion of aquaculture in the global fish market has been increasing. Nearly 50% of fish products come from aquaculture, which shows that the shift from fishing to aquaculture has been crucial. China is a major aquaculture country, accounting for nearly 70% of the world's total aquaculture output. However, there are so many kinds of fish diseases caused by pathogenic bacteria (Frans et al., 2008), such as *A. hydrophila, Pseudomonas fluorescens, Streptococcus spp*., *Edwardsiella ictaluri andVibrio spp*., (Vivas et al., 2004; Frans et al., 2008; Wang et al., 2010; Xi et al., 2011) which caused a severe economic loss in aquaculture throughout China (Feng, 2010).

A. hydrophila is a gram-negative rod-shaped bacterium belonging to the Aeromonas family, widely distributed in freshwater, sewage-contaminated water, sludge, soil, and foods. And It is also an important fish bacterial pathogen associated with hemorrhagic septicemia, fin and tail rot, epizootic ulcer syndrome, and other fish diseases (Larsen et al., 1977; Lu 1992). These diseases have caused high mortality in freshwater fish resulting in extensive losses around the world (Feng, 2010). So far, a large number of antibiotics and chemicals are added in feed to control *A. hydrophila* disease, which will result in drug resistance and residues and other issues, detrimental to the sustainable development of aquaculture, seafood safety, and human health (Ming et al., 2012). With increasing the demand for organic aquaculture, looking for a non-toxic side effects of green products to replace antibiotics in aquaculture has become more important, such as traditional herbal medicine.

Curcumin is a natural yellow acidic phenol extracted from curcuma genus (*Curcumalonga*) plant such as turmeric, curcuma (*Curcuma L*), and acruginous turmeric rhizome (*Rhizoma curcumae Aeruginosae*) (Aggarwal, et al., 2007). Some recent studies have demonstrated a wide range of pharmacological effects of dietary curcumin, such as elimination of free radicals (Toda et al., 1985) and antioxidant (Ruby et al., 1995), relief of inflammation (Gupta et al., 2011), antibacterial effects, and immunomodulation (Bhuvaneswari & Balasundaram, 2006; Bai et al., 2009; Ganguly et al., 2010). However, only in a few cases the mechanism has been elucidated among their wide biological activities. In particular, the antibacterial activity and mechanisms of action of curcumin against *A. hydrophila* and whether curcumin can replace antibioticshave been little reported.

Enrofloxacin is now widely used in the prevention and treatment of a variety of infectious animal diseases, as well as in aquatic animal disease prevention and control (Wang et al., 2010). However, as there is no enrofloxacin in animal tissue, in high quantities, enrofloxacin is toxic to the liver and kidneys (Vancutsem, 1990). Therefore, it is often used as a model contrast in scientific research.

To study about the possible mechanism of antibacterial activity curcumin against *A. hydrophila* and the comparison between curcumin and enrofloxacin*,* here we investigated the morphology of treated cells and the molecular mechanism of curcumin and enrofloxacin against *A. hydrophila*. Several possible mechanisms of action were proposed. Therefore, the aims of the present work are to investigate the mechanism of action of curcumin and enrofloxacin against *A. hydrophila*. Our results provide theoretical bases for future improvement of disease resistance in fish by using curcumin.

Materials and Methods

Microorganisms and chemicals/reagents.

A. hydrophila WJ2011BJ43, WJ2011BJ44, IB101, JG101, 4LNS301, CCH201, LNB101, CG101 were obtained from the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences. Through challenge experiments, we chose *A. hydrophila* WJ2011BJ44 because its virulence was the strongest. Curcumin and enrofloxacin (with a purity > 99%) were obtained from Feida Chemical Reagent Company (Xian, China). A Cell Apoptosis PI detection

kit was purchased from Beijing FanBo Biotech. Co. Ltd., China. UPLC grade methanol was purchased from Sigma–Aldrich (St. Louis, MO, USA). Luria-Bertani (LB) broth was self-configurable. The preparation method is shown in **Table 1**. All other reagents (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) were of analytical grade.

Table 1 Components of the Luria-Bertani Broth.

Luria-Bertani Broth*	(g/L)
Yeast extract	5
Peptone	10
Sodium chloride	5

Note: *Its ingredients were dissolved in 1000 mL distilled water. The medium contained agar (20%). The pH of the LB Medium was 7.5±0.1.

The minimal inhibitory concentration (MIC).

The antimicrobial activities of curcumin and enrofloxacin were determined using a twofold microdilution broth method (Naghmouchi et al., 2010). *A. hydrophila* WJ2011BJ44 was incubated in LB broth at 28 ℃ for 20 h. The curcumin and enrofloxacin were dissolved by the absolute ethyl alcohol, and the initial concentration of curcumin and enrofloxacin were 2 mg/ml and 3 mg/ml, respectively. Twofold serial dilutions of 200 ul of curcumin sample solution transferred into test tubes to final concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.782, 0.391and 0 ug/ml, which were previously filled with 1800 ul LB broth. In addition, twofold serial dilutions of 200 ul of enrofloxacin sample solution transferred into test-tubes to final concentrations of 300, 150, 75, 37.5, 18.75, 9.375, 4.69, 2.34,1.17, 0.59, 0.29, 0.15 and 0 ug/ml, which were previously filled with 1800 ul LB broth. And corresponding to the concentration of ethanol as the positive control. The bacterial suspension (5 ul) was added to each test tube to final concentration of 10^6 colony-forming units (CFU) cells/ml. Test tubes were incubated at 28℃ for 20 h. After incubation, microbial growth was determined by estimating the increased turbidity of each well, measured at 530 nm using an MK3 spectrophotometer microplate reader (ThermoFisher). The minimal inhibitory concentration (MIC) was calculated from the highest content of curcumin and enrofloxacin above that inhibited the growth of *A. hydrophila* WJ2011BJ44. The test of antibacterial activity was repeated three times.

Growth curve.

Aeromonas hydrophila WJ2011BJ44 was incubated in LB broth at 28℃ for 16 h. The bacterial suspension was made to final concentration of 10^6 CFU cells/ml. The curcumin and enrofloxacin solution were added into bacterial suspension and kept final concentrations of MIC, 2 MIC, 3 MIC, and 4 MIC curcumin or enrofloxacin, respectively. And then, bacterial suspensions were incubated at 28℃. In addition, the group without curcumin and enrofloxacin was the control group. Every hour of the incubation period, microbial growth was determined by estimating the increased turbidity of each well, measured at 530 nm using an MK3 spectrophotometer microplate reader (ThermoFisher) (Zhang et al., 2014). The experiment of grow curve was repeated three times.

Killing curve.

Aeromonas hydrophila WJ2011BJ44 was incubated in LB broth at 28 ℃ for 16 h. The bacterial suspension was made to final concentration of 10^7 CFU cells/ml. The curcumin and enrofloxacin solution were added into bacterial suspension and kept final concentrations of MIC, 2 MIC, and 4 MIC curcumin or enrofloxacin, respectively. Then bacterial suspensions were incubated at 28 ℃. In addition, the group without curcumin and enrofloxacin was the

control. Every 2 h of the incubation period, tenfold serial dilutions of cell suspensions were inoculated in LB AGAR medium and incubated at 28℃ for 20 h. After incubation, all the colonies were calculated. Then the test results of models were plotted separately as a Killing curve with Lg CFU as the ordinate and incubation time as the abscissa (Zhang et al., 2014). The killing curve test was repeated three times.

Bacterial membrane permeability.

Aeromonas hydrophila WJ2011BJ44 was grown to log phase in LB broth for 16 h at 28℃. The bacterial suspension was made to final concentration of 10^6 CFU cells/ml. The curcumin, enrofloxacin, and combination of both solutions (curcumin: enrofloxacin=1 : 1) were added into bacterial suspension, and the final concentration of the curcumin, enrofloxacin and combination of both were 2 MIC, respectively. Then all bacterial suspensions were incubated at 28 ℃. The control group was treated without curcumin and enrofloxacin. Every 2 h of the incubation period, the bacterial suspensions were centrifuged at 3000 rpm for 5 min at 4℃, and the supernatants were diluted at 20-fold (Hao et al., 2009). The concentration of released K^+ was measured by atomic absorption spectrometer (Spectr AA 220; VARIAN, USA). All analysis was carried out in triplicate.

Flow cytometric (FACS) analysis.

After the treatment of curcumin and enrofloxacin, the membrane integrity of *A. hydrophila* WJ2011BJ44 was determined by flow cytometric analysis using propidium iodide (PI) as a probe (Jiang et al., 2006). *A. hydrophila* WJ2011BJ44 was grown to log phase in LB broth and then mixed with curcumin, enrofloxacin, and a combination of both solutions, respectively. The final concentrations of the curcumin, enrofloxacin and their combination (curcumin: enrofloxacin=1 : 1) were 2 MIC, respectively. Then all bacterial suspensions were incubated at 28℃ for 4 h respectively. The bacterial suspensions were centrifuged at 3000 rpm for 5 min at 4℃, and *A. hydrophila* Cells were washed three times with sterile phosphate-buffered saline (PBS) and resuspended at a concentration of 10^6 CFU/ml in the same buffer. The treated cells were incubated with PI solution (50 ug/ml final concentration) at 37 ℃ for 30 min, followed by the removal of unbound dye through excessive washing with PBS. PI was excited at 488 nm using an argon laser and could result in fluorescence emission, measured by a 660 nm long-pass filter. The group of enrofloxacin was used as positive control and without treatment with curcumin and enrofloxacin as a negative control. Flow cytometry analysis was conducted using a FACScan instrument (Calibur, BO, USA). All analysis was carried out in triplicate.

Transmission electron microscopy.

To clarify the sterilization mechanism of curcumin against *A. hydrophila*, we treated *A. hydrophila* cells with curcumin, enrofloxacin, or their combination, and the ultrastructure of *A. hydrophila*cells of treatment was measured by using Transmission electron microscopy (Zhang et al., 2014). Exponential-phase bacteria were treated with 2 MIC of curcumin or enrofloxacin for 4 h at 37 ℃. Cells were harvested by centrifugation and washed twice with deionized water. After treatment, the bacterial pellets were fixed with 2.5% buffered glutaraldehyde for 1 h. The cells were then post-fixed in 1% buffered osmium tetroxide for 1 h, stained en bloc with 1% uranyl acetate, dehydrated in graded ethanol concentrations, and subsequently embedded in spur resin. The buffer used was 0.1 M sodium cacodylate (pH 7.4). Thin sections were prepared on Formvar copper grids and stained with 2% uranyl acetate and lead citrate (Friedrich et al., 2001). Enrofloxacin was used as a positive control and double-distilled water as a negative control. Microscopy was performed with transmission electron microscopy (H-7000; Hitachi, Japan) under standard operating conditions.

Data statistics and analysis.

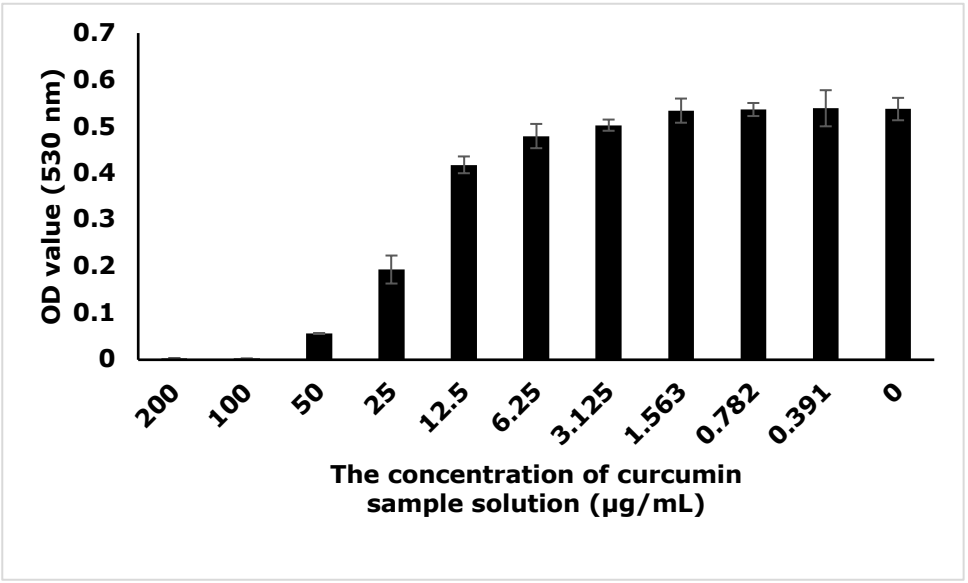
All data are presented as means ± SEM (standard error of the mean). Data were logarithmic transformed before subjecting to one-way analysis of variance (ANOVA) using SPSS 19.0. When the overall treatment effect was significantly different, Tukey's test was conducted to compare the means between the different treatment groups. The level of significant difference was set at $P < 0.05$.

Results

Antibacterial activities of curcumin and enrofloxacin.

Figure 1 indicated that the minimal inhibitory concentration (MIC) values of curcumin(**a**) and enrofloxacin (**b**) against *A. hydrophila* WJ2011BJ44 were 100 ug/ml and 9.375 ug/ml, respectively. As shown in **Figure 2**, results showed that the trend of the growth curve of *A. hydrophila* treated with different concentrations (from MIC to 4 MIC) of curcumin (**a**) or enrofloxacin (**b**) was similar and steady, and there was no significant difference on the growth curve of different groups of treatment. It turned out that curcumin or enrofloxacin completely inhibited the growth of *A. hydrophila,* even at low concentrations. In contrast, the bacteria in the control group continued to grow, completing the logarithmic phase of growth, stable phase, and decline phase.

a



b

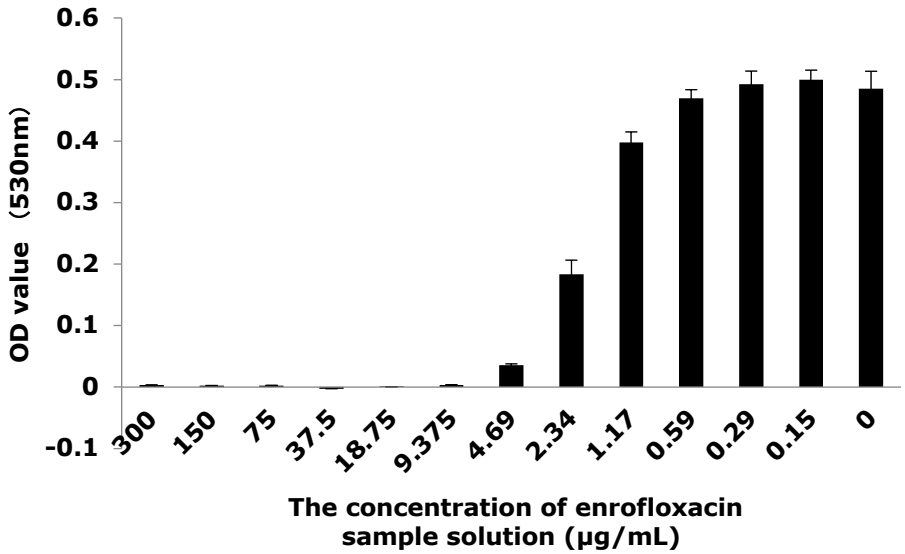
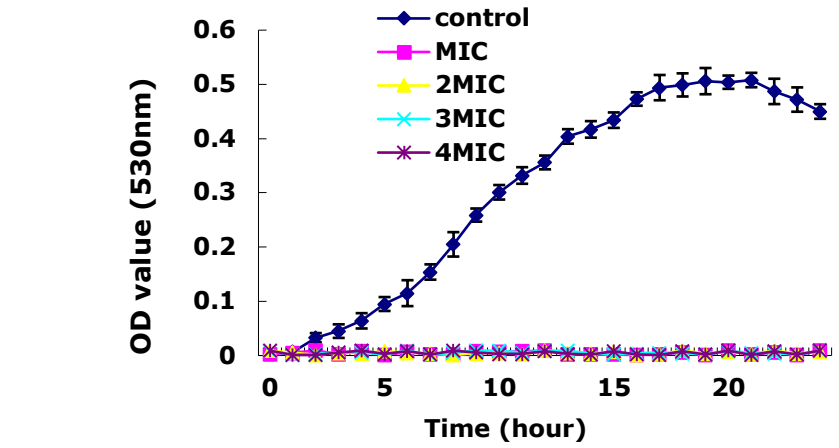


Figure 1 Antibacterial activities (MIC) of curcumin (a) and enrofloxacin (b) against *Aeromonas hydrophila.* Note: Data are expressed as means ± SEM（n = 3.



b

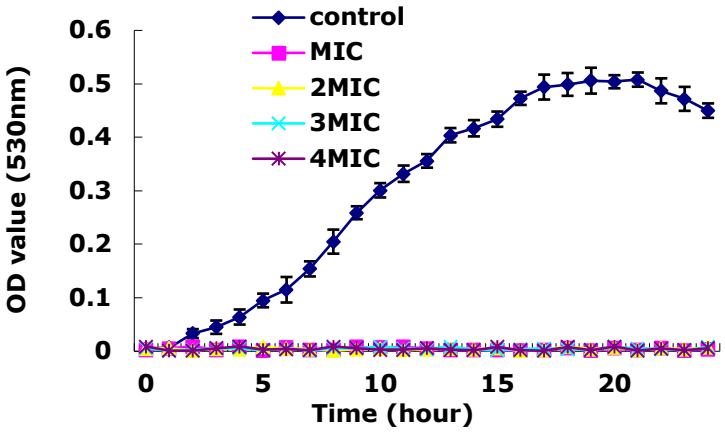


Figure 2 The effect of curcumin (a) and enrofloxacin (b) on the growth curve of *Aeromonas hydrophila*
Note: Data are expressed as means ± SEM (n = 3).

killing curve of curcumin and enrofloxacin.

The killing curve of curcumin (**a**) and enrofloxacin (**b**) on *A. hydrophila* are shown in **Figure 3**. Curcumin at a concentration of 2 MIC could completely kill *A. hydrophila* within 10 hours. However, curcumin at a concentration of 4 MIC could completely kill *A. hydrophila* within 8 hours. There were significant differences ($P < 0.05$) among different groups from 2 h to 8 h after *A. hydrophila* was treated with different concentrations of curcumin (**Figure 3a**). In addition, enrofloxacin at 4 MIC concentration can completely kill *A. hydrophila* within 8 hours, but enrofloxacin at MIC and 2 MIC concentration could not completely kill *A. hydrophila* within 10 hours. And significant differences ($P < 0.05$) were observed among all treatment groups from 2 h to 10 h (**Figure 3b**).

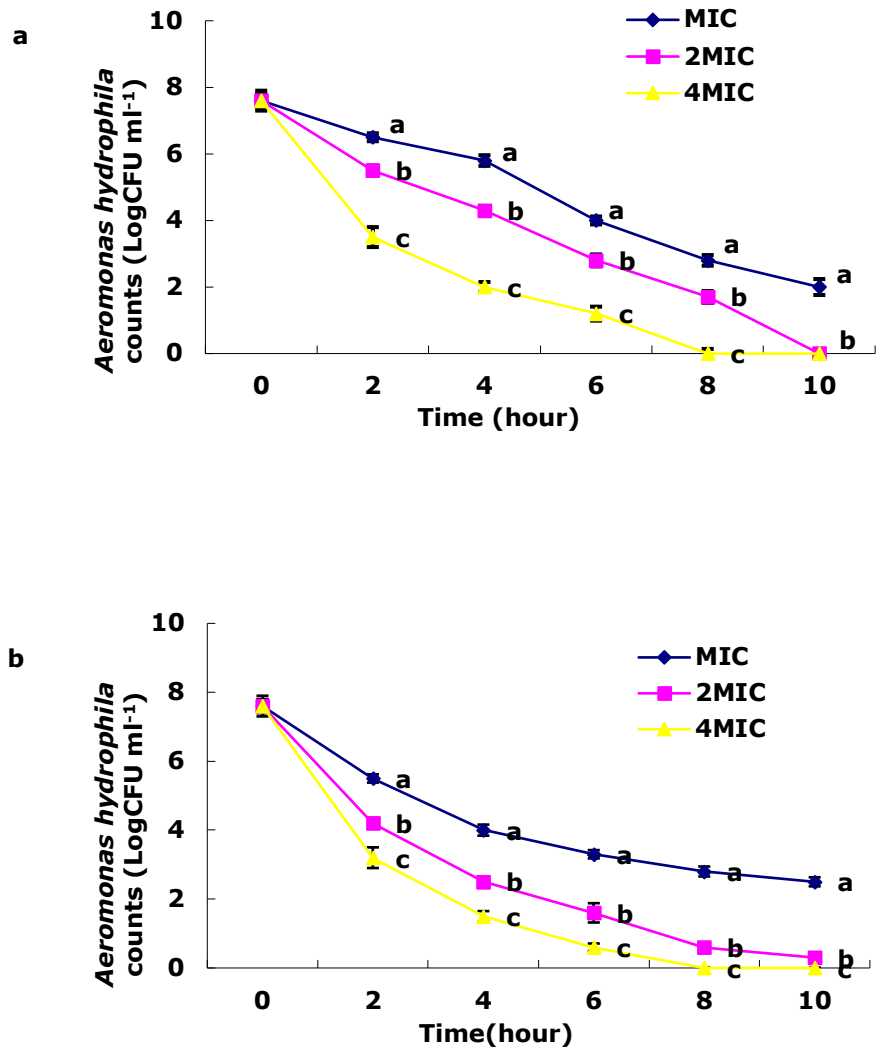


Figure 3 The effect of curcumin (a) enrofloxacin (b) on the killing curve of *Aeromonas hydrophila.* Values are means ± SEM of 3 replications. Means in the same index with different superscripts are significantly different (*P<0.05*).

Bacterial membrane permeability.

The effect of curcumin on the membrane permeability of *A. hydrophila* was investigated by measuring the number of potassium ions released from curcumin-treated cells. **Figure 4** indicated that a significant potassium efflux from curcumin-treated cells was induced after incubation. The K^+ efflux increased with increasing incubation time from 1 to 4 h; when the time was increased further, only slight changes were observed. There were significant differences ($P < 0.05$) in the K^+ concentration of bacteria cells among all treatment groups after incubation from 1 h to 8 h compared with that of the control. The highest K^+ concentration was observed in enrofloxacin of the 2 MIC group after incubation from 1 h to 8 h. In addition, at 2 to 8 h after incubation, the K^+ concentration in the enrofloxacin group was significantly higher than that in the curcumin group ($P < 0.05$), but had no significant difference from that in the compatibility group ($P > 0.05$). The membrane permeability of *A. hydrophila* cells

treated with drugs was: enrofloxacin treatment group > curcumin + enrofloxacin treatment group > curcumin treatment group > control group.

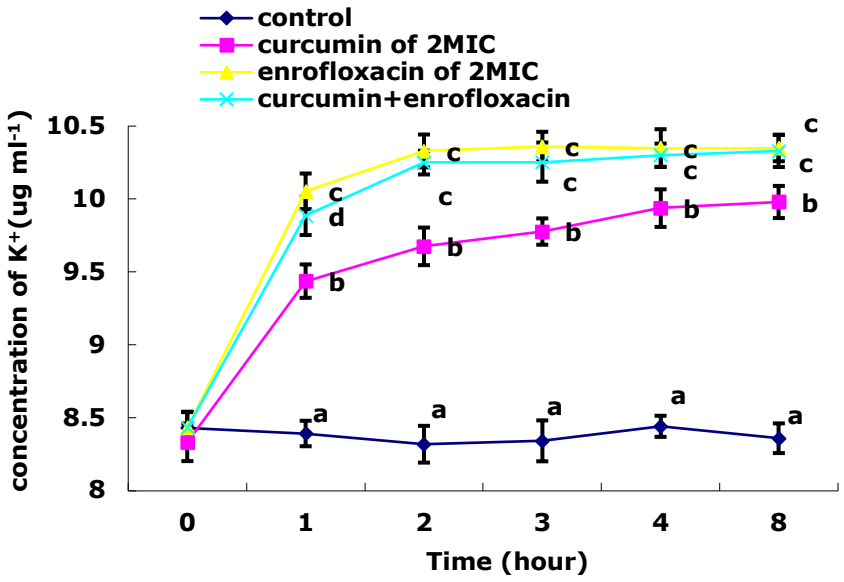


Figure 4 The effect of curcumin on bacterial membrane permeability of *Aeromonas hydrophila.* Values are means ± SEM of 3 replications. The Means in the same index with different superscripts are significantly different (*P<0.05*).

Flow cytometric (FACS) analysis.

Detection of internal PI in single cells can indirectly reflect the state of the cells and was analyzed via flow cytometry. As shown in **Figure 5**, in the absence of curcumin and enrofloxacin, 0.57 % of untreated control cells showed a PI fluorescence signal (**Figure 5a**), indicating viable cells excluding the PI dye. However, when treated with 2MIC curcumin and 2MIC enrofloxacin, 14.88% (**Figure 5b**) and 13.98 % (**Figure 5c**) of *A. hydrophila* cells labeled fluorescently, respectively, thereby indicating that curcumin induced PI influx into the cells as enrofloxacin did. But when treated with curcumin and enrofloxacin, the PI fluorescence signal of treated *A. hydrophila* cells was 18.03 (**Figure 5d**). The highest PI fluorescence signal was observed in the curcumin + enrofloxacin group (**Figure 5d**).

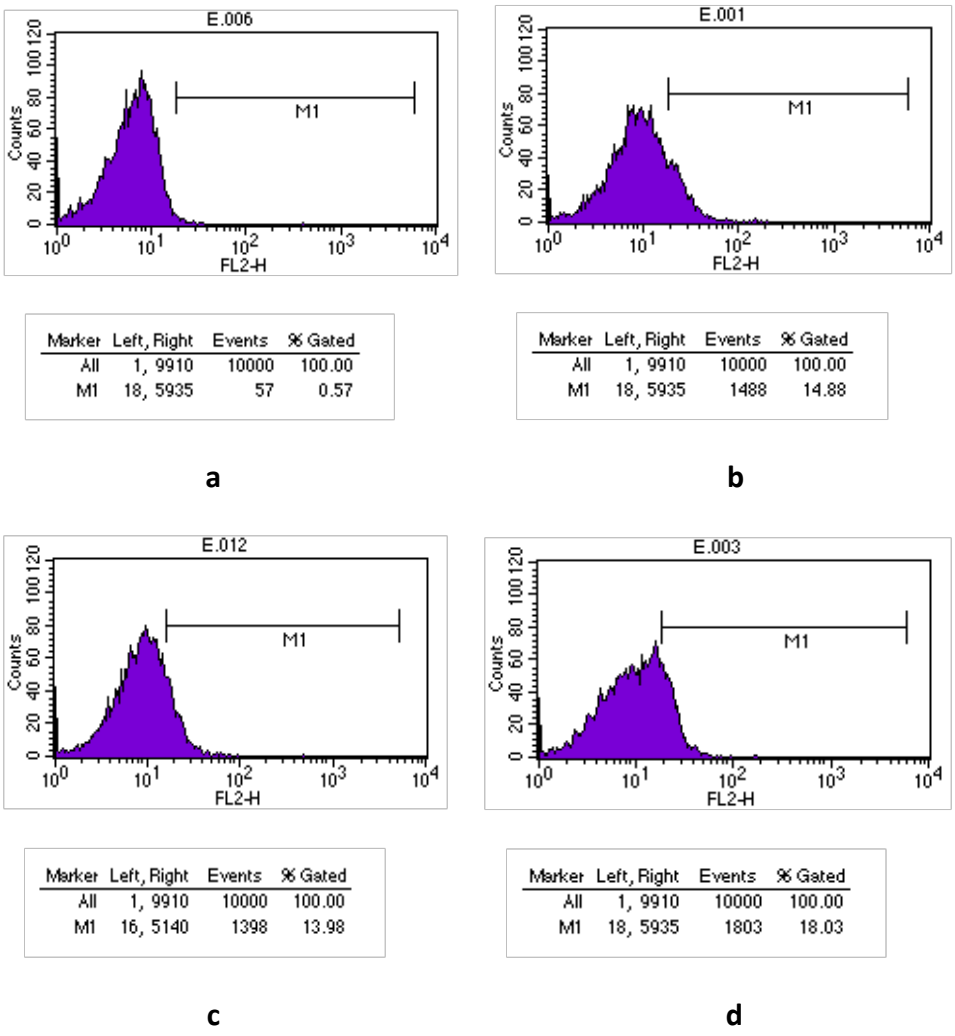


Figure 5 Flow cytometric measurement of the effects of curcumin and enrofloxacin. The increments of the log fluorescence signal represent the uptake of PI by the bacteria cells. Cells not treated with curcumin (**a**), cells treated with curcumin (**b**) or enrofloxacin (**c**), and cells treated with curcumin and enrofloxacin (**d**).

Transmission electron microscopy.

Transmission electron microscopy was used to observe the morphological changes of bacterial cells treated with curcumin. The electron micrographs are displayed in Figure 6. The cells in the control group remained intact, with smooth surfaces and no structural damage (**Figure 6a**). However, after 4 h of treatment with curcumin or enrofloxacin or their combination, the cells showed important morphological changes such as breakage of cell wall and membrane, and leakage of cellular cytoplasmic contents were also observed (**Figure 6b, c, d**). **Figure 6** indicated that the destruction of *A. hydrophila* cells treated with the combination of curcumin and enrofloxacin was the most serious compared with other groups.

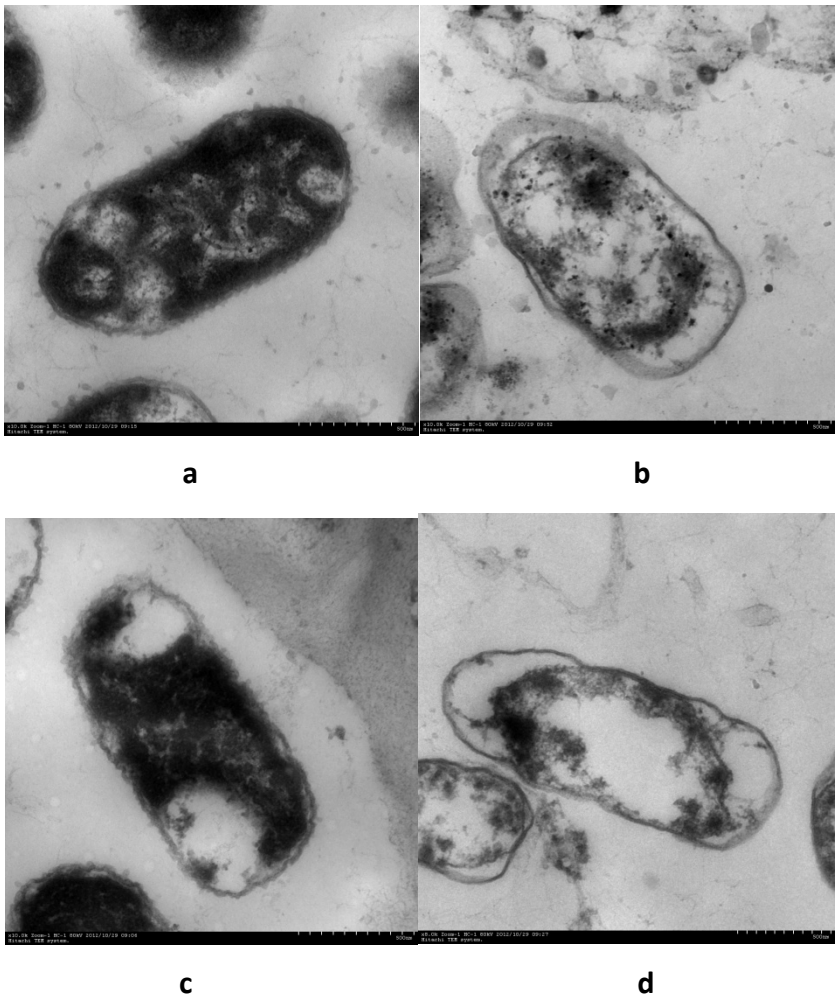


Figure 6 Transmission electron micrographs of the effects of curcumin and enrofloxacin. Cells not treated with curcumin (**a**), cells treated with curcumin (**b**) or enrofloxacin (**c**), and cells treated with curcumin and enrofloxacin (**d**).

Discussion

Analysis of the antibacterial activity of curcumin and enrofloxacin showed that the two drugs exhibited excellent antibacterial activity against *A. hydrophila*. In addition, the antibacterial activity of curcumin and enrofloxacin was positively related to concentrations of the two drugs, indicating that curcumin and enrofloxacin were similar to the main components inhibiting the growth of *A. hydrophila*. In the present study, the results showed that the minimum inhibitory concentration (MIC) of curcumin against *A. hydrophila* was 100 μg/mL, and 2 MIC and 4 MIC curcumin could completely kill *A. hydrophila* within 10 h and 8 h, respectively. In addition, the minimum inhibitory concentration (MIC) of enrofloxacin against *A. hydrophila* was 9.375 μg/mL, significantly different from curcumin against *A. hydrophila*. A reasonable explanation was that enrofloxacin was a small molecule compound, which had a high permeability to the bacterial cell wall, and could destroy the cell membrane, quickly and directly acting on the bacterial DNA to achieve the purpose of sterilization. However, curcumin, as turmeric extract, mainly played a regulatory role in aquatic animals. Curcumin could not penetrate the cell membrane of bacteria quickly due to its large molecular weight, so it had a slow effect on bacteria, which affected its inhibitory effect on bacteria (Wang et al., 2010).

Enrofloxacin has been widely used because of its strong antibacterial activity. However, with the long-term abuse of the drug in large doses, the biological body will produce drug residue and drug resistance, resulting in a significant decline in its antibacterial activity. To avoid the occurrence of these phenomena, it has become a hot research topic to find a natural antimicrobial agent to replace enrofloxacin with less toxic side effects and less drug residue and resistance.

Damage to the bacterial cell wall and cytoplasmic membrane might indicate a loss of structural integrity and impact the membrane's ability as a permeable barrier (Lu et al., 2011). When the bacterial membrane was damaged to a certain extent, small ions such as potassium and phosphate could be leached out, and some cytoplasmic constituents from the cells could be monitored. Therefore, we evaluated the effects of curcumin, enrofloxacin, and a combination of both on the membrane permeability of *A. hydrophila* cells by measuring the number of potassium ions released by cells treated with the drug. Our results showed that the increase in the amount of K^+ released from *A. hydrophila* cells after treatment provided evidence that curcumin and enrofloxacin increased the plasma membrane permeability, caused potassium ion leakage from treated cells (**Figure 4**) and then led to *A. hydrophila* cells death (Denyer, 1990; Larsen et al., 1977). One possible reason is that curcumin and enrofloxacin may form pores in the cell membrane, resulting in leakage of cell contents, destruction of membrane fluidity, loss of electrochemical potential, and failure of the cell to maintain normal osmotic pressure, and thus leading to cell death (Denyer, 1990; Bhuvaneswari & Balasundaram, 2006). It is also possible that the loss of intracellular potassium ions and other contents will block various metabolic pathways in the cell, reduce the activity of enzymes, and destroy the stability of the membrane (Denyer, 1990). In addition, the membrane permeability of *A. hydrophila* in the compatibility group was higher than that in the monomer group, indicating that the combination of curcumin and enrofloxacin increased the membrane permeability more easily than that of the monomer (Bai et al., 2009; Ganguly et al., 2010). However, the detailed mechanism needs to be further researched. Meanwhile, FACScan analysis showed that the fact was confirmed (**Figure 5**).

As a cationic nucleic acid dye, PI cannot enter living cells without cell membrane damage under normal conditions. In other words, normal cells and apoptotic cells refuse to stain PI in the case of non-fixation. In contrast, in necrotic cells, PI can enter the cell and bind to double-stranded nucleotides due to damage to membrane integrity (Ananta et al., 2004). According to these characteristics, to explore whether curcumin, enrofloxacin, and their combination have a damaging effect on the cell membrane of *A. hydrophila*, we can use PI cell activity staining to identify the degree of cell damage. In this experiment, the relative fluorescence intensity of bacteria treated with drugs was significantly higher than that of the control group, indicating that curcumin, enrofloxacin, and a combination of both could destroy the integrity of the bacterial membrane, and their mechanism of action was the same as increasing the permeability of the bacterial membrane. Among them, the damage degree of membrane integrity was the most serious after the combination treatment. In addition, morphological changes and leakage of cytoplasmic contents were also demonstrated by electron micrographs of *A. hydrophila* cells treated with curcumin, enrofloxacin, and a combination of both (**Figure 6**). The previous reports indicated that curcumin (Alves et al., 2004; Shan et al., 2008) and enrofloxacin (Efthimiadou et al., 2008) could bind and insert into the cell membrane, cause damage to cytoplasmic membrane integrity. The present study was in agreement with these results. In addition, the results showed that the combination of curcumin and enrofloxacin had the highest membrane permeability to *A. hydrophila*, indicating that curcumin could partially replace enrofloxacin as a bactericidal drug, which may be related to the synergistic effect between curcumin and enrofloxacin (Jiang et al., 2006). Detailed mechanisms need to be further researched.

Conclusion

In conclusion, these results indicated that a 2 MIC concentration of curcumin, 2 MIC concentration of enrofloxacin, combination of both (Both concentrations were 1MIC) could inhibit the growth of *A. hydrophila*, increase bacterial membrane permeability and damage bacterial cell membrane integrity. The compatibility group was the best for the antibacterial result against *A. hydrophila*. Our results showed that compared with the monomeric group, the compatibility group had the most enhanced anti-*A. hydrophila* ability, indicating that curcumin could be used as a bactericidal drug to replace enrofloxacin to some extent.

Acknowledgments

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