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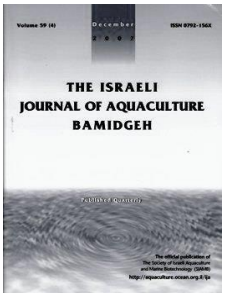

Comparative Study of Antibacterial Properties of Emodin and Enrofloxacin Against Aeromonas hydrophila

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Key words: emodin, *Aeromonas hydrophila*, antibacterial activity, bacterial membrane permeability

Abstract

Antibacterial properties of emodin, extracted from rhubarb, and enrofloxacin, against *Aeromonas hydrophila*, were assessed in this study. The minimum inhibitory concentration (MIC) values of emodin and enrofloxacin to fight *A. hydrophila* WJ2011BJ44 were found to be 100ug/ml and 9.375ug/ml, respectively. To understand the mechanisms of action of emodin and enrofloxcain against *A. hydrophila* we studied antibacterial activity, bacterial membrane permeability, and ultrastructure of *A. hydrophila* cells treated with emodin, enrofloxacin individually, and the combination of both. The results shown in the growth curve of *A. hydrophila* treated with different concentrations (from 0 MIC to 4 MIC) of emodin and enrofloxacin were similar and stable, and there was no significant difference in the growth curve of different treatment groups. There were significant differences in the K^+ concentration among all treatment groups from 1 h to 8 h after incubation compared with the control. The highest K^+ concentration was observed in the emodin+enrofloxacin group from 1 h to 8 h after incubation. PI fluorescence signal of untreated *A. hydrophila* cells and *A. hydrophila* cells treated with emodin, or enrofloxacin individually, or the combination of both were 0.89, 11.4, 13.98 and 18.3, respectively. The mortality of *A. hydrophila* cells treated with the combination of emodin and enrofloxacin was greatest compared with other groups. These results indicated that 2 MIC emodin, 2 MIC enroflxacin, and combination of 1 MIC emodin and 1 MIC enrofloxacin can inhibit the growth of *A. hydrophila*, increase bacterial membrane permeability, and damage cell membrane integrity. The combination of 1 MIC concentration emodin and 1 MIC concentration enrofloxacin produced the best antibacterial activity against *A. hydrophila*.

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Introduction

There is increasing interaction between aquaculture and fisheries for socio-economic reasons. The relevance of interaction between the two sectors is becoming more evident as the transition from fishing to farming has reached a pivotal point, as almost 50% of fish food supply now comes from aquaculture. China is a major aquaculture country, accounting for nearly 70% of aquaculture output worldwide. However, there are many types of fish diseases caused by pathogenic bacteria (Frans et al., 2008). These include the bacteria, *Aeromonas hydrophila* (Vivas et al., 2004), as well as fungi (Frans et al., 2008), viruses (Wang et al., 2012), and parasites (Xi et al., 2011), which have caused severe economic losses in aquaculture throughout the country (Feng, 2010).

Aeromonas hydrophila, a Gram-negative rod-shaped bacterium belonging to the Aeromonidae family, is widely distributed in fresh water, sewage-contaminated water, sludge, soil, and foods. *A. hydrophila* is an important bacterial pathogen and is associated with several fish diseases, such as hemorrhagic septicemia, fin and tail rot, and epizootic ulcerative syndrome (Larsen et al., 1977; Lu, 1992). These diseases have caused high mortality in freshwater fish resulting in extensive losses worldwide (Feng, 2010). Antibiotics and chemotherapeutics used to control these diseases can result in development of drug-resistant bacteria, environmental pollution, and residues in fish. In order to prevent disease and reduce side effects associated with antibiotics demand is increasing for organic aquaculture, and there is a growing interest in using natural products, functional carbohydrates (Sun et al., 2011), and plant extracts (Harkrishnan et al., 2008; Xie et al., 2008; Bhuvaneswari and Balasundaram, 2006)

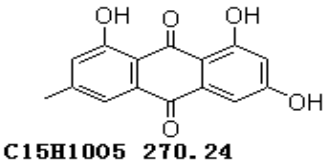


Fig. 1 Structural formula of emodin

Emodin (1, 3, 8-trihydroxy-6-methyl-anthraquinone), (Fig 1) one of the important bioactive compounds in rhubarb, has shown a wide variety of pharmacological properties — anti-inflammatory (Kuo et al., 2001), antioxidation (Iizuka et al., 2004), scavenging free radicals (Huang et al., 1995), antimicrobial (Wang et al., 2010), blood lipid reduction (Zhou et al., 2006), liver protection (Lin et al., 1996), immunity regulation (Wang et al., 1995) and antitumor activities (Wang et al., 2010). Among its wide biological activities, in only a few cases has the mechanism has been elucidated. The antibacterial activity and mechanisms of action of emodin against *A. hydrophila* have been little reported. Anthraquinone extract (main components, emodin, chrysophanol, and rhein) can promote growth, enhance immunity and resistance to high temperatures, of freshwater prawn *Macrobrachium rosenbergii* (Liu, et al., 2010), however little information has been obtained on the comparison between emodin and enrofloxacin.

Enrofloxacin (Fig. 2) 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).

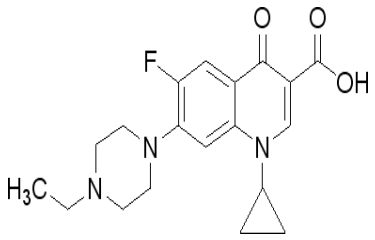


Fig. 2. Structural formula of enrofloxacin.

Emodin has been regarded as an immunostimulant that leads to an increase in non-specific immunity of fish (Xie et al., 2008), anti-oxidization enzyme activity (Xie, et al., 2008; Liu, et al., 2010), and disease resistance (Xie, et al., 2008).

The aim of this study was to investigate the mechanism of antibacterial activity of emodin and enrofloxacin against *A.hydrophila* and the comparison between emodin and enrofloxacin. We investigated the morphology of treated cells and the molecular mechanism of emodin and errofloxacin against *A. hydrophila*. Several possible mechanisms of action were proposed. Our results provide theoretical base for the use of emodin to increase disease resistance in fish in the future.

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. *A. hydrophila* WJ2011BJ44, was selected due to its virulence in preliminary challenge experiments. Emodin and enrofloxacin (purity>99%) were obtained from Feida Chemical Reagent Co. (Xian, China). A Cell Apoptosis PI detection kit was purchased from Beijing FanBo Biotech. Co. Ltd., China. UPLCgrade methanol was purchased from Sigma–Aldrich (St. Louis, MO, USA). All other reagents (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) were of analytical grade.

Antibacterial activity. The antimicrobial activities of emodin extracted from *Rheum officinale Bail* and enrofloxacin were determined by using a twofold micro-dilution broth method (Naghmouchi et al., 2006). *A. hydrophila* WJ2011BJ44 was grown to mid-log phase in Luria-Bertani (LB) broth at 28^0C for 20 h. The emodin and enrofloxacin were dissolved in absolute ethyl alcohol and the initial concentrations of emodin and enrofloxacin were 2 mg/ml and 3 mg/ml, respectively. Twofold serial dilutions of 200 ul of emodin sample solution were transferred into test-tubes to final concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.782, 0.391 and 0 ug/ml, which had been filled with 1800 ul LB broth. Twofold serial dilutions of 200 ul of enrofloxacin sample solution were transferred into test-tubes to make up 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, filled with 1800 ul LB broth and corresponding to the concentration of ethanol as a positive control. Bacterial suspension (5 ul) was then added into each test-tube to a final concentration of 10^6 colony-forming units (CFU) cell/ml. Test-tubes were incubated at 28^0C for 20 h. After incubation, microbial growth was determined by estimating the increased turbidity of each well, measured at 530 nm using a MK3 spectrophotometer microplate reader (ThermoFisher). The minimal inhibitory concentration (MIC) was calculated from the highest content of emodin and enrofloxacin above which growth of *A. hydrophila* WJ2011BJ44 was inhibited. The test of antibacterial activity was carried out in triplicate.

Growth curve. A. hydrophila WJ2011BJ44 was grown to log phase in LB broth at 28^0C for 16 h. Bacterial suspension was made up to a final concentration of 10^6 CFU cell/ml. The emodin and enrofloxacin solutions were added to the bacterial suspension and kept as final concentrations of 0 MIC (control), 2 MIC, 3 MIC and 4 MIC emodin and enrofloxacin, respectively. The bacterial suspension was incubated at 28^0C. The control group was not treated with either emodin or enrofloxacin. Microbial growth was determined hourly during the incubation period by estimating the increased turbidity of each well, measured at 530 nm using a MK3 spectrophotometer microplate reader (ThermoFisher). The growth curve experiment was repeated three times.

Mortality curve. A. hydrophila WJ2011BJ44 was grown to log phase in LB broth at 28^0C for 16 h. Bacterial suspension was made to a final concentration of 10^7 CFU cell /ml. The emodin and enrofloxacin solutions were added to the bacterial suspension and kept at final concentrations of 0 MIC, 2 MIC and 4 MIC emodin and enrofloxacin, respectively. Then bacterial suspensions were incubated at 28^0C. Every 2 h during the incubation period, tenfold serial dilutions of cell suspensions were inoculated in LB AGAR medium, incubated at 28^0C for 20 h. After incubation, all the colonies were counted. The test results of models were plotted separately as a mortality curve with Lg CFU as the ordinate and culturing time as the abscissa. The mortality curve test was repeated three times (Fig 5).

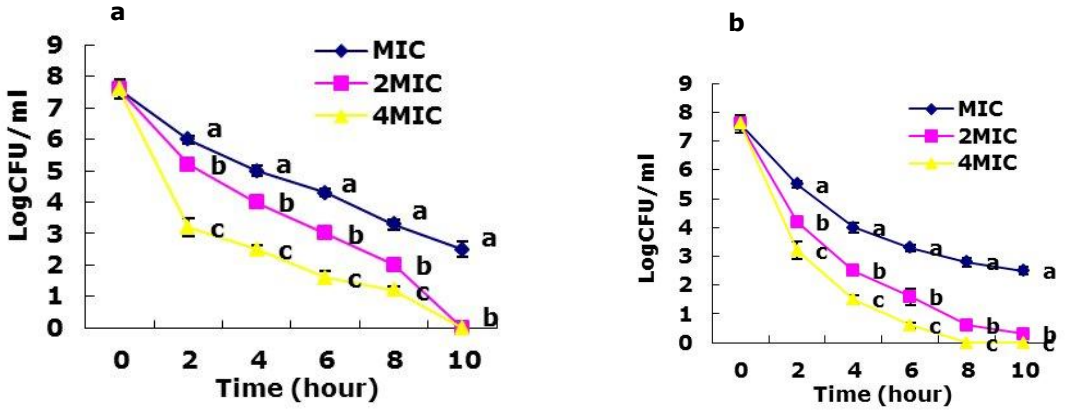


Fig. 5 The effect of emodin (a) enrofloxacin (b) on mortality curve of *Aeromonas hydrophila.*
Note: Data are expressed as means ± SEM (n = 3). Diverse little letters show significant differences (P < 0.05) in different dosage groups of each sampling point in Duncan's multiple range test.

Bacterial membrane permeability. A. hydrophila WJ2011BJ44 was grown to mid-log phase in LB broth for 16 h at 28^{0}C. Bacterial suspension was made up to a final concentration of 10^6 CFU cell /ml. The emodin, enrofloxacin, and combination of both solutions were added to the bacterial suspension. Final concentrations of emodin, and enrofloxacin, were 2 MIC respectively, and the combination of both was also 2 MIC (1 MIC emodin and 1 MIC enrofloxacin). All bacterial suspensions were then incubated at 28^{0}C. The control group treatment was emodin and enrofloxacin. Every 2 h of incubation period, the bacterial suspensions were centrifuged at 3000 rpm for 5 min at 4^{0}C, and the supernatants were diluted 20-fold (Hao et al., 2009). The concentration of released K^+ was measured by an atomic absorption spectrometer (Spectr AA 220; VARIAN, USA). All analysis was carried out in triplicate (Fig.6).

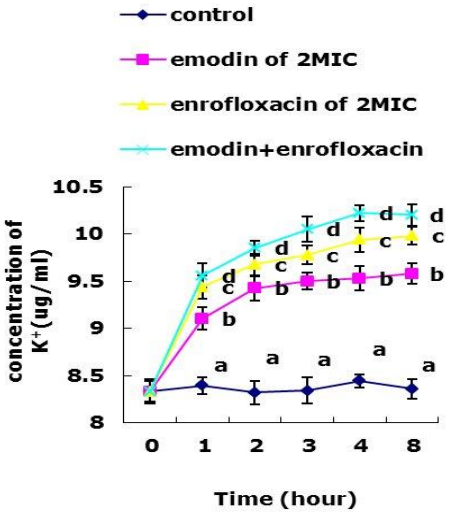


Fig. 6 The effect of emodin on bacterial membrane permeability of *Aeromonas hydrophila.*
Note: Data are expressed as means ± SEM (n = 3). Diverse lower case letters show significant differences (P < 0.05) in different dosage groups of each sampling point in Duncan's multiple range test.

Flow cytometric (FACS) analysis. After treatment with emodin and enrofloxacin, the membrane integrity of *A. hydrophila* WJ2011BJ44 was determined by flow cytometric analysis using propidium iodide (PI) as a probe (Jang et al., 2006). *A. hydrophila* WJ2011BJ44 was grown to log phase in LB broth and then mixed with emodin, enrofloxacin, and a combination of both solutions. Final concentrations of the emodin, enrofloxacin, and their combination were 2 MIC, respectively (The combination was of 1 MIC emodin and 1 MIC enrofloxacin). All bacterial suspensions were incubated at 28^{0}C for 4 h. *A. hydrophila* cells were washed three times with sterile phosphate-buffered saline (PBS), and re-suspended 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^{0}C for 30 min, then thoroughly washed with PBS to remove unbound dye. PI was excitated at 488 nm using an argon laser, and the resulting fluorescence emission was measured by a 660 nm long-pass filter. Enrofloxacin was used as a positive control, and the negative control

received no emodin or enrofloxacin. Flow cytometry analysis was conducted using a FACScan instrument (Calibur, BO, USA). All analysis was carried out in triplicate.

Scanning electron microscopy. In order to clarify the sterilization mechanism of emodin against *A. hydrophila*, we treated *A. hydrophila* cells with emodin, enrofloxacin, or a combination of both. The ultrastructure of treated *A. hydrophila* cells was measured using Scanning electron microscopy. *A. hydrophila* WJ2011BJ44 was grown to log phase in LB broth, and subjected to the same flow cytometric (FACS) analysis. *A. hydrophila* cells were collected by centrifugation (3000rmp, 3min) and washed twice with deionized water. After treatment, the bacterial pellets were fixed with 2.5% buffered glutaraldehyde for 3 h. The *A. hydrophila* cells were dehydrated in graded ethanol concentrations for 10 min each time, dehydrated twice in absolute ethyl alcohol for 10 min each time, and subsequently exchanged with graded tert-butanol concentrations for 5 min each time. After treatment, the cells were submerged in tert-butanol, and subsequently dried using a Hep-2 critical evaporator. Finally, samples were sprayed using a sputter coater and observed using scanning electron microscopy (S-3000N; Hitachi, Japan) under standard operating conditions. The group without emodin and enrofloxacin was the control (Fig 7).

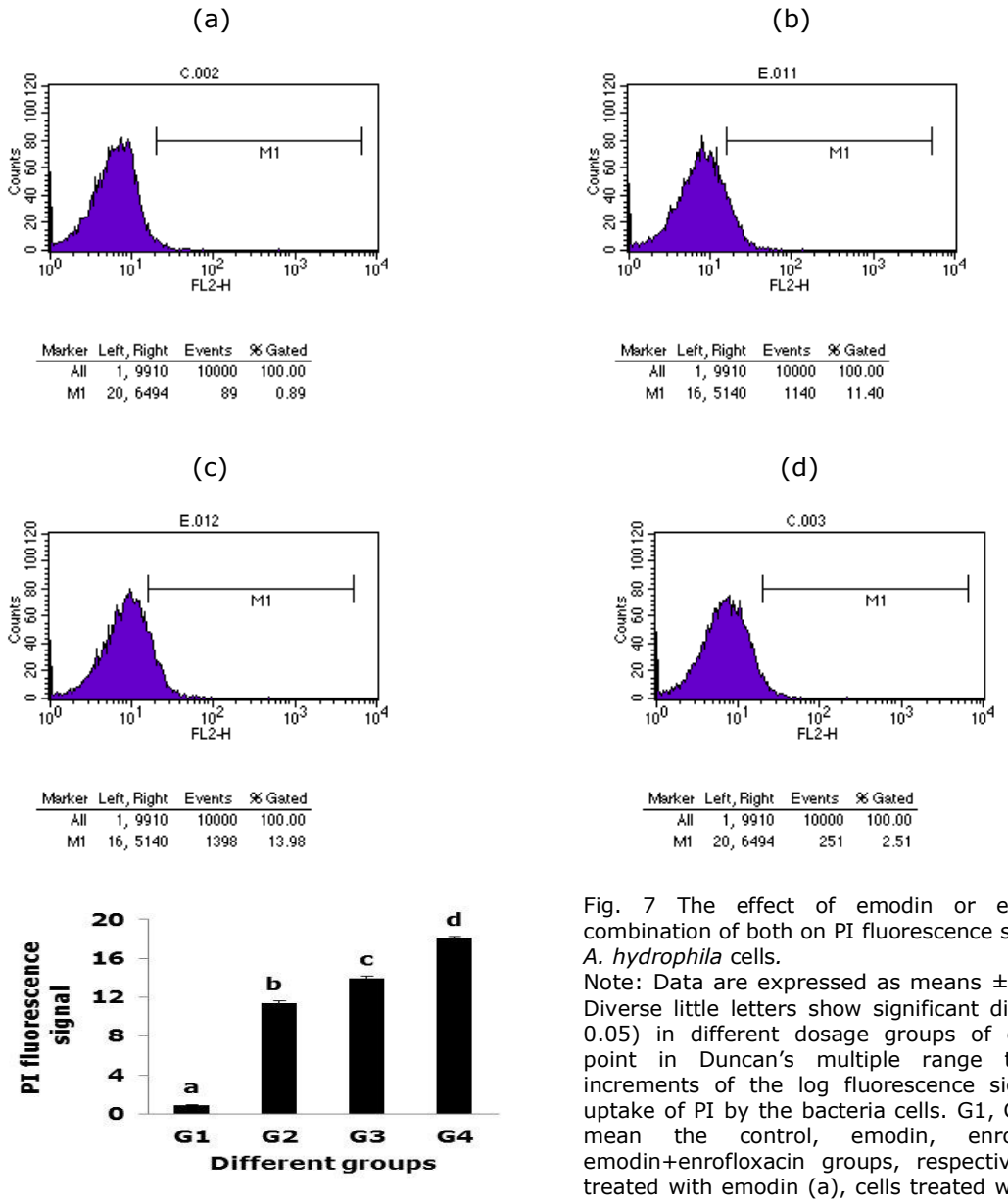


Fig. 7 The effect of emodin or enrofloxacin or combination of both on PI fluorescence signal of *A. hydrophila* cells.

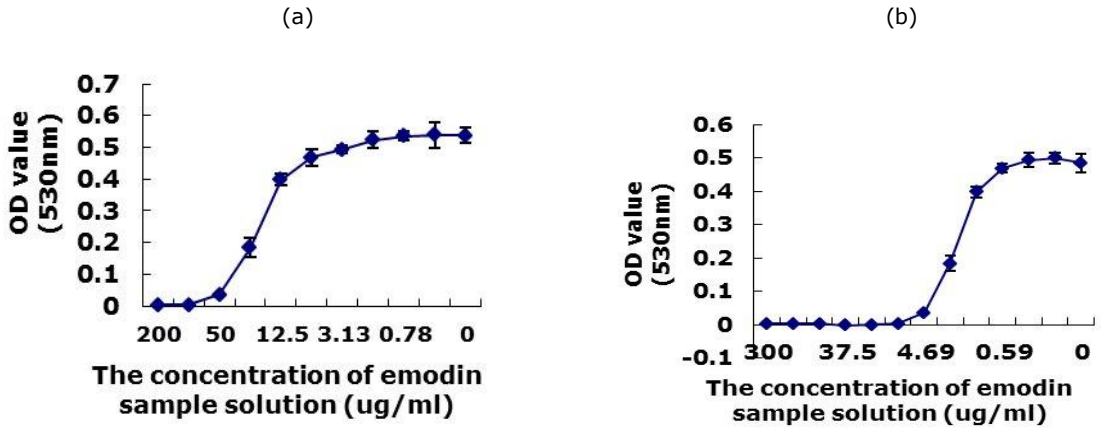
Note: Data are expressed as means ± SEM (n = 3). Diverse little letters show significant differences (P < 0.05) in different dosage groups of each sampling point in Duncan's multiple range test (A). The increments of the log fluorescence signal represent uptake of PI by the bacteria cells. G1, G2, G3 and G4 mean the control, emodin, enrofloxacin and emodin+enrofloxacin groups, respectively. Cells not treated with emodin (a), cells treated with emodin (b) or enrofloxacin (c), and cells treated with emodin and enrofloxacin (d).

Data statistics and analysis. All data are presented as means ± S.E. (standard error of the mean). Data were transformed logarithmically before being subjected to one-way analysis of variance (ANOVA) using SPSS 13.0. 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 emodin and enrofloxacin. The antibacterial activities of emodin and enrofloxacin on *A. hydrophila* are shown in Fig. 3.

Fig. 3 Antibacterial activities (MIC) of emodin (a) and enrofloxacin (b) against *Aeromonas hydrophila*



Note: Data are expressed as means ± SEM (n = 3).

The minimal inhibitory concentration (MIC) values of emodin and enrofloxacin against *A. hydrophila* WJ2011BJ44 were 100 ug/ml and 9.375 ug/ml, respectively. Fig. 4 indicated that the trend of growth curve of *A. hydrophila* treated with different concentrations (from MIC to 4 MIC) emodin (a) and enrofloxacin (b) were similar and steady, and there was no significant difference on the growth curve of the different treatment groups.

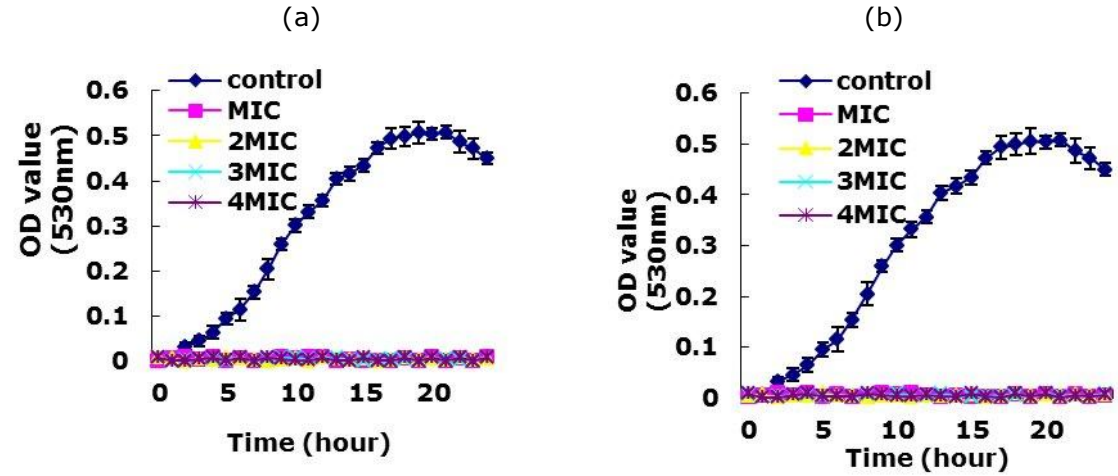


Fig. 4 The effect of emodin (a) and enroflxacin (b) on growth curve of *Aeromonas hydrophila*
Note: Data are expressed as means ± SEM (n = 3).

Results showed that emodin (a) at concentrations of 2 MIC and 4MIC can kill *A. hydrophila* within 10 hours. There were significant differences ($P < 0.05$) between different groups from 2 h to 8 h after *A. hydrophila* was treated with emodin (Fig.5a). In addition, enrofloxacin at a concentration of 4 MIC can kill *A. hydrophila* within 8 hours (Fig. 5b). Significant differences ($P < 0.05$) were observed among all treatment groups from 2 h to 10 h.

Bacterial membrane permeability. Significant potassium efflux from bacterial cells was induced after incubation, and K^+ efflux increased with increasing incubation time from 1 to 4 h; only slight changes were observed after more time. There were significant differences ($P < 0.05$) in the K^+ concentration of bacterial cells among all treatment groups from 1 h to 8 h after incubation compared with the control. The highest K^+ concentration was observed in emodin+enrofloxacin group from 1 h to 8 h after incubation. In addition, the K^+ concentration of *A. hydrophila* cells treated with enrofloxacin was significantly ($P < 0.05$) higher than that of *A. hydrophila* cells treated with emodin. Therefore, the membrane permeability of *A. hydrophila* cells treated was highest in the emodin+enrofloxacin treatment group; next was the enrofloxacin treatment group, followed by the emodin treated group. The lowest was the control group.

Flow cytometric (FACS) analysis. Detection of internal PI in single cells can indirectly reflect the state of the cells and this was analyzed using flow cytometry. The PI fluorescence signal of untreated *A. hydrophila* cells in the control group was 0.89 (Fig. 7a). However, when *A. hydrophila* was treated with 2MIC emodin, and 2MIC enrofloxacin, the PI fluorescence signal of treated *A. hydrophila* cells was 11.4 (Fig. 7b), and 13.98 (Fig. 7c), respectively. When treated with the combination of emodin and enrofloxacin, PI fluorescence signal of treated *A. hydrophila* cells was 18.3 (Fig. 7d). The highest PI fluorescence signal was observed in emodin+enrofloxacin group (Fig. 7A).

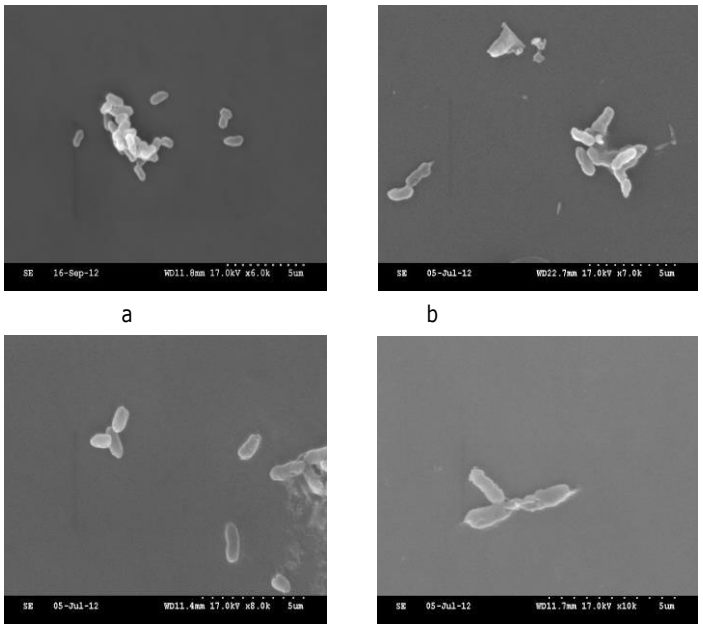


Fig. 8 Scanning electron micrographs of the effects of emodin and enrofloxacin. Cells not treated with emodin (a), cells treated with emodin (b) or enrofloxacin (c), and cells treated with emodin and enrofloxacin (d).

Scanning electron microscopy. Untreated *A. hydrophila* cells remained intact and showed a smooth surface in the control group and the structure of the untreated (control) *A. hydrophila* cells were not affected (Fig. 8a) but 4 h after treatment with emodin, or enrofloxacin, or both combined, the *A. hydrophila* cells showed important morphological changes such as breakage of cell wall and membrane (Fig. b, c, d). The destruction of *A. hydrophila* cells treated with the combination of emodin and enrofloxacin was more serious compared with other groups (Fig. 8).

Discussion

After analysis both emodin and enrofloxacin exhibited excellent antibacterial activity against *A. hydrophila* and the activity of the two drugs was positively related to their concentrations (even low concentrations, indicating that emodin and enrofloxacin were major antibacterial components against the growth of *A. hydrophila*. In addition, emodin and enrofloxacin at the concentration of 2 MIC and 4 MIC was shown to kill bacteria within 10 h after the incubation of *A. hydrophila* cells. These results were consistent with previous reports which indicated that emodin has the same antibacterial activity as enrofloxacin (Wang et al., 2010; Chen et al., 1962). This can be seen in Wuchang bream (Ming et al., 2012). It is feasable that emodin and enrofloxacin

could directly affect the growth of, and may even kill *A. hydrophila* cells. The present study indicated that the concentration of 2 MIC or 4 MIC emodin and enrofloxacin had the greatest potential to kill *A. hydrophila*.

Damage to the bacterial cell wall and cytoplasmic membrane might indicate loss of structural integrity and affect the membrane's ability as a permeable barrier. When the bacterial membrane was damaged, small ions such as potassium and phosphate could leach out, and cytoplasmic constituents from the cells could be monitored. Therefore, the effects of emodin, enrofloxacin, and combination of both, on the membrane permeability of *A. hydrophila* cells were investigated by measuring the amount of potassium ions released from drug-treated cells. In this experiment, results showed that the increase in the amount of K^+ released from *A. hydrophila* cells after treatment confirmed that emodin and enrofloxacin increased the permeability of the plasma membrane, causing potassium ion leakage from treated cells ultimately destroying the *A. hydrophila* cells (Denyer, 1990). This was confirmed by FACScan analysis. To investigate whether damage to the plasma membrane improved the antibacterial effect of emodin, enrofloxacin, and the combination of both, PI was added to cells which were incubated with the drugs individually and combined. PI is a fluorochrome that intercalates into nucleic acid as a viability marker, by penetrating cells and staining them only when membrane integrity is lost (Ananta et al., 2004). Morphological changes and leakage of cytoplasmic contents were also observed in electron micrographs of *A. hydrophila* cells treated with emodin, enrofloxacin and combination of both. Reports indicated that emodin (Alves et al., 2004; Shan et al., 2008) and enrofloxacin (Efthimiadou et al., 2008) could bind and enter the cell membrane, causing damage to the cytoplasmic membrane. The present study confirmed these results.

All results showed that bacterial membrane permeability of *A. hydrophila* cells treated with the combination of emodin + enrofloxacin was highest. This indicated that emodin could partially replace enrofloxacin as a bactericidal drug. A synergistic effect was observed between emodin and enrofloxacin. Further research is needed to understand the mechanism involved. Results from the present investigation conclusively indicate that emodin and enrofloxacin increase membrane permeability of *A. hydrophila* and cause leakage of bacterial intracellular contents. The death of *A. hydrophila* cells might be the result of cell content leakage or the initiation of autolytic processes. The combination of emodin + enrofloxacin significantly increased membrane permeability of *A. hydrophila* cells compared with other treatments.

In conclusion, results indicate that 2 MIC concentration emodin, 2 MIC concentration enroflxacin, or a combination of 1 MIC concentration emodin + 1 MIC concentration enrofloxacin, inhibit the growth of *A. hydrophila*, increase bacterial membrane permeability, and damage bacterial cell membrane integrity. Our results indicate that the combination of 1 MIC concentration emodin + 1 MIC concentration enrofloxacin are optimal (concentration ratio=1MIC:1MIC), have the best antibacterial result, and can enhance resistance against *A. hydrophila*. Emodin may to some extent replace enrofloxacin as a bactericidal drug. The underlying mechanisms of emodin and enrofloxacin against *A. hydrophila* is not yet understood and control of *A. hydrophila* requires further study in aquaculture.

Acknowledgments

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