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ORIGINAL ARTICLE

Aquaculture

Antibacterial properties of anthraquinones extracted from rhubarb against *Aeromonas hydrophila*

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Abstract Antibacterial properties of crude extract from rhubarb and its major bioactive compounds against *Aeromonas hydrophila* were assayed. Major bioactive compounds (anthraquinone derivatives) in rhubarb collected from different cultivation areas were determined by ultra-performance liquid chromatography (UPLC); the antibacterial activity [minimum inhibitory concentration (MIC)] of rhubarb was positively related to the anthraquinone content ($r = 0.9306$, $P < 0.01$). The MIC values of five anthraquinones against *A. hydrophila* were found to be in the range 50–200 μg/ml. Action-mode studies showed that anthraquinones (emodin) inhibits cellular functions by binding to cell DNA after penetrating the cell membrane, resulting in cell death. The present study suggests that anthraquinones extracted from rhubarb have potential use as antimicrobials for control of *A. hydrophila*.

Keywords Rhubarb · *Aeromonas hydrophila* · UPLC · Antimicrobial property

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Introduction

Aeromonas hydrophila is a Gram-negative rod-shaped bacterium belonging to the Aeromonidae, a family that is widely distributed in fresh water, sewage-contaminated water, sludge, soil, and foods. *A. hydrophila* is an important bacterial fish pathogen and is associated with several diseases of fish, such as hemorrhagic septicemia, fin and tail rot, and epizootic ulcerative syndrome [1, 2]. These diseases have caused high mortality in freshwater fish, resulting in extensive losses around the world. Antibiotics and chemotherapeutics used to control these diseases can result in the development of drug-resistant bacteria, environmental pollution, and residues in fish. With the increasing demand for organic aquaculture, there has been growing interest in using natural products in aquaculture to prevent diseases for their lesser side-effects than antibiotics [3–6].

Rhubarb is an important Chinese herbal medicine (called *Dahuang*) and has been widely used as a plant medicine for treatment of blood stagnation, constipation, and mental and renal disorders, as well as a purgative agent, in China for a long time. In rhubarb, anthraquinone derivatives [emodin, chrysophanol, rhein, aloe-emodin, and physcion (Fig. 1) and their glucosides] are thought to be the major active components, having many different biological and pharmacological properties such as antioxidant [7], antibacterial [8], antiviral [9], antifungal [10], anti-atherosclerotic [11], and anticancer activities [12]. Due to their biological effects, increasing attention is being paid to these compounds. Recent literature has shown that rhubarb can promote nonspecific immune system functions in fish and prawns to prevent pathogenic infection, mitigate the negative effects of crowding stress, and promote growth [6, 13]. None of these previous studies, however, screened

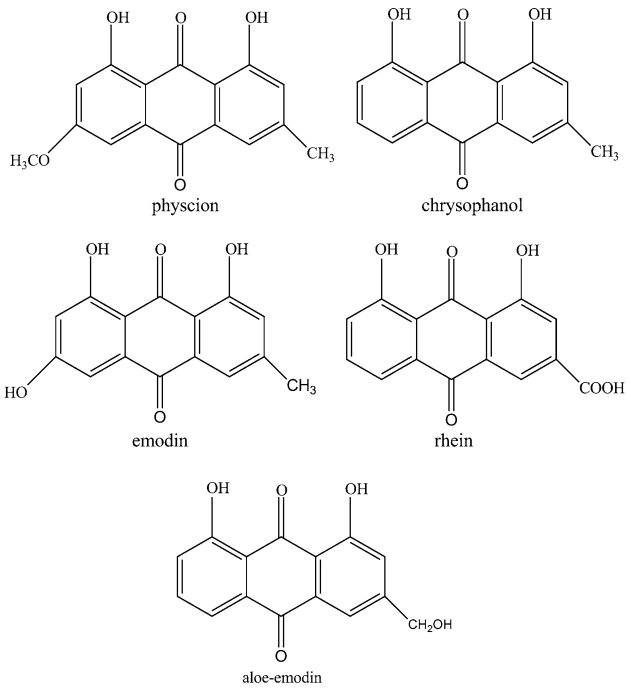


Fig. 1 Structures of five anthraquinones

antibacterial activity of rhubarb and its major components against *A. hydrophila* in vitro. Meanwhile, there have been few reports and discussion on the mechanisms of action of antimicrobial components.

Therefore, the aims of the present work are: (1) to investigate the antibacterial activities of crude extract from rhubarb and its major components against *A. hydrophila*, (2) to detect the contents of five anthraquinones in rhubarb collected from different cultivation areas, and (3) to investigate the mechanism of action of anthraquinones against *A. hydrophila*.

Materials and methods

Microorganisms and chemicals/reagents

Aeromonas hydrophila TPS-30, BSK-10 were obtained from Zhejiang Institute of Freshwater Fisheries, and *A. hydrophila* IB101, JG101, 4LNS301, CCH201, LNB101, CG101 were obtained from the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences. Eight rhubarb samples were purchased in Chinese markets near production areas of *Rheum* species. Emodin, chrysophanol, rhein, aloe-emodin, and physcion (with purity >99%) were obtained from Kemiou Chemical Reagent Company (Shanghai, China). A Spin Column Genomic DNA isolation kit was purchased from Bio Basic Inc., Canada. A Cell Apoptosis PI detection kit was purchased from Nanjing KeyGen Biotech. Co. Ltd., China. Ethidium bromide (EB) was obtained from Amersco Inc. (Solon, OH, USA). UPLC-grade methanol was purchased from Sigma–Aldrich (St. Louis, MO, USA). All other reagents (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) were of analytical grade.

Extraction of anthraquinones

Dried rhubarb was ground into fine powder and passed through a sieve (60 mesh). Rhubarb powder (10.0 g) was mixed with 100 ml 80% ethanol, and extraction was carried out at 80°C for 2 h and repeated three times. The extracts were combined, filtered, and then concentrated using a rotary evaporator at 40°C under vacuum and lyophilized using a freeze-dryer (LGJ-10D; Four-Ring Science Instrument Beijing Co., Ltd., China). The freeze-dried sample of crude extract was stored at 4°C until use.

UPLC analysis

Ultra-performance liquid chromatography (UPLC) analyses were carried out using an UPLC apparatus equipped with a Waters Acquity PDA detector (Waters, USA) and an Acquity UPLCTM BEH C18 column (100 mm × 2.1 mm, particle size 1.7 μm; Waters, USA). The column oven temperature was fixed at 45°C. The eluents were: A, water 0.1% formic acid; B, acetonitrile/methanol (20:80, v/v). The gradient program was as follows: 10–30% B (15 min), 30–100% B (18 min) at constant flow of 0.3 ml/min. The peaks of the anthraquinone compounds were monitored at 280 nm. UV–Vis absorption spectra were recorded online from 200 to 600 nm during UPLC analysis.

Extraction of *A. hydrophila* genomic DNA

Genomic DNA from *A. hydrophila* TPS-30 was extracted using the Spin Column Genomic DNA isolation kit. The purity of the extracted DNA was checked by the absorbance ratio A_{260}/A_{280} ($OD_{260}/OD_{280} = 1.83$). DNA concentration was determined from the absorbance at 260 nm ($A_{260} = 1.0$ OD for 50 μg/ml) using a UV-2100 spectrophotometer (UNIC) [14].

Antibacterial activity (MIC)

The antimicrobial activities of the rhubarb extracts and its major components (anthraquinone derivatives) were determined by using a twofold microdilution broth method [15]. *A. hydrophila* were grown to mid-log phase in LB broth for 16 h at 37°C. Twofold serial dilutions of 80 μl of test samples were transferred to test-tubes to final concentrations of 6400, 3200, 1600, 800, 400, 200, 100, 50, 25,

12.5, 6.25, and 0 μg/ml, which were previously filled with 1900 μl LB medium. Bacterial suspension (20 μl) was then added to each test-tube to final concentration of 10^6 colony-forming units (CFU)/ml. Test-tubes were incubated at 37°C for 24 h. After incubation, microbial growth was determined by estimating the increased turbidity of each well, measured at 630 nm using a UV-2100 spectrophotometer microplate reader (UNIC). The MIC was calculated from the highest dilution showing complete inhibition of the tested strain. All analysis was carried out in triplicate, and the median value of each triplicate was used for data analysis.

Bacterial membrane permeability

Aeromonas hydrophila TPS-30 was grown to mid-log phase in LB for 16 h at 37°C, and cells were collected, washed, and resuspended in 1 ml deionized water (absorbance at 630 nm was adjusted to 0.2). The emodin sample solution (10 μl) of 2 MIC concentrations was added in test-tubes, and incubated at 37°C for various times. Then, the cell suspensions were centrifuged at 10000 rpm for 10 min, and the supernatants were diluted at 100-fold [16]. The amount of released K^+ was measured by atomic absorption spectrometer (Spectr AA 220; VARIAN, USA).

Transmission electron microscopy

Exponential-phase bacteria were treated with 2 MIC of emodin for 4 h at 37°C. 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 [17]. Penicillin was used as positive controls, and double-distilled water as negative controls. Microscopy was performed with a transmission electron microscopy (H-7000; Hitachi, Japan) under standard operating conditions.

Flow cytometric analysis

Membrane integrity after emodin treatment was determined by flow cytometric analysis using propidium iodide (PI) as a probe [18]. *A. hydrophila* TPS-30 was grown to log phase in LB and mixed with emodin at concentration of 2 MIC for 4 h at 37°C. Cells were washed three times with phosphate-buffered saline (PBS), and resuspended at concentration of 10^6 CFU/ml in the same buffer. The emodin-treated cells were incubated in PI solution (50 μg/ml final concentration) for 30 min at 37°C, followed by removal of unbound dye through excessive washing with PBS. PI was excited at 488 nm using an argon laser, and the resulting fluorescence emission was collected through a 660-nm long-pass filter. Penicillin was used as positive controls, and double-distilled water as negative controls. Flow cytometry analysis was conducted using a FACScan instrument (Calibur, BO, USA).

Fluorescence measurement

The fluorescence spectrum of emodin in the absence and presence of *A. hydrophila* genomic DNA was measured using a F-7000 fluorophotometer (Hitachi, Japan) at room temperature with excitation at 290 nm (kex = 290 nm). The change of fluorescence spectra from 360 to 560 nm was measured as increasing concentrations (0, 5.0, 10.0 and 20 μg/ml) of DNA were added to emodin with a fixed concentration (of 200 μg/ml) at room temperature [19]. Tris–HCl buffer (10 mM, pH 7.2) was used as blank solution for all samples.

Competitive binding of emodin and EB with bacterial DNA

Fluorescence measurements of competitive binding assays were carried out using a F-7000 fluorophotometer (Hitachi, Japan). DNA was dissolved in 2 ml Tris–HCl buffer (10 mM, pH 7.2) to final concentration of 10 μg/ml; 10 μl EB (2 μM) solution was added to the DNA solution, and the EB–DNA solution was placed in a thermostated water bath at 37°C for 10 min. Varying concentrations of emodin (0, 50, 100, and 200 μg/ml) were then added to the EB–DNA solution, and the fluorescence spectra were measured for each test solution after 30 min of incubation at 37°C. The solutions were excited at 535 nm, and spectra were recorded from 550 to 720 nm [20, 21]. Tests were performed in a 1-cm-path-length quartz cell.

KI fluorescence quenching

The experiment was performed according to methods described by Guo et al. [19] and Song et al. [22], with some modifications. The concentration of emodin was adjusted to 200 μg/ml; potassium iodide (KI) was dissolved in Tris–HCl buffer (10 mM, pH 7.2) to final concentrations of 0, 1, 2, 4, 6, 8, and 10 mmol. Varying concentrations of KI were then added to emodin-containing solutions in the absence or presence of DNA (10 μg/mL). Emission spectra were scanned from 360 to 560 nm with fixed excitation wavelength of 290 nm. Data were plotted according to the Stern–Volmer equation [23]

$F_0/F = 1 + K_{SV}[Q]$,

where F_0 and F are the fluorescence intensities in the absence and the presence of the DNA, respectively. K_{SV} is the Stern–Volmer quenching constant, and [Q] is the concentration of quencher.

Results

Determination of anthraquinones in rhubarb

Ultra-performance liquid chromatography analysis results of the crude extract of rhubarb are shown in Table 1 and Fig. 2. The total contents of five anthraquinones obtained from rhubarb from different cultivation areas ranged from 5.87 ± 0.30 to 24.86 ± 0.81 mg/g. The antibacterial activity (MIC) of rhubarb was positively related to the anthraquinone content ($r = 0.9306$, $P < 0.01$).

Antibacterial activity of anthraquinones

The antibacterial activities (MIC) of five anthraquinones are shown in Table 2. The MIC values of the five anthraquinones against *A. hydrophila* ranged from 50 to 200 μg/ml, the general order of their antibacterial activity being: emodin = rhein = aloe-emodin > physcion = chrysophanol. Considering the antibacterial activity and content of emodin, emodin was chosen as a candidate anthraquinone derivatives for study of the antibacterial mechanism.

Bacterial membrane permeability

The effect of emodin on the membrane permeability of *A. hydrophila* was investigated by measuring the amount of potassium ions released from emodin-treated cells. When the bacterial membrane is damaged, to a certain extent, small ions such as potassium and phosphate tend to leach out, and cytoplasmic constituents released from the cell were monitored. Figure 3 shows that a significant potassium efflux from bacteria cells was induced after incubation, and the K^+ efflux increased with increasing incubation time from 0.5 to 4 h; when time was increased further, only slight changes was observed. The increase in the amount of K^+ released from *A. hydrophila* after treatment provides evidence that emodin probably acted on the plasma membrane by increasing permeabilization, causing ion leakage from the cell.

Transmission electron microscopy

Transmission electron microscopy was used to observe the morphological changes of bacterial cells treated with emodin. The electron micrographs are displayed in Fig. 4. Control cells of nontreated bacteria remained intact and showed a smooth surface (Fig. 4a). However, after 4 h of treatment, the cells showed important morphological changes such as breakage of cell wall and membrane, and leakage of cellular cytoplasmic contents was also observed (Fig. 4b, c), which was similar to in previous studies [25].

Flow cytometric analysis

To investigate whether the antibacterial effect of emodin was induced by damage to the plasma membrane, the cells were incubated with emodin and PI. PI is a fluorochrome that intercalates into nucleic acid as a viability marker, which is supposed to penetrate cells and stain them only when membrane integrity is lost [26]. Detection of internal PI in single cells was analyzed via flow cytometry. As shown in Fig. 5, in the absence of emodin, 97.15% of untreated control cells showed no PI fluorescence signal (Fig. 5a), indicating viable cells excluding the PI dye. However, when treated with emodin and penicillin, 71.65% and 81.5% of *A. hydrophila* cells were labeled fluorescently

Table 1 The relationship between antibacterial activity and anthraquinone content (mg/g) of rhubarb from different cultivation areas

Sample no.	Cultivation areas	Physcion	Chrysophanol	Emodin	Rhein	Aloe-emodin	Total	MIC (mg/ml)
1	Gansu Prov.	1.54 ± 0.04	12.44 ± 0.42	4.18 ± 0.09	3.81 ± 0.015	2.23 ± 0.08	23.57 ± 0.79	0.78 ± 0
2	Sichuan Prov.	0.63 ± 0.01	5.63 ± 0.22	0.44 ± 0.02	0.028 ± 0.001	0.22 ± 0.01	6.95 ± 0.23	3.12 ± 0
3	Gansu Prov.	1.86 ± 0.07	13.93 ± 0.64	3.17 ± 0.09	2.99 ± 0.12	2.91 ± 0.13	24.86 ± 0.81	0.78 ± 0
4	Shanxi Prov.	1.26 ± 0.05	9.55 ± 0.56	1.38 ± 0.05	0.23 ± 0.004	1.40 ± 0.08	12.82 ± 0.64	1.56 ± 0
5	Yunnan Prov.	1.70 ± 0.06	8.52 ± 0.41	1.57 ± 0.06	0.11 ± 0.004	1.58 ± 0.07	13.48 ± 0.63	1.56 ± 0
6	Neimeng Prov.	1.33 ± 0.04	8.01 ± 0.46	1.41 ± 0.07	0.16 ± 0.003	1.44 ± 0.06	12.35 ± 0.54	1.56 ± 0
7	Gansu Prov.	1.80 ± 0.05	12.11 ± 0.49	4.12 ± 0.15	3.55 ± 0.13	2.18 ± 0.10	23.76 ± 0.96	0.78 ± 0
8	Guangxi Prov.	0.10 ± 0.002	0.78 ± 0.04	1.27 ± 0.06	2.71 ± 0.11	1.01 ± 0.04	5.87 ± 0.30	3.12 ± 0

Results are expressed as mean ± SD ($n = 3$). Strain, *A. hydrophila* TPS-30

MIC minimum inhibitory concentration

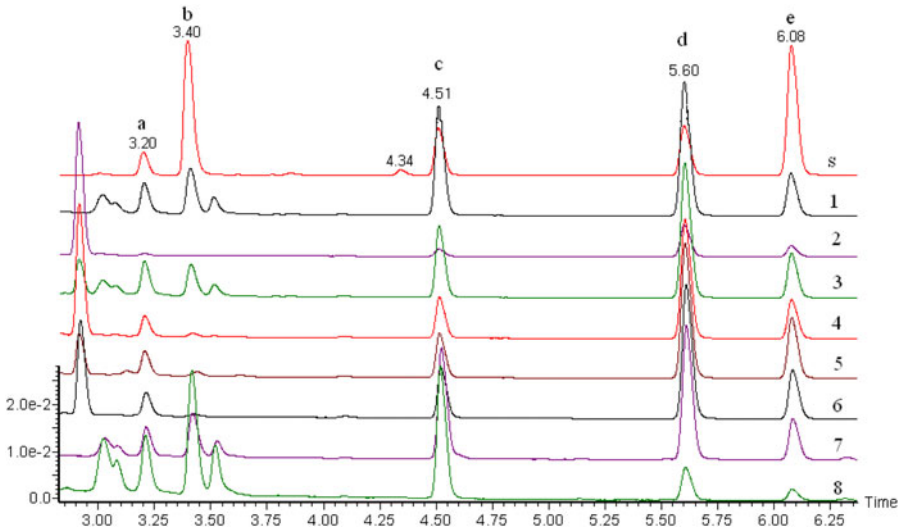


Fig. 2 Chromatograms of standard solution (S) and extract of rhubarb obtained in sample 1, 2, 3, 4, 5, 6, 7, 8: *a* aloe-emodin, *b* rhein, *c* emodin, *d* chrysophanol, and *e* physcion

Table 2 Antibacterial activities of five anthraquinones against *Aeromonas hydrophila*

Strain	MIC (μg/ml)				
	Physcion	Chrysophanol	Emodin	Rhein	Aloe-emodin
A. hydrophila IB101	200	200	50	50	50
A. hydrophila JG101	200	200	50	50	50
A. hydrophila TPS-30	200	200	50	50	50
A. hydrophila BSK-10	200	200	50	50	50
A. hydrophila 4LNS301	200	200	50	50	50
A. hydrophila CCH201	200	200	50	50	50
A. hydrophila LNB101	200	200	50	50	50
A. hydrophila CG101	200	200	50	50	50

Results are expressed as mean ± SD ($n = 3$)

MIC minimum inhibitory concentration

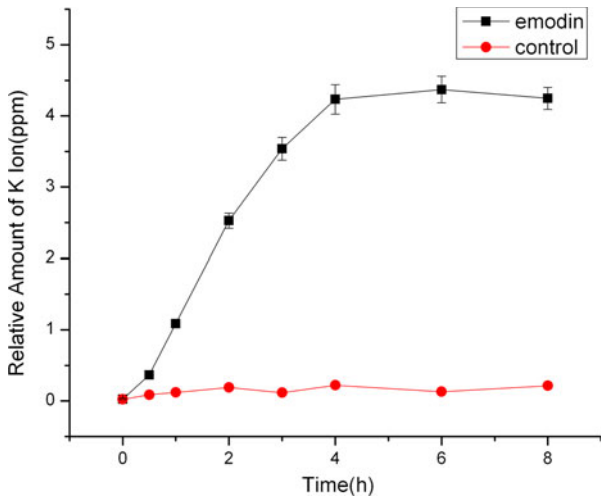


Fig. 3 Effect of emodin on the amount of K^+ released from *A. hydrophila* TPS-30. Cells were treated with emodin for predetermined times, and the relative amounts of K^+ released from the cells were measured

after 4 h of incubation, respectively (Fig. 5b, c), thereby indicating that emodin induced PI influx into the cells.

Fluorescence spectra study

The spectrophotometric titrations of emodin with *A. hydrophila* genomic DNA, in the concentration range of 0–20 μg/ml, provided information about the emodin–DNA interaction mode. As shown in Fig. 6, emodin has strong intrinsic fluorescence; in the absence of DNA, the wavelength maximum of emodin was about 425 nm when excited at 290 nm. When increasing concentrations (5.0, 10, and 20 μg/ml) of DNA were added to the emodin solution, the fluorescence intensity of emodin gradually decreased. This could be due to intercalation of emodin into the base pairs of the DNA helix, resulting in electric charge transfer and change of excited electronic states, which would lead to lower fluorescence [19]. An emission decrease is widely recognized as an indication of interactions between drugs and DNA [27].

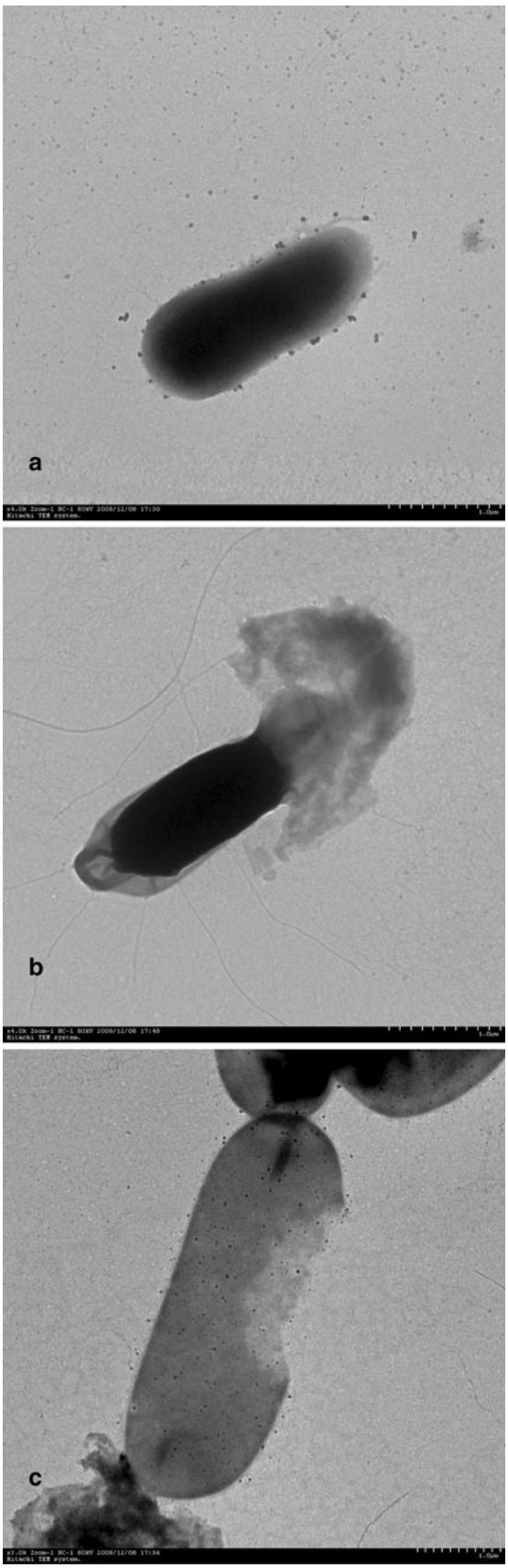


Fig. 4 Transmission electron microscopy observations of *A. hydrophila* TPS-30 treated with emodin (**b**) or penicillin (**c**, positive control), and untreated (**a**)

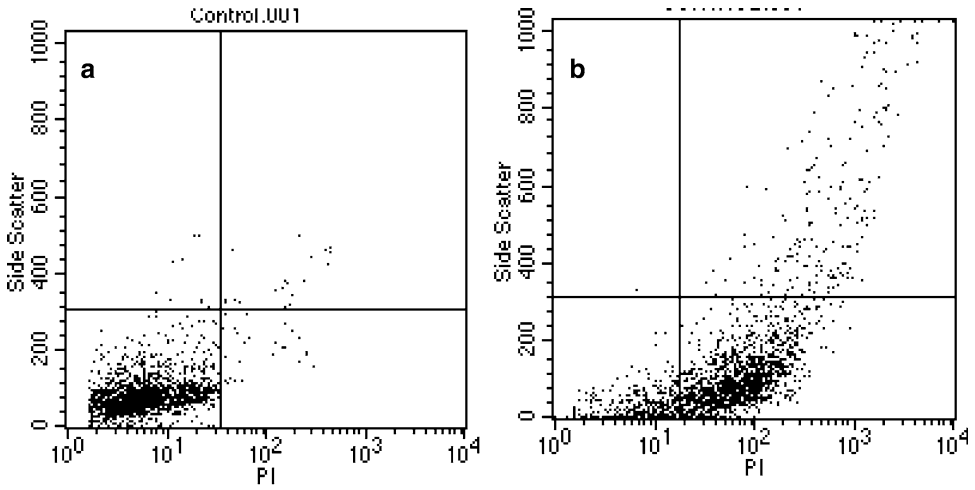
Competitive binding of emodin and EB with bacterial DNA

To confirm the mode of interaction of DNA with emodin, a competitive binding experiment was carried out using EB as a probe. EB is one of the most sensitive fluorescent probes that can bind with DNA. Fluorescence of free EB is low, but intense fluorescence is emitted after binding with DNA, due to intercalation between adjacent base pairs within the double helical structure of DNA. This enhanced fluorescence can be quenched when it coexists with a reagent molecule that undergoes a similar reaction. This can be used to monitor the binding mode, thereby indicating the ability of a compound to prevent intercalation of EB into DNA [28]. Accordingly, the experiment was carried out by titrating the EB–DNA system with emodin. When the concentration of emodin was increased, a remarkable fluorescence decrease of the EB–DNA system was observed at the maximum of 590 nm (Fig. 7). This phenomenon indicated that EB was partially replaced by emodin in the EB–DNA system, and EB was released from a hydrophobic environment into the water solution. The result suggested that emodin binds to DNA in the intercalating mode [21, 29].

KI fluorescence quenching

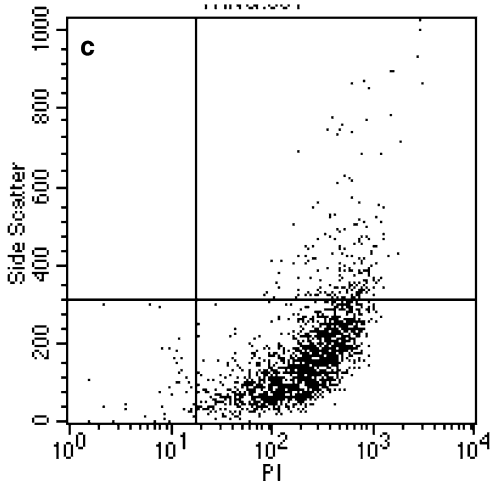
The I^- ion is a dynamic quenching agent, and the mode of action between small molecules and DNA can be determined by evaluating the effect of the I^- ion on the quenching of fluorescence of small molecules. When a small molecule inserts into the bases of DNA, these bases (in the DNA double helix structure) together with the negatively charged phosphor-diester skeleton, inhibit the action of the anion quencher located close to small molecules, resulting in a weakening of the quenching effect of the I^- ion [30].

Figure 8 shows Stern–Volmer plots of the KI quenching effect in the absence and presence of DNA. Quenched fluorescence yielded $Y = 1.0151 + 0.069X$, $r = 0.998$ and $Y = 1.0015 + 0.045X$, $r = 0.998$, respectively. The quenching constant of I^- to emodin was 0.069 L/mmol, but the quenching constant of the KI–emodin system in the presence of DNA was 0.045 L/mmol. From Fig. 8, it can be seen that, in the absence DNA, increasing the KI concentration caused efficient quenching of the fluorescence of emodin in a concentration-dependent manner. In the presence of DNA, however, KI showed less effective



Quad	Events	% Gated	% Total
UL	10	0.50	0.50
UR	19	0.95	0.95
LL	1943	97.15	97.15
LR	28	1.40	1.40

Quad	Events	% Gated	% Total
UL	2	0.10	0.10
UR	198	9.90	9.90
LL	367	18.35	18.35
LR	1433	71.65	71.65



Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	207	10.35	10.35
LL	43	2.15	2.15
LR	1750	87.50	87.50

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

quenching of emodin fluorescence than that observed in the absence of DNA. This phenomenon suggests that emodin binds to DNA, possibly in the intercalating mode. The intercalation leads to a decrease in the collision frequency of quenching molecules, so DNA plays a protective role [22].

Discussion

Analysis of the antibacterial activity of rhubarb showed that the crude extract exhibited excellent antibacterial activity against *A. hydrophila* and the antibacterial activity (MIC) of rhubarb was positively related to the anthraquinone content ($r = 0.9306$, $P < 0.01$), which indicated that anthraquinones was a major antibacterial component in rhubarb against the growth of *A. hydrophila*. However, based on their average MICs against *A. hydrophila* (50–200 μg/ml) (Table 2), five anthraquinones showed different antibacterial activities. Comparisons of the activities of the five anthraquinones revealed that the effects of emodin, rhein, and aloe-emodin against all bacterial strains were higher than those of physcion and chrysophanol. This was consistent with the results of

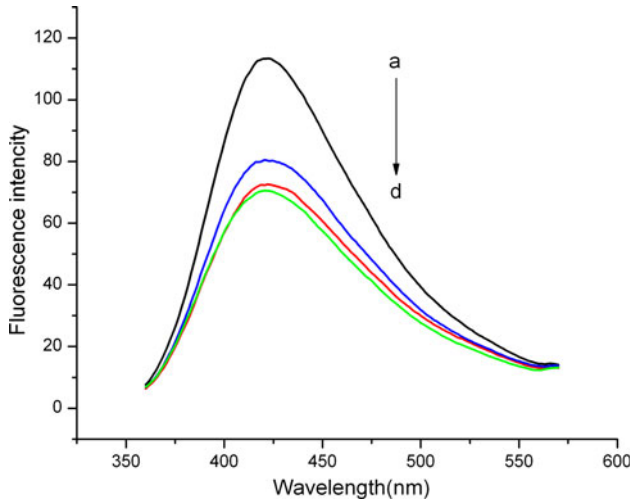


Fig. 6 Fluorescence spectra of emodin in the absence (**a**) and presence of *A. hydrophila* genomic DNA (**b–d**) in Tris buffer (pH 7.2). Total concentration of emodin: 200 μg/ml. Cell path length: 1 cm. **a** 0, **b** 5.0, **c** 10, **d** 20 μg/ml DNA

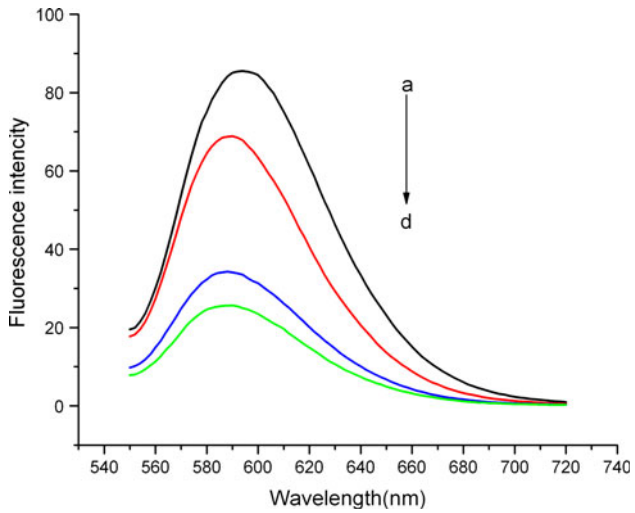


Fig. 7 Competitive binding of emodin and EB with *A. hydrophila* genomic DNA: **a** control (2 μM EB + 10 μg/ml DNA), **b** 2 μM EB + 10 μg/ml DNA + 50 μg/ml emodin, **c** 2 μM EB + 10 μg/ml DNA + 100 μg/ml emodin, and **d** 2 μM EB + 10 μg/ml DNA + 200 μg/ml emodin

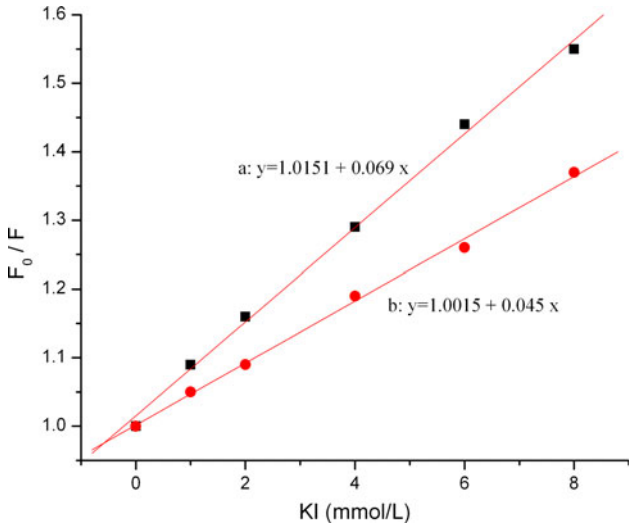


Fig. 8 Stern–Volmer plots for quenching of emodin fluorescence on sequential addition of KI in the absence (**a**) or presence (**b**), of DNA; DNA = 10.0 μg/ml; EGCG = 200 μg/ml

previous reports [8, 24], which suggested that the antibacterial activity of these anthraquinone derivatives might be related to the type of substituent groups on the molecular structure. All of these anthraquinone derivatives have the same hydroxyanthraquinone nucleus composed of two ketone groups at C9 and C10 and two hydroxyl groups at C1 and C8, while different groups are substituted at C3 and C6 of the phenyl ring (Fig. 1). Three anthraquinones (rhein, emodin, and aloe-emodin) have polar substituent carboxyl, hydroxyl, and hydroxymethyl groups at C3, C6, and C3, respectively. It was reported that the presence of polar functional group (carboxyl, hydroxyl, and hydroxymethyl) can increase antibacterial activity [8, 24]. Although physcion and chrysophanol also have hydroxyl groups at C1 and C8 (Fig. 1), the apolar methyl and weakly polar methoxyl in chrysophanol and physcion might weaken their antibacterial activity.

Emodin, one of the important bioactive compounds in rhubarb, has shown a wide variety of pharmacological activities, such as anti-inflammatory [31], antioxidant [7], antimicrobial [8], and antitumor activities [32]. Among their wide biological activity, only in a few cases has their molecular mechanism been elucidated. In particular, the antibacterial activity and mechanisms of action of emodin against *A. hydrophila* have been little reported. To learn about the possible mechanism of antibacterial activity against *A. hydrophila*, here we investigated the morphology of treated cells and the molecular mechanism of emodin–DNA interactions. Several possible mechanisms of action were proposed.

Damage to the bacterial cell wall and cytoplasmic membrane might indicate loss of structural integrity and of the membrane's ability to act as a permeable barrier. In our experiments, FACScan analysis showed that emodin increased the plasma membrane permeability for influx of ONPG into cells (Fig. 5), and caused large leakage of potassium ions from treated cells (Fig. 3). Moreover, morphological changes and leakage of cytoplasmic contents were also demonstrated by electron micrographs of *A. hydrophila* cells treated with emodin (Fig. 4). All results elucidated that emodin increased membrane permeabilization and caused leakage of intracellular contents. Cell death might be the result of cell contents leakage or the initiation of autolytic processes [33].

The assays reported herein and previous reports [25, 34] indicated that emodin can bind and insert into the cell membrane, leading to loss of cytoplasmic membrane integrity. What then does emodin do inside the cell? Can it act on intracellular targets in bacteria? Previous studies have demonstrated that anthraquinone derivatives of Chinese rhubarb could inhibit macromolecular synthesis in cells [35, 36], so it was also hypothesized to target intracellular processes in bacteria beyond membrane permeabilization. Therefore, we investigated the molecular mechanism of the bactericidal activity of emodin on *A. hydrophila* genomic DNA. Interestingly, fluorescence spectroscopic studies showed that emodin could bind with the phosphate group of DNA and intercalate into the base pairs of the DNA helix, suggesting that DNA may be a target for the antibacterial activity of this anthraquinone, which might affect replication and transcription, repress expression, and even lead to cell death [36].

In addition, the antimicrobial activity of emodin might involve other modes of action. Previous studies have demonstrated that anthraquinone derivatives could inhibit the activities of nicotinamide adenine dinucleotide (NADH) oxidase and succinate oxidase of mitochondria [37]. Therefore, we propose that emodin might inhibit electron transfer of the respiratory chain, substrate oxidation, and dehydrogenation processes in the bacteria. As a result, such inhibition could lead to uncoupling of oxidative phosphorylation, restraining of active transport, and loss of pool metabolites [33, 36].

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