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Protective Effect of Polygoni Cuspidati Radix and Emodin on *Vibrio vulnificus* Cytotoxicity and Infection

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***Vibrio vulnificus*, a good model organism of bacterial septicemia, causes fatal septicemia manifesting a fulminating course and a high mortality rate within days. In order to identify new natural substances preventing *V. vulnificus* infection, a plant library was screened for inhibiting cytotoxicity to host cells by using Trypan blue staining and LDH assay. We found that Polygoni Cuspidati Radix potently suppressed the acute death of HeLa and RAW264.7 cells in a dose dependent manner. Further studies revealed that Polygoni Cuspidati Radix inhibited *V. vulnificus* growth and survival in HI broth and seawater, respectively. We confirmed that Polygoni Cuspidati Radix contained high level of emodin by thin layer chromatography (TLC). Emodin showed direct antibacterial activity against *V. vulnificus*. In addition, emodin prevented the morphologic damages and acute death of HeLa cells caused from *V. vulnificus*. The safety of Polygoni Cuspidati Radix and emodin to host cells was confirmed by MTT assay. Polygoni Cuspidati Radix and emodin protected mice from *V. vulnificus* infection.**

***Keywords*:** *V. vulnificus* infection, emodin, Polygoni Cuspidati Radix, cytotoxicity, antibacterial activity

Vibrio vulnificus (*V. vulnificus*), a halophilic estuarine bacterium causes fatal septicemia and necrotic wound infection (Tacket *et al.*, 1984; Oliver, 2005). This pathogen is a good model organism of bacterial septicemia, because the bacterial infection shows wide pathogenic spectrum, which resulted in a fulminating course and a high mortality rate (over 50%) within days (Tacket *et al*., 1984). Suggested virulence factors of *V. vulnificus* are as follow; an RtxA1 toxin (Lee *et al.*, 2007; Kim *et al.*, 2008), an extracellular haemolysin/cytolysin (Kreger and Lockwood, 1981; Gray and Kreger, 1986), an elastolytic protease (Kothary and Kreger, 1987; Miyoshi *et al.*, 1987), a phospholipase A2 (Testa *et al.*, 1984), a polysaccharide capsule (Wright *et al.*, 1990), and siderophores (Simpson and Oliver, 1983). We have recently reported that *V. vulnificus* due primarily to RtxA1 toxin caused cytoskeletal rearrangement, acute cytotoxicity, haemolysis, and lethality to mice (Kim *et al*., 2008).

From the high throughput screening over a plant extract library, we found that the ethanol extract of Polygoni Cuspidati Radix inhibited significantly *V. vulnificus* cytotoxicity. Polygoni Cuspidati Radix was reported to show antiallergic effect (Lim *et al.*, 2007), and antiviral activity (Wang *et al.*, 1999). Piceid, resveratrol, emodin-8-O-beta-D-glucoside, and emodin were analyzed as the major constituents of the herb medicine (Yi *et al.*, 2007). We confirmed that emodin, 6-methyl-1,3,8-trihydroxyanthraquinone, was a major component of Polygoni Cuspidati Radix used in this study by thin layer chromatography (TLC). The therapeutic properties of emodin have been reported on cancer (Huang *et al.*, 2008), hepatitis (Ding *et al.*, 2008), and inflammation (Kumar *et al.*, 1998). Emodin has also been reported to show antibacterial activities against MRSA and *Helicobacter pylori* (Wang and Chung, 1997; Hatano *et al.*, 2005) and antiviral activities (Shuangsuo *et al.*, 2006; Hsiang and Ho, 2008; Lin *et al.*, 2008). In the present study, we investigated the effects of Polygoni Cuspidati Radix and emodin on *V. vulnificus*-induced cytotoxicity to host cells and infection to mice.

Materials and Methods

Bacterial strains and reagents

MO6-24/O is a clinical isolate of *V. vulnificus* (Reddy *et al.*, 1992), and CMM1368 is MO6-24/O with pBAD24::GFP (Dhakal *et al.*, 2006). *V. vulnificus* strains inoculated in 2.5% NaCl Heart Infusion (HI) broth were grown in a 37°C shaking incubator at 200 rpm. To produce a GFP-expressing *V. vulnificus* strain, CMM1368 was cultured overnight in HI broth with 0.2% arabinose at a 37°C shaking incubator.

Ethanol extracts from herb medicines were purchased from Plant Diversity Research Center, Daejeon, Korea. The extracts were dissolved in a stock concentration of 100 mg/ml using 50% DMSO and 50% ethanol solution. Emodin was purchased from Sigma (USA). All other reagents were purchased from commercial sources.

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Effects on *V. vulnificus*-induced cytotoxicity

HeLa cells in 96 well microplates were preincubated in serum-free DMEM culture media (GIBCO Invitrogen, USA) with each plant ethanol extracts for 2 h, and then *V. vulnificus* was inoculated into the cells at a multiplicity of infection (MOI) of 100. After 90 min of incubation at 37°C, the supernatants were removed, and then the remaining HeLa cells were stained with Trypan blue solution (Sigma). The plant extracts showing suppressive effects on *V. vulnificus* cytotoxicity were selected, and the quantitative effects were further assessed by lactate dehydrogenase (LDH) assay using a CytoTox96^{TM} Non-Radioactive cytotoxic assay kit (Promega, USA) as described elsewhere (Kim *et al.*, 2003). Briefly, *V. vulnificus* was inoculated to HeLa cells at an MOI 100 for 90 min, 2 h after the preincubation of Polygoni Cuspidati Radix or emodin with the host cells. LDH released in supernatant was assayed as a marker of the cytotoxicity. To test whether Polygoni Cuspidati Radix and emodin may affect directly on host cells or not, HeLa cells preincubated with them were washed to remove the drugs before *V. vulnificus* treatment. Effects of Polygoni Cuspidati Radix and emodin were also tested on *V. vulnificus*-induced cytotoxicity to RAW264.7 cell by LDH assay.

Thin Layer Chromatography (TLC)

To address that Polygoni Cuspidati Radix used in this experiment contains emodin in high level, TLC was conducted using a standard emodin (Sigma). Polygoni Cuspidati Radix and emodin dissolved in ethanol were applied to silica gel (60 F254 TLC plates with 2.0 mm thickness, Merck, Germany). The silica gel plates were developed with a buthanol/water/ethanol mixture (3:1:1) to a distance of about 7 cm. After air-drying, the silca gel plates were sprayed with 10% sulfuric acid, and then heated on hot plate.

Confocal microscopy

Live cell images were obtained from HeLa cells in 8-well Chambered Coverglass w/cvr #1 German borosilicate (Nalge Nunc International, USA) in accordance with the method described previously (Dhakal *et al*., 2006). HeLa cells were preincubated in serum-free DMEM medium with or without emodin for 2 h, and then *V. vulnificus* expressing GFP (CMM1368) was inoculated into the HeLa cells at an MOI 100 for 50 min. After washing, the HeLa cells were stained with wheat germ agglutinin (WGA)-Alexa fluor 594 conjugate (Molecular Probes Invitrogen, USA) to visualize the plasma membrane. Confocal images were acquired using a laser scanning confocal microscope (Radiance 2100, BIO-RAD, USA).

Effects on *V. vulnificus* growth and survival in HI broth and seawater

V. vulnificus was grown overnight in a 37°C shaking incubator at 200 rpm. The diluted bacterial suspension was inoculated into 96-well microplates with different concentrations of the tested agents, and then was incubated at a 37°C incubator overnight. The bacterial growth was determined by measuring absorbance at 600 nm using an ELISA leader (Power Wave ×340, BIO-TEK INSRTUMENTS, INC, USA).

In order to test the effect on *V. vulnificus* survival in seawater, the bacterial overnight culture in HI broth was washed with PBS, diluted in filter-steriled seawater (10^8 CFU/ml) with or without Polygoni Cuspidati Radix, and then cultured in a 37°C incubator for 3 days. The bacterial numbers in the seawater were counted after diluted culture media (10 μl) was dropped on HI agar plate and cultured overnight.

MTT assays of cellular viability

Cell viability was measured by 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) assay. HeLa cells were cultured in 96 well microplates overnight and washed with serum-free culture medium. The cells were treated with Polygoni Cuspidati Radix or emodin for 4 h and further incubated with MTT (0.5 mg/ml) for 3 h at a 37°C, 5% CO_2 incubator. The insoluble formazan product was solubilized with DMSO, and then was determined by colorimetric analysis at 540 nm using an ELISA reader. Cell viability was expressed as a percentage of those cells treated with PBS alone.

Effect on mice lethality caused by *V. vulnificus*

V. vulnificus log phase cells (2×10^6 CFU) were administered to SPF 8-week-old CD-1 mice via intraperitoneal route. The mice were pretreated with Polygoni Cuspidati Radix (200 mg/kg) or emodin (20 mg/kg) twice, 1 day and 2 h before *V. vulnificus* treatment. Five mice were tested at each group, and the infected mice were observed for 48 h. All animal procedures were conducted in accordance with the guidelines of the Animal Care and Use Committee of Chonnam National University.

Statistical analysis

All values are given Mean±SEM. Statistical comparisons were made using the Student's t-test. All experiments were repeated three times, and the results from a representative experiment are shown.

Results

Polygoni Cuspidati Radix and emodin protect significantly host cells from *V. vulnificus*-induced cytotoxicity and morphologic damages.

Live *V. vulnificus* is highly cytotoxic to host cells (Kim and Rhee, 2003; Kim *et al*., 2008). In order to identify new substances suppressing *V. vulnificus* cytotoxicity, we screened over 300 herb medicines for protecting HeLa cells from *V. vulnificus* in 96 well microplates by using Trypan blue staining. It was identified that Polygoni Cuspidati Radix prevented HeLa cells from *V. vulnificus*-induced cytotoxicity (data not shown). The quantitative inhibitory effect of Polygoni Cuspidati Radix was tested on *V. vulnificus*-induced cytotoxicity by LDH assay, and the herb medicine inhibited significantly the cytotoxicity in a dose dependent manner (Fig. 1A). In addition, Polygoni Cuspidati Radix protected RAW264.7 cells from *V. vulnificus*-induced cytotoxicity (Fig. 1B).

Emodin, 6-methyl-1,3,8-trihydroxyanthraquinone, (Fig. 1C) was reported as a major constituent of the herb medicine (Yi *et al*., 2007). We confirmed that Polygoni Cuspidati Radix used in this study contained high level of emodin by using

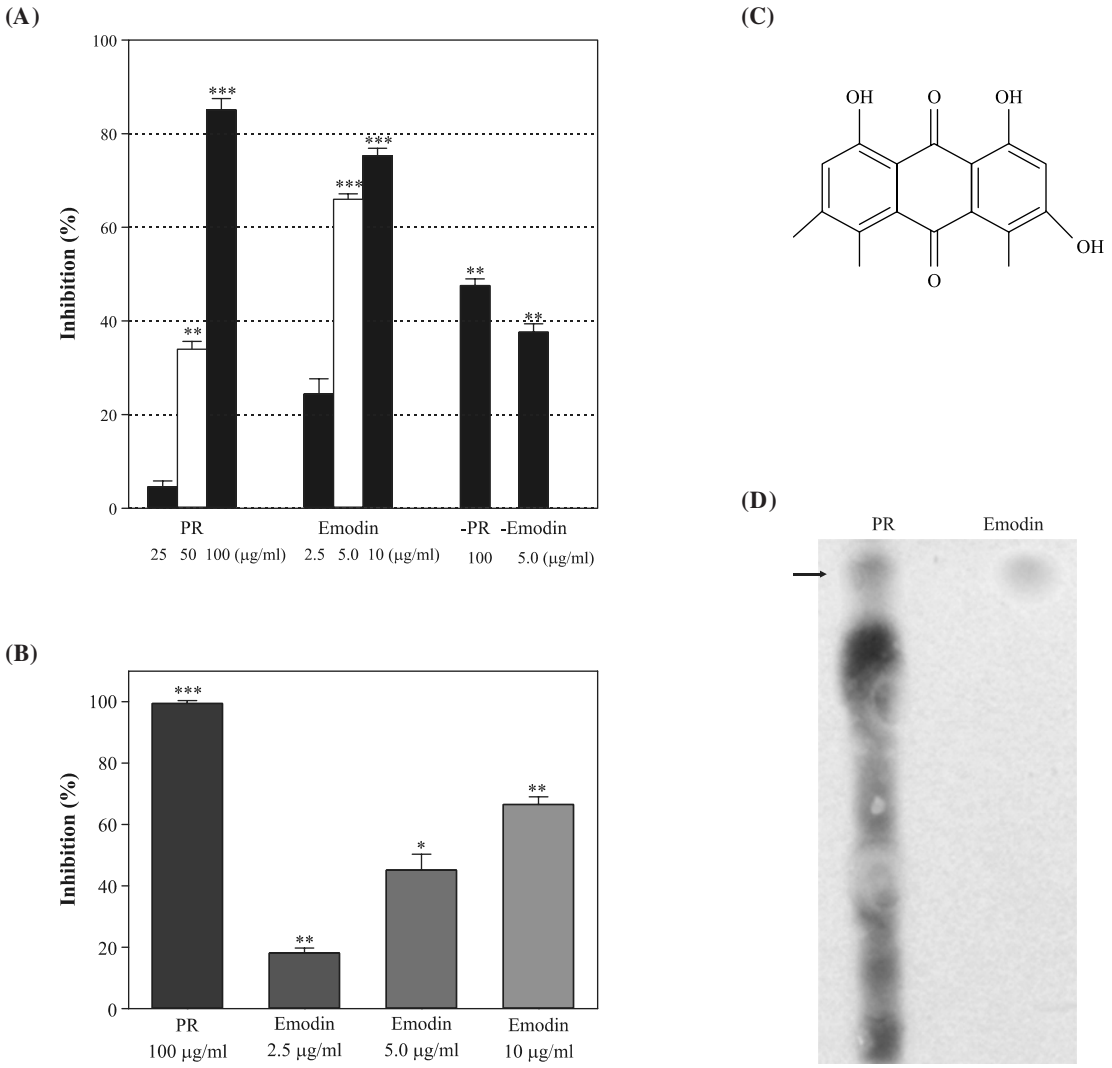

Fig. 1. Effects on *V. vulnificus*-induced cytotoxicity to host cells. (A) *V. vulnificus* was treated to HeLa cells at an MOI 100 for 90 min, 2 h after the preincubation of Polygoni Cuspidati Radix (PR) or emodin with the host cells. LDH released in supernatant was assayed as a marker of the cytotoxicity. To test whether Polygoni Cuspidati Radix and emodin may directly affect host cells or not, HeLa cells preincubated with the drugs were washed to remove the drugs before *V. vulnificus* inoculation (designated as -PR, -Emodin). The data indicate the mean and SEM from four experiments (**$P<0.01$, ***$P<0.001$). Polygoni Cuspidati Radix and emodin inhibited *V. vulnificus*-induced cytotoxicity to HeLa cells in a dose dependent manner. (B) Polygoni Cuspidati Radix and emodin were tested on *V. vulnificus*-induced cytotoxicity to RAW264.7 cell by LDH assay. (C) Emodin, a component of Polygoni Cuspidati Radix has a structure of 6-methyl-1,3,8-trihydroxyanthraquinone. (D) Thin layer chromatography (TLC) was conducted to detect emodin in Polygoni Cuspidati Radix. Polygoni Cuspidati Radix and emodin dissolved in ethanol were applied to silica gel, and developed with a buthanol/water/ethanol mixture (3:1:1). After air-drying, the plates were sprayed with 10% sulfuric acid, and then heated on hot plate. Arrow indicates yellow colored emodin showing Rf of 0.95.

TLC (Fig. 1D). Retardation factor (Rf) value of emodin was 0.95 in a developing solution of buthanol/water/ethanol mixture (3:1:1). We also found that emodin suppressed significantly the *V. vulnificus*-induced cytotoxicity to HeLa and RAW264.7 cells in a dose dependent manner (Fig. 1A and B). In addition, Polygoni Cuspidati Radix and emodin inhibited the cytotoxicity on the HeLa cells even after the removal of the agents by washout after the incubation with the host cells for 2 h (Fig. 1A, designated as -PR and -Emodin).

We investigated the effect of emodin on the morphologic damage of host cells by *V. vulnificus*. In order to produce a GFP-expressing *V. vulnificus* strain, CMM1368 was cultured overnight in HI broth with 0.2% arabinose at 37°C. The bacterial green fluorescence decreased by the incubation with Polygoni Cuspidati Radix or emodin, but the bacterial morphology was not changed after incubation with them (Fig. 2A). HeLa cells treated with GFP-expressing *V. vulnificus* were stained with WGA conjugated with Alexa fluor 594 to monitor the morphologic changes of host cells. *V. vulnificus* caused the rounding and shrinkage of HeLa cells at a time window of 50 min, which were prevented by emodin (Fig. 2B).

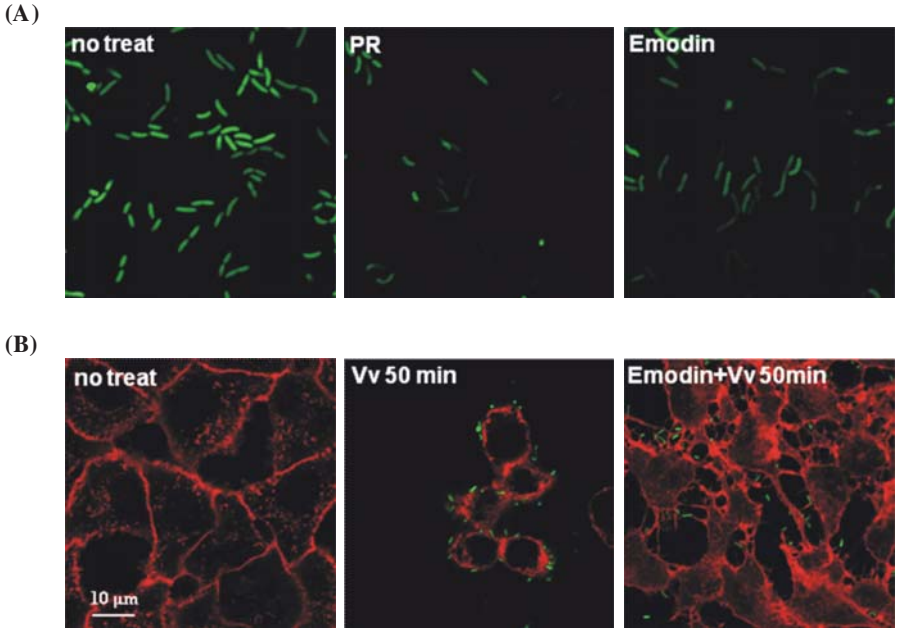

Fig. 2. Effects on the morphologic changes of HeLa cells caused by *V. vulnificus.* (A) MO6-24/O with pBAD24::GFP (CMM1368) was cultured in HI broth with 0.2% arabinose at a 37°C shaking incubator to produce GFP-expressing *V. vulnificus* strain. Polygoni Cuspidati Radix (PR) and emodin did not affect the bacterial morphology. (B) HeLa cells inoculated with *V. vulnificus* expressing GFP (designated as Vv) were stained with Alexa fluor 594 conjugated WGA (red). The fluorescence images were acquired by using a laser scanning confocal microscope. Emodin prevented the HeLa cells from being rounded and shrinkaged by *V. vulnificus* at a time window of 50 min.

Polygoni Cuspidati Radix and emodin inhibit significantly *V. vulnificus* growth and survival in HI broth and seawater, respectively

The effects of Polygoni Cuspidati Radix and emodin were studied on *V. vulnificus* growth and survival in HI broth and seawater. Polygoni Cuspidati Radix and emodin inhibited significantly *V. vulnificus* growth in HI broth in a dose dependent manner (Fig. 3A). To test whether Polygoni Cuspidati Radix inhibits the survival of *V. vulnificus* in seawater, the microbe was incubated in filter-steriled seawater with or without Polygoni Cuspidati Radix. Polygoni Cuspidati Radix at concentrations of 50 and 100 μg/ml inhibited sig-

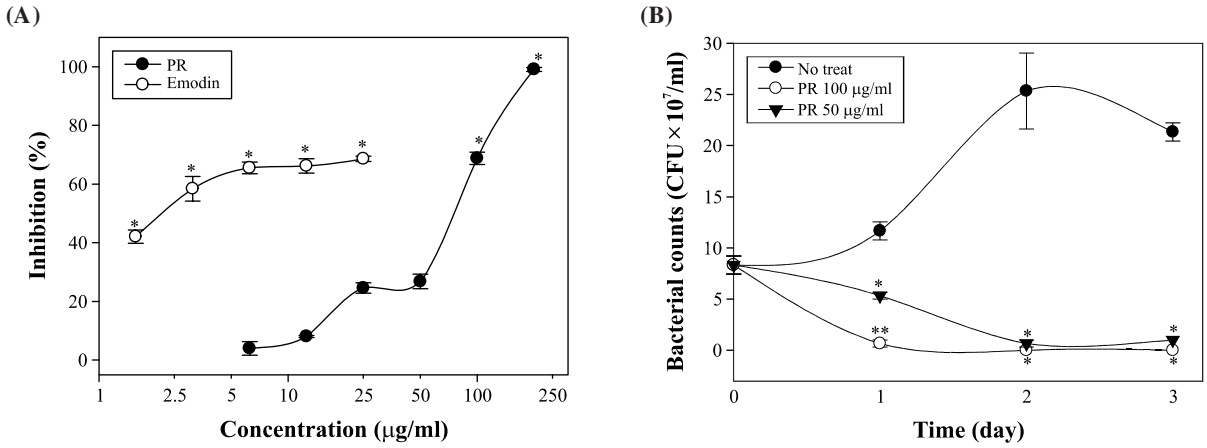

Fig. 3. Effects on *V. vulnificus* growth and survival in HI broth and seawater. (A) Effect of Polygoni Cuspidati Radix (PR) and emodin was tested on *V. vulnificus* growth in HI broth. *V. vulnificus* grown overnight was diluted in HI broth with the tested agents, and cultured in 37°C incubator overnight. The bacterial growth was determined by measuring absorbance at 600 nm using ELISA leader. Polygoni Cuspidati Radix and emodin inhibited *V. vulnificus* growth in HI media in a dose dependent manner. (B) The bacterial overnight culture was diluted in filter-steriled seawater (10^8 CFU/ml) with or without PR, and cultured in a 37°C incubator for 3 days. Bacterial numbers in the seawater were counted after diluted culture media (10 μl) was dropped on HI agar plate and cultured overnight. Polygoni Cuspidati Radix inhibited significantly *V. vulnificus* survival in seawater. The data indicate the mean and SEM from three experiments (*P<0.01, **P<0.001).

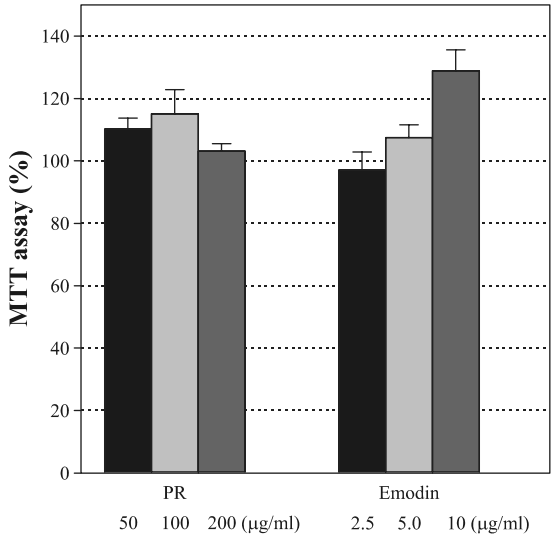

Fig. 4. MTT assays of cellular viability. Cell viability was measured by MTT assay. HeLa cells were cultured in 96 well microplates overnight and washed with serum-free culture medium. The cells were treated with Polygoni Cuspidati Radix (PR) or emodin for 4 h and further incubated with MTT for 3 h at 37°C. The insoluble formazan product was solubilized with DMSO, and then was determined by colorimetric analysis at 540 nm. Cell viability was expressed as a percentage of those cells treated with PBS alone. Polygoni Cuspidati Radix and emodin did not affect host cell viability.

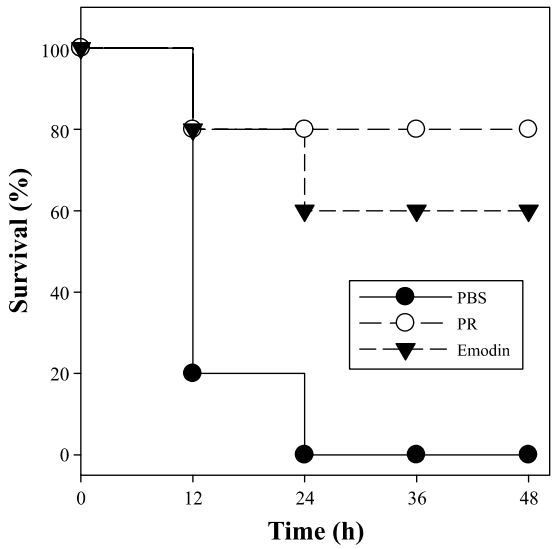

Fig. 5. Polygoni Cuspidati Radix and emodin protect mice from *V. vulnificus* infection. *V. vulnificus* were administered to 8-week-old CD-1 mice via intraperitoneal route. The mice were pretreated with Polygoni Cuspidati Radix (PR) or emodin twice, 1 day and 2 h before *V. vulnificus* injection. Polygoni Cuspidati Radix and emodin protected mice from *V. vulnificus* challenge.

nificantly the bacterial survival in seawater (Fig. 3B).

Safety of Polygoni Cuspidati Radix and emodin

Safety of Polygoni Cuspidati Radix and emodin on host cells was tested using MTT assay. HeLa cells were treated with Polygoni Cuspidati Radix or emodin for 4 h, and further incubated with MTT for 3 h at a 37°C incubator. Cell viability was expressed as a percentage of mock cells without any treatment. Figure 4 shows that Polygoni Cuspidati Radix and emodin did not affect the host cell viability.

Polygoni Cuspidati Radix and emodin protected mice from *V. vulnificus* infection

Effect of Polygoni Cuspidati Radix and emodin was studied on mice lethality caused by *V. vulnificus* infection. *V. vulnificus* were administered to SPF 8-week-old CD-1 mice via intraperitoneal route. Some group mice were pretreated via intraperitoneal route with Polygoni Cuspidati Radix or emodin twice, 1 day and 2 h before *V. vulnificus* injection. We observed difficulties in breathing, bristled fur, and decreased activity in *V. vulnificus*-infected mice. The symptoms were much milder in mice injected with Polygoni Cuspidati Radix and emodin. *V. vulnificus* caused mice to be sacrificed within 1 day, which was protected by the pretreatment of Polygoni Cuspidati Radix (200 mg/kg) or emodin (20 mg/kg) (Fig. 5).

Discussion

V. vulnificus, a serious opportunistic human pathogen, frequently causes fatal septicemia with a rapid progress, resulting in a mortality rate of more than 50% within a few days. Treatment of *V. vulnificus* septicemia has been limited by an acute and fulminating course of the pathogen. Numerous *V. vulnificus* isolates were found to be resistance to antibiotics routinely prescribed for the pathogen infections (Radu *et al.*, 1998; Baker-Austin *et al.*, 2008). The present study shows that Polygoni Cuspidati Radix and its constituent, emodin have significant protective effects on *V. vulnificus* cytotoxicity and infection. First, the pathogen caused the acute cytotoxicity to HeLa and RAW264.7 cells, which was potently prevented by Polygoni Cuspidati Radix and emodin (Fig. 1A and B). It was also observed that emodin protected the host cells from be rounded and damaged by the pathogen (Fig. 2B). In addition, Polygoni Cuspidati Radix and emodin inhibited significantly *V. vulnificus* growth and survival in HI broth and seawater, respectively (Fig. 3A and B). Taken together, Polygoni Cuspidati Radix and emodin protected mice from *V. vulnificus* infection (Fig. 5). The safety of Polygoni Cuspidati Radix and emodin was confirmed by MTT assay (Fig. 4). These results suggest that Polygoni Cuspidati Radix and emodin have a possibility to be developed as new therapeutic agents against *V. vulnificus* infection.

We screened natural products that have suppressive effects on *V. vulnificus* cytotoxicity. Polygoni Cuspidati Radix showed excellent protective effect of our expectation. Subsequently, we tried to identify active anti-cytotoxic component of Polygoni Cuspidati Radix, and emodin was proved to be a responsible active component by our present study. However, Polygoni Cuspidati Radix showed more potent activity than purified emodin on antibacterial effect and mice protective effect against *V. vulnificus* challenge (Fig. 3A and 5). We suggest that other components of Polygoni Cuspidati Radix such as piceid and resveratrol might protect mice from *V.*

vulnificus infection. This should be clarified by further studies. Polygoni Cuspidati Radix, through synergistic effect of emodin and other components, might protect mice more efficiently than purified emodin did. In other experiment, we tested resveratrol, one of known major components of Polygoni Cuspidati Radix, whether it could inhibit *V. vulnificus* cytotoxicity and infection. Resveratrol did not have significant inhibitory effect on *V. vulnificus* growth (data not shown). However, various beneficial effects of resveratrol reported in the literature might have exerted synergistic effects with emodin.

Emodin has been reported to inhibit the growth of MRSA and *H. pylori* (Wang and Chung, 1997; Hatano *et al*., 2005). Our results indicated that Polygoni Cuspidati Radix inhibited the growth of *V. vulnificus* and MRSA, but did not affect the growth of *Shigella flexeneri*, *O157*, *Enterotoxigenic E. coli* (*ETEC*), and *Streptococcus mutans* (data not shown). We suggest that Polygoni Cuspidati Radix and emodin have direct antibacterial activity on *V. vulnificus*, which might be a major mechanism of emodin protecting host from *V. vulnificus*. In addition, NF-κB inhibition and anti-inflammatory activity of emodin (Kumar *et al.*, 1998) might be considered as its other possible mechanisms. Because the antibacterial activity of emodin can mask the other possible effects of emodin on host cell in *V. vulnificus* cytotoxicity test, we did not test the effect of emodin on NF-*κ*B inhibition and inflammation. This should be clarified by further studies. Emodin was also reported to induce apoptosis in cancer cells (Huang *et al*., 2008). In the present study, emodin at concentrations from 2.5 to 10 μg/ml protected HeLa cells from being damaged by *V. vulnificus* (Fig. 2B). The anticancer effect of emodin has been evaluated after treatment for several days. In contrast, we tested the effect of emodin on *V. vulnificus* cytotoxicity just after several h incubation, because *V. vulnificus* causes acute cell death within 2 h. Therefore, emodin itself might not have affected host cell morphology and viability in our cytotoxicity assay model.

We first identified that Polygoni Cuspidati Radix and emodin inhibit significantly *V. vulnificus* growth and infection. Any natural product having that effect could be used to remove *V. vulnificus* in aquarium. Polygoni Cuspidati Radix showed excellent protective effect of our expectation. Because emodin was reported to have beneficial effect on host cells, we suppose that combination therapy of emodin and other antibiotics may help patients to be efficiently treated from *V. vulnificus* septicemia. These results suggest that Polygoni Cuspidati Radix and emodin have a potential as a safe and effective therapeutic target for bacterial infectious diseases.

Acknowledgements

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