Characterization and evaluation of antimicrobial activity of actinonin against foodborne pathogens

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Abstract This study revealed the antimicrobial properties of actinonin against major foodborne pathogens, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Staphylococcus aureus*, and *Vibrio vulnificus*. Among them, actinonin caused growth defect in *S. Typhimurium* and *V. vulnificus*. Minimal inhibitory concentration (MIC) values of actinonin were determined by broth microdilution methods. The MICs of actinonin were ≤ 0.768 $\mu\text{g/ml}$ for *S. Typhimurium* and ≤ 0.192 $\mu\text{g/ml}$ for *V. vulnificus*. Susceptibility to actinonin in both pathogens was measured by colony-forming ability and disc diffusion test. The results showed actinonin had antimicrobial activity against *S. Typhimurium* and *V. vulnificus* in a dose-dependent manner. The inhibitory effects on swarming motility were determined, and cytotoxicity of each pathogen against HeLa cells was decreased significantly by actinonin treatment. Furthermore, actinonin showed an antimicrobial efficacy in food models infected with these pathogens. These results demonstrate that actinonin is potentially an effective agent for food sanitization or preservation.

Keywords Actinonin · Foodborne pathogens · Antimicrobial agent

Introduction

Foodborne diseases remain a serious and growing problem throughout the world despite advances in food safety. Multiple factors such as changes in climate, eating habits, and increase of the elderly population, which is highly susceptible to disease, contribute to the circumstances regardless of foodborne pathogens [1]. In addition, foodborne pathogens have developed decreased susceptibility or even resistance due to widespread use of antibiotics [1]. Therefore, the battle against foodborne pathogens has risen to the point at which the need for novel antimicrobial agents is urgent [2].

Actinonin is a natural pseudo-peptides in the acidic component produced by a strain of *Streptomyces* spp. and *Actinomyces* spp. isolated from soil [3]. It has been reported that actinonin inhibits human peptide deformylase (*Homo sapiens* PDF, or HsPDF), which is a functional enzyme in mitochondria required for growth of normal or cancer cells [4]. Similarly, there are a few reports that actinonin may also control the activity of PDF in bacteria. [4, 5]. In bacterial cells, PDF removes *N*-formyl groups before the action of methionine aminopeptidase (MAP), removing methionine in the *N*-terminal methionine excision (NME) pathway, and as *N*-formyl group and methionine of the newly synthesized polypeptides are removed sequentially by PDF and MAP, various mature proteins for bacterial growth can be produced [4, 5]. As PDF plays an important role in the post-translational modification of peptides in bacteria, there are several reports that actinonin can control bacterial growth effectively by inhibiting bacterial PDF activity in *Haemophilus influenzae*, *Moraxella catarrhalis*, *Neisseria gonorrhoeae*, and *Vibrio anguillarum* [5, 6]. However, other studies have reported that there are several kinds of bacteria, including *Bacillus subtilis* and *Streptococcus pneumoniae*, that show resistance to actinonin [7, 8]. These results suggest that the

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antimicrobial spectrum of actinonin against pathogens is not clearly established, although actinonin is expected to inhibit the growth of bacteria.

The aim of the present study was to investigate the antimicrobial activity of actinonin toward the most frequently reported foodborne pathogens, including *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*), *Staphylococcus aureus*, and *Vibrio vulnificus*. By assessing the growth inhibition of these foodborne pathogens, *S. Typhimurium* and *V. vulnificus* were determined to be sensitive to actinonin. The effect of actinonin against 2 pathogens was examined and evaluated by determining the minimal inhibitory concentrations (MICs), colony-forming ability test, disc diffusion test, swarming motility test, and cytotoxicity assay. Antimicrobial activity of actinonin was also confirmed using food models that were infected with *S. Typhimurium* and *V. vulnificus*, respectively. Consequently, all results obtained provide a new insight into the antimicrobial activity of actinonin, and may contribute to the development of a novel agent that will improve the microbiological safety of food.

Materials and methods

Bacterial strains and reagents

E. coli O157:H7 NCTC 12079, *S. Typhimurium* SL1344, *S. aureus* Newman, and *V. vulnificus* CMCP6 were cultivated in Luria–Bertani (LB; Difco Inc., Detroit, MI, USA) containing 0.5% NaCl at 37 °C, or 30 °C for *V. vulnificus* CMCP6. *L. monocytogenes* KCTC 13064 was cultivated in Brain Heart Infusion (BHI, Difco) medium at 37 °C. Each culture was harvested by centrifugation ($3150 \times g$, 5 min) and washed twice with phosphate-buffered saline (PBS, pH 7.2). The final cell pellets were resuspended in PBS to retain proper cell concentration, and suspensions of each strain were used for inoculation study.

Actinonin was purchased from Abcam (Cambridge, UK). Stock solution (50 mM) was prepared by dissolving 5 mg actinonin in 259 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) or 94% ethanol (Daejung, Siheung, Korea). Actinonin was dissolved in DMSO for use in bacterial growth inhibition, antimicrobial activity, swarming motility inhibition, and cytotoxicity inhibition experiments, and dissolved in 94% ethanol for food model experiments.

Growth inhibition test

Foodborne pathogens grown overnight were seeded in LB broth and cultured until the absorbance at 600 nm reached 0.8 (log phase). Then, 90 µM of actinonin in DMSO or DMSO

only (control) group was added to the cultures. Absorbance (Optical density, O.D.₆₀₀) of the cultures was measured every 2 h using a UV/Vis spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA).

Assessment of antimicrobial activity of actinonin against *S. Typhimurium* and *V. vulnificus*

Determining the minimal inhibitory concentrations (MIC), colony-forming ability test and disc diffusion test were performed to assess the antimicrobial activity of actinonin upon *S. Typhimurium* and *V. vulnificus*.

MICs of actinonin were determined using a broth microdilution method [9]. Actinonin (concentration range, 0–2 µM) was added to the wells of 96-well plates containing the same CFUs of *S. Typhimurium* and *V. vulnificus*. After 16 h culture, the absorbance (O.D.₆₀₀) of each well was measured. The MIC was defined as the lowest concentration of actinonin that decreased the visible turbidity statistically.

The colony-forming ability test was performed essentially as described in previous studies [9]. *S. Typhimurium* and *V. vulnificus* were inoculated in 96-well plates with 5.91×10^7 CFU (colony-forming units) and 7.10×10^7 CFU per well, respectively. Then, actinonin (concentration range, 0–750 µM) was added to each well. After actinonin challenge for 4 and 8 h, the bacterial culture from each well was diluted 10-fold with PBS. Then, 40 µl of the diluted bacteria was spread onto LB agar plates, and CFUs were counted following overnight incubation.

A test for disc diffusion was conducted by the standard method of the Clinical and Laboratory Standards Institute (CLSI) [10]. A 0.1 ml aliquot of each strain was spread over Mueller–Hinton agar (Difco), and sterilized filter-paper discs saturated with 50 µl of varying concentrations of actinonin (0–2 mM) were placed on the surface. The plates were incubated overnight at 37 °C for *S. Typhimurium* and 30 °C for *V. vulnificus*. Diameters of the inhibition zones surrounding the discs were measured for each actinonin concentration in triplicate.

Swarming motility test

Inhibition of *S. Typhimurium* and *V. vulnificus* swarming motility was determined based on the method described by Na et al. [11]. Semi-solid agar plates containing 0.5% bactoagar with actinonin (0–5 µM) were used. The distance of swarming migration was measured 20 h after inoculation.

Cytotoxicity assay

The cytotoxicity of *S. Typhimurium* and *V. vulnificus* to HeLa cells was determined by lactate dehydrogenase

(LDH) assay using a CytoTox96 Non-Radioactive cytotoxicity assay kit (Promega, Madison, WI, USA), as described elsewhere [12]. HeLa cells were grown in T75 cell culture flasks (SPL Life Sciences, Pocheon, Korea) containing Dulbecco's Modified Eagles Medium (DMEM, Thermo Fisher Scientific) that was supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C with 5% CO₂. The HeLa cells were seeded into each well of a 24-well culture plate (6.0×10^5 cells/well) and cultured overnight. The cells were washed with antibiotic-free DMEM. To examine the effect of actinonin, 15 µM of actinonin was added to HeLa cells 2 h before bacterial infection. Then, HeLa cells were inoculated with *S. Typhimurium* and *V. vulnificus* at a multiplicity of infection (MOI) of 20. After the designated incubation time for each group, released LDH activity was determined by measuring the absorbance (O.D.₄₉₀) using a UV/Vis spectrophotometer (Thermo Fisher Scientific). The value of LDH activity of each experimental group was subtracted by the one of the cells only treated by actinonin.

Decontamination of food samples inoculated with *S. Typhimurium* and *V. vulnificus*

Fresh lettuce (*Lactuca sativa*) and webfoot octopus (*Amphioctopus fangsiao*) were purchased from a local market in Daejeon, South Korea, and then used immediately. A 10.00 ± 0.30 g portion of the food samples were sliced with a sterile stainless steel blade and treated with UV irradiation for 30 min in a laminar flow biosafety hood with UV-C lamps to eliminate normal flora. Sterilized lettuce slices were spot-inoculated with 20 µl of *S. Typhimurium* to an initial cell concentration of $\sim 3.86 \times 10^6$ CFU/g. Sterilized webfoot octopus samples were dampened with 150 ml of *V. vulnificus* at an initial cell concentration of $\sim 4.64 \times 10^6$ CFU/g.

The inoculated samples were air-dried for 1 h in a laminar flow biosafety hood to allow attachment of the pathogens. Then, each sample was cleansed in a bag containing 20 ml of distilled water or washing solution containing various concentrations of actinonin (0 - 400 µM). The samples were homogenized by a stomacher laboratory blender (Interscience, Saint-Nom-la-Bretèche, France) for 3 min. The lettuce and webfoot octopus rinsates from the bags were diluted 10³-fold and 10⁴-fold, and 100 µl aliquots of rinsates were spread onto LB agar plates. After overnight incubation at 37 °C for *S. Typhimurium* and 30 °C for *V. vulnificus*, the logarithmic scales of CFU/g were determined.

Statistical analysis

All the experiments were repeated three times and the values were calculated as the mean $\pm$ standard deviation.

One-way analysis of variance (ANOVA) was performed by using Statistical Analysis System (SAS) software for statistical comparison.

Results and discussion

Actinonin inhibits the growth of *S. Typhimurium* and *V. vulnificus*

The effects of actinonin on the growth of foodborne pathogens were observed by measuring absorbance (O.D.₆₀₀) every 2 h. The presence of actinonin at a concentration of 90 µM caused no growth inhibition of *E. coli* O157:H7, *L. monocytogenes*, and *S. aureus* [Fig. 1(B), (C) and (E)], whereas the growth of *S. Typhimurium* and *V. vulnificus* were clearly inhibited by actinonin treatment. In particular, the antimicrobial efficacy against *V. vulnificus* was higher than that against *S. Typhimurium* [Fig. 1(D), (F)].

Natural compounds have been utilized as alternative antimicrobial agents to eliminate the side effects of conventional antibiotics or sanitizers that are composed of synthetic chemical compounds [13]. Among them, natural antimicrobial peptides have been revealed to control pathogens efficiently in many studies [14]. There are two types of antimicrobial peptides, ribosomally synthesized and non-ribosomally synthesized peptides [15]. Ribosomally synthesized peptides are produced by all species, whereas non-ribosomally synthesized peptides are produced primarily by bacteria [16]. Antimicrobial pseudopeptides, including actinonin, are non-ribosomal synthesized antimicrobial peptides from bacteria. Actinonin is usually derived from *Streptomyces* spp. [6], and it is known to have inhibition activity against PDF, which is an essential enzyme in almost prokaryotic and eukaryotic cells [4]. However, our results demonstrate that of the 5 foodborne pathogens that have active PDF, only *S. Typhimurium* and *V. vulnificus* showed actinonin-induced growth inhibition. Therefore, it was unexpected that only two of the five foodborne pathogens belonged to the antimicrobial spectrum of actinonin (Fig. 1). The growth of *B. subtilis*, *E. coli*, *S. pneumoniae* was not inhibited by actinonin while it inhibited the growth of *H. influenzae*, *M. catarrhalis*, *N. gonorrhoeae*, *S. aureus*, *S. epidermidis* according to the previous studies (Fig. 2). In addition to the reported antimicrobial spectrum of actinonin, the growth inhibition result on foodborne pathogens from this study provides more information on its spectrum.

In previous reports, actinonin repressed the growth of several strains, including *H. influenzae* ATCC 51907, *M. catarrhalis* BBH18, *N. gonorrhoeae* ATCC 700825, *S. aureus* ATCC 25923, and *S. epidermidis* ATCC 35984. It

Fig. 1 The growth inhibition of actinonin against *E. coli* O157:H7, *L. monocytogenes*, *S. Typhimurium*, *S. aureus* and *V. vulnificus*. **(A)** Chemical structure of actinonin; Actinonin ($C_{19}H_{35}N_3O_5$) is a pseudo-tripeptide hydroxamate inhibitor derived from L-prolinol and its *n*-pentyl side chain binds hydrophobic subsite of peptide deformylase (PDF), which inhibits the activity of PDF that removes N-formyl groups before the action of methionine aminopeptidase (MAP) in N-terminal methionine excision (NME) pathway from

was also revealed that activity of PDF in these strains was decreased by actinonin [5, 17]. However, actinonin showed no antimicrobial effect on *E. coli* O157:H7 NCTC 12079, *L. monocytogenes* KCTC 13064, and *S. aureus* Newman in this study. *B. subtilis* 168, *E. coli* K-12, and *S. pneumoniae* ATCC 49619 have also been reported to be resistant to actinonin [5, 7, 8]. Thus, BLAST searches of the genomes of actinonin-sensitive/resistant strains were performed to infer the mode of growth inhibition caused by actinonin, and putative PDFs were retrieved and compared (Fig. 2).

eubacteria. **(B)–(F)** five foodborne pathogens were incubated overnight and subcultured in 20 ml of LB broth media until absorbance (O.D.₆₀₀) reached 0.8. Then, growth of the control group and the experimental group with 90 μM actinonin was assessed by measuring the absorbance (O.D.₆₀₀) every 2 h for 8 h post actinonin treatment. All values are mean ± standard deviation of three repeated processes, and double stars express significant *p* values (***p* < 0.01)

Using previous studies and our results, we could not find any distinct motifs in PDF sequences in actinonin-sensitive/resistant strains. This suggest that actinonin-resistant strains have a unique mechanism of antimicrobial resistance, such as a mutation in *fnt*, which encodes Methionyl-tRNA formyltransferase (FMT), or an efflux-pump.

FMT adds the formyl group to the nonformylated methionyl initiator tRNA (Met-tRNA_i) before the action of PDF in ribosome-mediated functional protein synthesis [17]. If functional FMT is not produced, PDF activities

Strains	Growth inhibition	References	Catalytic domains of PDF		
			I	II	III
<i>H. influenzae</i> ATCC 51907 <i>def</i>	+	Chen et al.	GIGLAAPQ	EGCLS	QHEIDHLNG
<i>M. catarrhalis</i> BBH18 <i>def</i>	+	Chen et al.	GIGLAATQ	EGCLS	QHEMDHLNG
<i>N. gonorrhoeae</i> ATCC 700825 <i>defB</i>	+	Chen et al.	GIGLAATQ	EGCLS	QHELDHLMG
<i>S. aureus</i> ATCC 25923 <i>defB</i>	+	Chen et al.	GVGLAAPQ	EGCLS	QHEIDHLNG
<i>S. epidermidis</i> ATCC 35984 <i>def</i>	+	Chen et al.	GVGLAAPQ	EGCLS	QHEIDHLNG
<i>S. Typhimurium</i> SL1344 <i>def</i>	+	This study	GIGLAATQ	EGCLS	QHEMDHLVG
<i>V. vulnificus</i> CMCP6 <i>def1</i>	+	This study	GIGLAATQ	EGCLS	QHELDHLQG
<i>V. vulnificus</i> CMCP6 <i>def2</i>	+	This study	GVGLAAPQ	EGCLS	QHEIDHLSG
<i>B. subtilis</i> 168 <i>defA</i>	–	Duroc et al.	GVGLAAPQ	EGCLS	QHEMDHLDG
<i>B. subtilis</i> 168 <i>defB</i>	–	Duroc et al.	GVGLAAPQ	EGCLS	QHEIDHLNG
<i>E. coli</i> K-12 <i>def</i>	–	Chen et al.	GIGLAATQ	EGCLS	QHEMDHLVG
<i>E. coli</i> O157:H7 NCTC 12079 <i>def</i>	–	This study	GIGLAATQ	EGCLS	QHEMDHLVG
<i>L. monocytogenes</i> KCTC 13064 <i>def</i>	–	This study	GIGLAAPQ	EGCLS	QHEIDHLNG
<i>S. aureus</i> Newman <i>defB</i>	–	This study	GVGLAAPQ	EGCLS	QHEIDHLNG
<i>S. pneumoniae</i> ATCC 49619 <i>defB</i>	–	Margolis et al.	GVGLAAPQ	EGCLS	QHEIDHLNG

Fig. 2 Alignment of the homologous PDF domains of various bacteria. The catalytic domains of PDF that are essential to *def* encoded peptide deformylase for protein synthesis in five foodborne pathogens from this study and other actinonin-sensitive/resistant strains from previous studies were identified [5, 7, 17]. I, II and III are

the motifs that comprise the catalytic domain. + and – indicate whether the growth of bacteria was inhibited by actinonin or not (+ inhibited, – not inhibited). Grey highlights mean the amino acid residue is not exactly the same but, it still retains amino acid specific functions

decrease in the functional protein synthesis pathway. Thus, actinonin would have difficulty interacting with PDF. However, in this case, *fnt* mutant should have an obvious impaired growth phenotype [17]. *E. coli* O157:H7, *L. monocytogenes*, and *S. aureus* showed normal growth rate during treatment with actinonin in the current study (Fig. 1), which suggests there is no *fnt* mutation in these strains.

There are several reports that most actinonin-resistant strains have well-known efflux-pump systems, including the multidrug efflux-pump AcrAB in *E. coli* O157:H7, the ubiquitous efflux-pumps MdrL and Lde in *L. monocytogenes* KCTC 13064, and the multidrug transporter NorA in *S. aureus* Newman. These pumps can extrude various antibiotic agents such as macrolides and cefotaxime, heavy metals, ethidium bromide, and fluoroquinolone compounds [18–20]. This suggests that efflux-pump systems may play an important role in actinonin-resistance. In particular, *S. aureus* Newman was resistant to actinonin (Fig. 1), contrary to a previous report that showed *S. aureus* ATCC 25923 was sensitive to actinonin [5]. This implies that the efflux-pump systems of the two *S. aureus* subspecies may have different features.

Measurement of the susceptibility of *S. Typhimurium* and *V. vulnificus* to actinonin

For more precise determination of antimicrobial activity, the susceptibility of *S. Typhimurium* and *V. vulnificus* to actinonin was assessed by MICs determination, a test for colony-forming ability and a test for disc diffusion.

To determine the MICs of actinonin in *S. Typhimurium* and *V. vulnificus*, a broth micro-dilution method was used. When actinonin was not present, the mean absorbance (O.D.₆₀₀) of *S. Typhimurium* and *V. vulnificus* were 0.370 ± 0.079 and 0.370 ± 0.109 , respectively. The absorbance (O.D.₆₀₀) of *S. Typhimurium* decreased in a concentration-dependent manner and statistical difference was observed at 2 μ M of actinonin, while the actinonin concentration that causes significant decrease in absorbance (O.D.₆₀₀) of *V. vulnificus* statistically was 0.5 μ M. Therefore, the MICs of actinonin against *S. Typhimurium* and *V. vulnificus* were ≤ 2 μ M (0.768 μ g/ml) and ≤ 0.5 μ M (0.192 μ g/ml), respectively, and the MIC of *S. Typhimurium* were higher than that of *V. vulnificus* (Table 1).

Previous studies have reported that there are many kinds of antimicrobial agents from a variety of sources that inhibit the growth of *S. Typhimurium* and *V. vulnificus* [21]. The MIC values of these antimicrobial agents against *S. Typhimurium* and *V. vulnificus* were determined by previous studies, but their values were higher than the values of actinonin against both pathogens. For example, the MICs of thymol and cinnamaldehyde, which are major

constituents in the essential oil of the plants *Thymus vulgaris* and *Cinnamomum cassia*, canine cathelicidin and lactoferricin B from placental mammals and bovine, and chrisin from *Lactococcus lactis* against *S. Typhimurium* SGI1 (*tet* A), ATCC 13311, CMCC 50013 and #98 were reported as 0.37 (thymol for SGI1), 0.33 (cinnamaldehyde for SGI1), 0.005 (canine cathelicidin for ATCC 13311), 0.032 (lactoferricin B for CMCC 50013), and 0.5 mg/ml (chrisin for #98), respectively [22–27]. The MICs of curcumin and eugenol extracted from *Curcuma longa* and sweet basil, pleurocidin-amide and tachyplesin from winter flounder and Japanese horseshoe crab, and CAP-1 from *Pseudomonas* spp., against *V. vulnificus* MTCC 1145, LA624 FDA/GC5L and ATCC 27562 were reported as 0.3 (curcumin for MTCC 1145), 0.45 (eugenol for MTCC 1145), 0.005 (pleurocidin-amide for LA624 FDA/GC5L), 0.0025 (tachyplesin for LA624 FDA/GC5L), and 0.136 mg/ml (CAP-1 for ATCC 27562), respectively [28–31]. Compared to the MICs of agents reported previously with considering their CFU/ml ($\leq 1 \times 10^8$), our results (*S. Typhimurium*: ≤ 0.768 μ g/ml, *V. vulnificus*: ≤ 0.192 μ g/ml) demonstrate that actinonin may be the most effective known antimicrobial natural compound against *S. Typhimurium* and *V. vulnificus*.

Actinonin reduced the colony-forming ability of *S. Typhimurium* and *V. vulnificus* in a dose-dependent manner (Table 1). The reduction levels of *S. Typhimurium* were 0.025, 0.103, 0.107, 0.573, 0.719, and 1.216 log CFU/ml after 4 h incubation with 2–750 μ M of actinonin compared with the control. In subjects with *S. Typhimurium* treated with actinonin for 4 h, the mean numbers of log CFU/ml were decreased significantly at 500 μ M of actinonin. After an 8 h incubation with actinonin, the reduction levels of *S. Typhimurium* were 0.430, 0.640, 0.764, 1.511, 3.266, and 4.278 log CFU/ml compared with control, and the lowest concentration of actinonin that significantly reduced the colony-forming ability of *S. Typhimurium* was 20 μ M. Likewise, the reduction levels of *V. vulnificus* were 0.917, 1.152, 1.034, 1.458, 1.558, and 3.705 log CFU/ml after 4 h incubation with 2–750 μ M of actinonin. After 8 h incubation, the reduction levels of *V. vulnificus* were 1.322, 3.978, 4.591, 5.043, 5.488, and 5.586 log CFU/ml, respectively. The lowest concentration of actinonin that significantly reduced the colony-forming ability of *V. vulnificus* was 2 μ M for both incubation times.

Results of the disc diffusion test are shown in Table 1. The inhibition zone on *S. Typhimurium* was formed by 1 mM and 2 mM of actinonin with average diameters of 14.00 mm and 18.66 mm, respectively. The inhibition zone on *V. vulnificus* started to appear at 0.1 mM of actinonin and clearly formed at 1 and 2 mM of actinonin with average diameters of 25.16 mm and 31.66 mm, respectively.

Table 1 Evaluation of antimicrobial activity

Actinonin concentration (μM)	Strains			
	<i>S. Typhimurium</i>		<i>V. vulnificus</i>	
MICs test	Optical density (O.D. _{600 nm})		Optical density (O.D. _{600 nm})	
0 ¹	0.370 ± 0.079 ^{2a}		0.370 ± 0.109 ^a	
0.125	0.331 ± 0.054 ^a		0.331 ± 0.054 ^a	
0.25	0.307 ± 0.097 ^a		0.307 ± 0.097 ^a	
0.5	0.261 ± 0.042 ^a		0.111 ± 0.013 ^b	
1	0.261 ± 0.046 ^a		0.110 ± 0.014 ^b	
2	0.141 ± 0.012 ^b		0.105 ± 0.025 ^b	
Colony forming test	Log CFU/mL		Log CFU/mL	
	4 h	8 h	4 h	8 h
0	8.228 ± 0.159 ^a	8.168 ± 0.230 ^a	8.056 ± 0.128 ^a	7.820 ± 0.299 ^a
2	8.203 ± 0.135 ^a	7.738 ± 0.310 ^{ab}	7.139 ± 0.524 ^b	6.498 ± 0.173 ^b
20	8.125 ± 0.290 ^a	7.528 ± 0.140 ^b	6.904 ± 0.521 ^b	3.842 ± 0.350 ^c
50	8.121 ± 0.317 ^a	7.404 ± 0.291 ^b	7.022 ± 0.591 ^b	3.229 ± 0.324 ^d
100	7.655 ± 0.342 ^{ab}	6.657 ± 0.241 ^c	6.598 ± 0.347 ^b	2.777 ± 0.274 ^{de}
500	7.509 ± 0.289 ^{bc}	4.902 ± 0.113 ^d	6.498 ± 0.173 ^b	2.332 ± 0.155 ^{ef}
750	7.012 ± 0.577 ^c	3.890 ± 0.428 ^e	4.351 ± 0.141 ^c	2.234 ± 0.317 ^f
Disc diffusion	Diameter (mm)		Diameter (mm)	
0	0 ^a		0 ^a	
0.02	0 ^a		0 ^a	
0.1	0 ^a		14.833 ± 1.041 ^b	
0.2	0 ^a		16.167 ± 0.764 ^b	
1	14.000 ± 1.000 ^b		25.167 ± 0.764 ^c	
2	18.667 ± 1.528 ^c		31.667 ± 4.726 ^d	

¹ Control (0 μM): The same amount of DMSO was added to the control of each strain

² All values are mean ± standard deviation of three repeated processes. Different superscripts indicate degree of significant difference ($p < 0.05$)

All these results revealed that actinonin has potent antimicrobial activity against *S. Typhimurium* and *V. vulnificus*. In addition, the susceptibilities of both pathogens to actinonin were determined, and *V. vulnificus* was more sensitive to actinonin than *S. Typhimurium* in all experiments.

Actinonin causes defects in swarming motility of *S. Typhimurium* and *V. vulnificus*

It has been demonstrated that swarming plays an important role in attachment and colonization of pathogens [32]. We investigated the effects of actinonin on the swarming motility of *S. Typhimurium* and *V. vulnificus* on semi-solid agar plates containing various concentration of actinonin. As shown in Fig. 3, *S. Typhimurium* and *V. vulnificus* swarming was repressed in a dose-dependent manner by actinonin. The swarming motility decline of *S. Typhimurium* was observed from 0.05 μM of actinonin, and it was inhibited completely at 5 μM. Similarly, 0.05 μM of

actinonin inhibited the swarming motility of *V. vulnificus* significantly. 0.1 μM decreased 60% of that, and we could not observe the swarming at 5 μM. In current study, the MICs of actinonin against *S. Typhimurium* and *V. vulnificus* were ≤2 and ≤0.5 μM, respectively. Although 0.05 μM of actinonin was lower than MICs, both *S. Typhimurium* and *V. vulnificus* swarming was significantly inhibited at this concentration (Fig. 3). These suggest actinonin has the decreasing effect on swarming motility regardless of antimicrobial activity.

Actinonin decreases the cytotoxicity of *S. Typhimurium* and *V. vulnificus*

S. Typhimurium and *V. vulnificus* have highly cytotoxic activity to host cells [33, 34]. To assess the inhibitory effect of actinonin on cytotoxicity caused by *S. Typhimurium* and *V. vulnificus*, HeLa cells were treated with actinonin before pathogen infection, and then LDH activities were determined (Fig. 4). The LDH for DMSO-treated cells reached a

Fig. 3 Effect of actinonin on swarming motility of *S. Typhimurium* and *V. vulnificus*. The inhibition of swarming motility of *S. Typhimurium* and *V. vulnificus* was determined by measuring the diameter of swarming zones on actinonin-treated semi-solid agar plates (0.5% agar) containing different concentrations of actinonin (0, 0.05, 0.1, 0.5, 2, 5 μM). An aliquot of 5 μl of *S. Typhimurium* or *V. vulnificus* was inoculated onto each plate and the diameter was

measured after 20 h incubation at 37 °C for *S. Typhimurium* and 30 °C for *V. vulnificus*. **(A)** Halo images of swarming plates containing different concentrations of actinonin were taken. **(B)** Diameters of swarming migrations of *S. Typhimurium* and *V. vulnificus* were measured. All values are mean $\pm$ standard deviation of three repeated processes, and double stars express significant p values (** $p < 0.01$)

maximum level 225 min after *S. Typhimurium* infection. However, actinonin-treated cells had a 51.68% reduction in LDH activity compared to DMSO-treated cells. After 135 min after *V. vulnificus* infection, actinonin-treated cells showed a 69.70% reduction in LDH compared to DMSO-treated cells. These results show actinonin decreased cytotoxicity in HeLa cells caused by *S. Typhimurium* and *V. vulnificus*.

Actinonin inactivates *S. Typhimurium* and *V. vulnificus* in food models

Actinonin was shown to cause a significant defect of growth in *S. Typhimurium* and *V. vulnificus* in the present study. Thus, it may be an alternative to overcome challenges related to *S. Typhimurium* and *V. vulnificus* contamination in food. If it is to be used successfully in the food industry, the evaluation of actinonin efficacy in food models was required.

S. Typhimurium and *V. vulnificus* were inoculated on lettuce and webfoot octopus. After 1 h incubation and washing with distilled water, counts of *S. Typhimurium* and *V. vulnificus* amounted to 6.791 ± 0.088 and 7.378 ± 0.127 log CFU/g. When food samples were

washed with distilled water containing 94% ethanol as a control solution, the recovered log CFU/g of *S. Typhimurium* was 6.795 ± 0.053 and that of *V. vulnificus* was 7.336 ± 0.071 . However, washing with distilled water containing actinonin (50–400 μM) dissolved in 94% ethanol showed the reduction of contaminated pathogens in food samples in a dose-dependent manner, as shown in Table 2. The 400 μM actinonin treatment reduced the amount of *S. Typhimurium* and *V. vulnificus* to 5.113 ± 0.275 and 6.796 ± 0.062 log CFU/g, respectively, and the reduction levels of contaminated *S. Typhimurium* and *V. vulnificus* was 1.903 and 0.756 log CFU/g under this concentration compared with untreated samples, respectively. Compared to washing with distilled water and control solution (distilled water containing ethanol), the concentration of actinonin that significantly inactivated *S. Typhimurium* and *V. vulnificus* on food samples was 50 μM. Based on these results, actinonin was effective in inactivation of *S. Typhimurium* on lettuce and *V. vulnificus* on webfoot octopus. In general, the efficacy of many chemical and naturally occurring antimicrobials may be reduced by certain food components [35]. Therefore, in addition to the antimicrobial efficacy of actinonin demonstrated by this study, further investigation of actinonin

Fig. 4 Determination of lactate dehydrogenase (LDH) release from HeLa cell infected with *S. Typhimurium* and *V. vulnificus* in the presence or absence condition of actinonin. HeLa cells were infected by *S. Typhimurium* (A) or *V. vulnificus* (B) at MOI 20, and supplemented with DMSO or actinonin (15 μ M). Then, LDH release from the cells was determined after infection. All values are mean $\pm$ standard deviation of three repeated processes. A star and double stars express significant *p* values (**p* < 0.05; ***p* < 0.01)

Table 2 The antimicrobial activity of actinonin against *S. Typhimurium* and *V. vulnificus* in food models

Treatment	<i>S. Typhimurium</i> (log CFU/g) from lettuce	<i>V. vulnificus</i> (log CFU/g) from webfoot octopus
Untreated	7.016 $\pm$ 0.110 ^{2a}	7.552 $\pm$ 0.174 ^a
D.W. ¹	6.791 $\pm$ 0.088 ^a	7.378 $\pm$ 0.127 ^{ab}
Concentration of actinonin in washing solution		
0 μ M	6.795 $\pm$ 0.053 ^a	7.336 $\pm$ 0.071 ^b
50 μ M	6.336 $\pm$ 0.074 ^b	7.267 $\pm$ 0.050 ^c
100 μ M	5.346 $\pm$ 0.357 ^c	7.080 $\pm$ 0.110 ^c
200 μ M	5.054 $\pm$ 0.173 ^c	6.787 $\pm$ 0.128 ^d
400 μ M	5.113 $\pm$ 0.275 ^c	6.796 $\pm$ 0.062 ^d

¹ D.W: distilled water

² All values are mean $\pm$ standard deviation of three repeated processes. Different superscripts indicate degree of significant difference (*p* < 0.05)

appropriate to the sensory profile of foods and composition of the food system is required.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

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