



## Research paper

# Genipin inhibits MMP-1 and MMP-3 release from TNF- $\alpha$ -stimulated human periodontal ligament cells



Satoru Shindo<sup>a</sup>, Yoshitaka Hosokawa<sup>a,\*</sup>, Ikuko Hosokawa<sup>a</sup>, Kazumi Ozaki<sup>b</sup>, Takashi Matsuo<sup>a</sup>

<sup>a</sup> Department of Conservative Dentistry, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima, Tokushima, 770-8504, Japan

<sup>b</sup> Department of Oral Health Care Promotion, School of Oral Health and Welfare, Faculty of Dentistry, The University of Tokushima, Tokushima, Tokushima, Japan

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## ABSTRACT

Genipin, the aglycon of geniposide found in gardenia fruit has long been considered for treatment of inflammatory diseases in traditional oriental medicine. Genipin has recently been reported to have some pharmacological functions, such as antimicrobial, antitumor, and anti-inflammatory effects. The aim of this study was to examine whether genipin could modify matrix metalloproteinase (MMP)-1 and MMP-3, which are related to the destruction of periodontal tissues in periodontal lesion, expression in tumor necrosis factor (TNF)- $\alpha$ -stimulated human periodontal ligament cells (HPDLCs). Genipin prevented TNF- $\alpha$ -mediated MMP-1 and MMP-3 productions in HPDLCs. Moreover, genipin could suppress not only extracellular signal-regulated kinase (ERK) and Jun-N-terminal kinase (JNK) phosphorylations but also AMP-activated protein kinase (AMPK) phosphorylation in TNF- $\alpha$ -stimulated HPDLCs. Inhibitors of ERK and AMPK could inhibit both MMP-1 and MMP-3 productions. Moreover, we revealed the ERK inhibitor suppressed AMPK phosphorylation in TNF- $\alpha$ -stimulated HPDLCs. These data provide a new mechanism through which genipin could be used for the treatment of periodontal disease to prevent MMPs expression in periodontal lesion.

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## 1. Introduction

Periodontitis is a chronic inflammatory disease characterized a complex interaction between periodontopathic bacteria and the host inflammatory response resulting in release of inflammatory cytokines leading to the destruction of periodontal connective tissues and alveolar bone [1,2].

Matrix metalloproteinase (MMPs) derived from periodontal cells play an important role in the destruction of periodontal tissues [3]. Especially, MMP-1 is thought to be a key player of the breakdown of periodontal tissues [4]. MMP-1 is the major proteolytic enzyme that can degrade collagens type I and III, which are the most massive protein components of the extracellular matrix in periodontal tissues [5]. It has been reported that MMP-1 level was enhanced in periodontal lesions [6]. MMP-3 has a pivotal role in

activating latent MMPs including pro-MMP-1, -8 and -9 [7]. Therefore, MMP-3 is an important MMP to control the destruction of connective tissues in periodontium. Previous reports showed that MMP-3 was highly expressed in periodontal lesion [8,9], and inflammatory cytokines could induce MMP-3 production in periodontal resident cells [10,11].

Genipin, the metabolite of geniposide, is a natural product present in the fruit of *Gardenia jasminoides*. It has been reported that genipin has anti-inflammatory [12], anti-oxidative [13], anti-cancer effects [14]. We previously reported that genipin could inhibit CCL20 and IL-6 productions from IL-1 $\beta$ -stimulated HPDLCs [15]. Our report showed that genipin has anti-inflammatory effects on cytokine-stimulated HPDLCs. However, the effect of genipin on MMPs production in HPDLCs is still uncertain.

The aim of this study was to examine the effect of genipin on MMP-1 and MMP-3 releases from TNF- $\alpha$ -stimulated HPDLCs which are the major cells in periodontal tissues. Moreover, we investigated whether genipin treatment modified the activation of mitogen-activated protein kinases (MAPKs) and AMP-activated protein kinase (AMPK) pathways in TNF- $\alpha$ -stimulated HPDLCs.

\* Corresponding author. Tel./fax: +81 886 33 7340.

E-mail address: [hosokawa@tokushima-u.ac.jp](mailto:hosokawa@tokushima-u.ac.jp) (Y. Hosokawa).

## 2. Materials and methods

### 2.1. Cell culture

Human periodontal ligament cells (HPDLCs) were obtained from Lonza Walkersville Inc. (Walkersville, MD, USA) and grown in the Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics (penicillin G: 100 units/ml, streptomycin: 100 µg/ml) at 37 °C in humidified air with 5% CO<sub>2</sub>. Cells were used between passage numbers 5 and 10.

### 2.2. MMP-1, MMP-3, and TIMP-1 release from HPDLCs

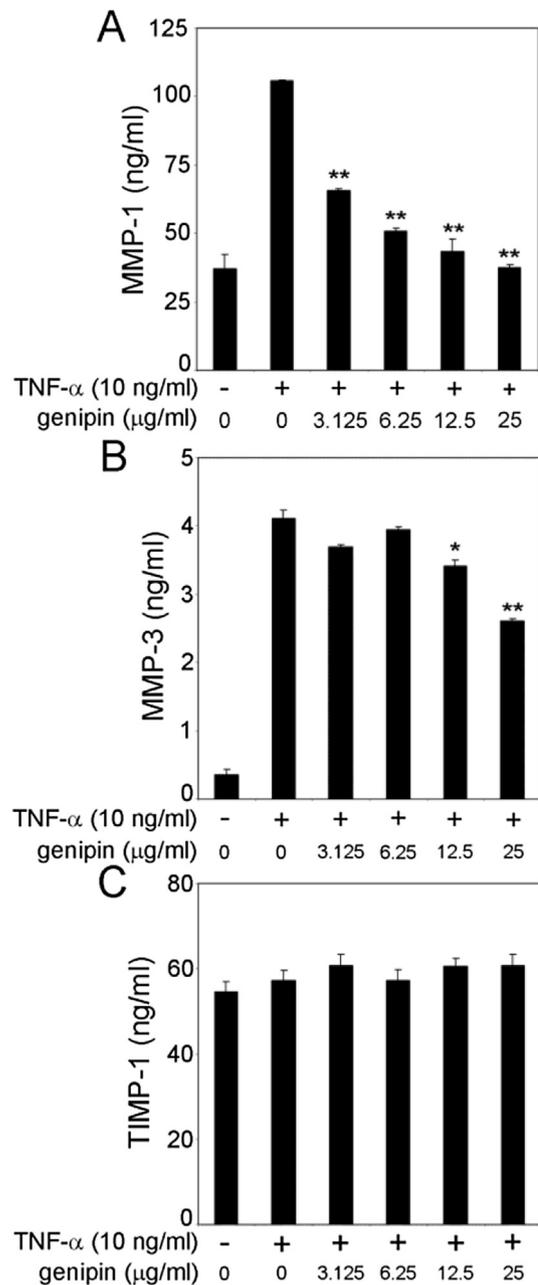
The HPDLCs were stimulated with recombinant human TNF- $\alpha$  (10 ng/ml; Peprotech, Rocky Hill, NJ, USA) for 24 h. The supernatants from the HPDLCs were collected, and the MMP-1, MMP-3, and TIMP-1 concentrations of the culture supernatants were measured in triplicate with ELISA. Duoset (R&D systems, Minneapolis, MN, USA) was used for the determination. All assays were performed according to the manufacturer's instructions, and MMPs levels were determined using the standard curve prepared for each assay. In selected experiments, the HPDLCs were cultured for 1 h in the presence or absence of Genipin (3.125, 6.25, 12.5, or 25 µg/ml; Cayman Chemical, Ann Arbor, MI, USA), SB203580 (10 µM; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PD98059 (10 µM; Cayman Chemical), SP600125 (10 µM; Enzo Life Sciences, Plymouth Meeting, PA, USA), or compound C (0.1 µM, 1 µM, or 10 µM; Calbiochem, La Jolla, CA, USA) prior to their incubation with TNF- $\alpha$  (10 ng/ml).

### 2.3. Western blot analysis

To confirm the TNF- $\alpha$ -induced phosphorylation of signal transduction molecules, western blot analysis was performed. HPDLCs stimulated by TNF- $\alpha$  (10 ng/ml) with or without genipin (12.5 or 25 µg/ml) or PD98059 (10 µM) pretreatment for 1 h were washed once with cold PBS, before being incubated on ice for 30 min with cell lysis buffer (Cell signaling technology, Danvers, MA, USA) supplemented with protease inhibitors cocktail (Sigma). After removal of debris by centrifugation, the protein concentrations of the lysates were quantified with the Bradford protein assay using IgG as a standard. A 20 µg protein sample was loaded onto a 4–20% SDS-PAGE gel, before being electrotransferred to a PVDF membrane. The phosphorylations of p38 MAPK, ERK, JNK, or AMPK were examined using phospho-p38 MAPK rabbit monoclonal antibody (Cell signaling technology), phospho-ERK rabbit monoclonal antibody (Cell signaling technology), phospho-JNK rabbit monoclonal antibody (Cell signaling Technology), phospho-AMPK rabbit monoclonal antibody (Cell signaling Technology), p38 MAPK rabbit monoclonal antibody (Cell signaling technology), ERK rabbit monoclonal antibody (Cell signaling Technology), JNK rabbit monoclonal antibody (Cell Signaling Technology), AMPK rabbit monoclonal antibody (Cell signaling technology), or GAPDH rabbit monoclonal antibody (Cell signaling technology) according to the manufacturer's instructions. Protein bands were visualized by incubation with the HRP-conjugated secondary antibody (Sigma), followed by detection using the ECL system (GE Healthcare, Uppsala, Sweden).

### 2.4. Statistical analysis

Statistical significance was analyzed using the Student's *t* test. *P* values <0.05 were considered significant in Figs. 1 and 2.

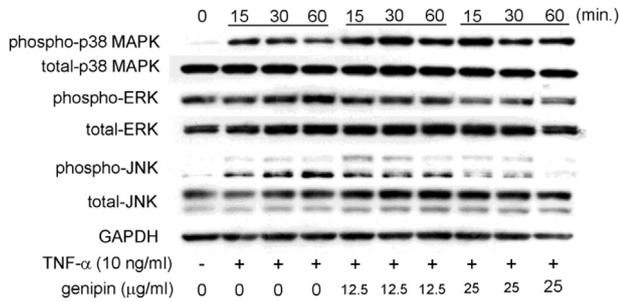


**Fig. 1.** Effects of genipin on MMP-1, MMP-3, and TIMP-1 productions in TNF- $\alpha$ -stimulated HPDLCs. HPDLCs were pretreated with genipin (3.125, 6.25, 12.5, or 25 µg/ml) for 1 h, and then the HPDLCs were stimulated with TNF- $\alpha$  (10 ng/ml), and the supernatants were collected after 24 h. The expression levels of MMP-1 (A), MMP-3 (B), and TIMP-1 (C) in the supernatants were measured using ELISA. The results are shown as the mean and SD of one representative experiment performed in triplicate. The error bars show the SD of the values. \* = *P* < 0.05, \*\* = *P* < 0.01 significantly different from the TNF- $\alpha$ -stimulated HPDLCs without genipin.

## 3. Results

### 3.1. The effect of genipin on MMP-1 and MMP-3 productions from TNF- $\alpha$ -stimulated HPDLCs

We first investigated whether genipin was able to modulate MMP-1 and MMP-3 productions in TNF- $\alpha$ -stimulated HPDLCs as shown in Fig. 1, genipin pretreatment significantly inhibited both MMP-1 and MMP-3 productions from TNF- $\alpha$ -stimulated HPDLCs in a dose-dependent manner. On the other hand, genipin treatment did not change TIMP-1 production from HPDLCs.



**Fig. 2.** Effects of genipin on the TNF- $\alpha$ -induced p38 MAPK, ERK, and JNK phosphorylations in HPDLCs. The cultured cells were pretreated with genipin (12.5 or 25  $\mu$ g/ml) for 60 min and then stimulated with 10 ng/ml TNF- $\alpha$  for 15, 30, or 60 min. The cell extracts were subjected to SDS-PAGE followed by Western blotting analysis with antibodies against phospho-specific p38 MAPK, p38 MAPK, phospho-specific ERK, ERK, phospho-specific JNK, JNK, or GAPDH. Each photograph is representative of the results in 3 separate experiments.

### 3.2. Effects of genipin on MAPKs activations in TNF- $\alpha$ -stimulated HPDLCs

It has been reported that TNF- $\alpha$  could activate MAPKs pathways in HPDLCs [16]. Therefore, we examined the effect of genipin on MAPKs phosphorylations in TNF- $\alpha$ -stimulated HPDLCs. Fig. 2 shows that ERK and JNK phosphorylations in TNF- $\alpha$ -stimulated HPDLCs were inhibited by genipin treatment. On the other hand, genipin treatment did not change p38 MAPK activation.

### 3.3. Effects of MAPKs inhibitors on MMP-1 and MMP-3 release from TNF- $\alpha$ -stimulated HPDLCs

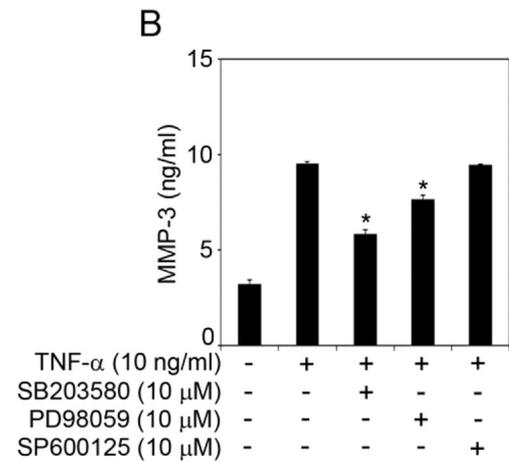
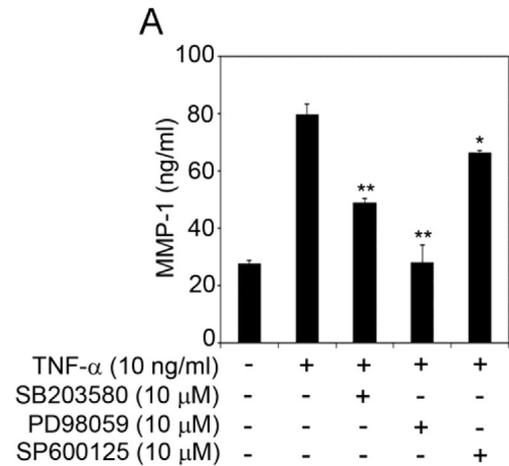
Next, we examined the roles of MAPKs on MMPs releases in TNF- $\alpha$ -stimulated HPDLCs because we revealed that genipin could modulate MAPKs activations in Fig. 2. We used inhibitors of MAPKs to examine whether MAPKs pathways are related to MMP-1 and MMP-3 release from HPDLCs. Fig. 3 shows that MMP-1 production from TNF- $\alpha$ -stimulated HPDLCs was inhibited by SB203580 (p38 MAPK inhibitor), PD98059 (ERK inhibitor) and SP600125 (JNK inhibitor) though the inhibitory effect of SP600125 (JNK inhibitor) was weak. MMP-3 release was decreased by p38 MAPK and ERK inhibitors. Judging from the data, the ERK inhibition by genipin treatment is involved in reduction of MMP-1 and MMP-3 release from TNF- $\alpha$ -stimulated HPDLCs.

### 3.4. Effects of genipin on AMPK activation in TNF- $\alpha$ -stimulated HPDLCs

Next, we examined the effects of genipin on AMPK phosphorylation in TNF- $\alpha$ -stimulated HPDLCs because some researchers reported that AMPK pathway is related to MMPs production [18,19]. Fig. 4 shows that TNF- $\alpha$  stimulation induced AMPK phosphorylation in HPDLCs, and genipin treatment apparently inhibited the phosphorylation of AMPK in TNF- $\alpha$ -stimulated HPDLCs.

### 3.5. Effects of the AMPK inhibitor on MMP-1 and MMP-3 productions in TNF- $\alpha$ -stimulated HPDLCs

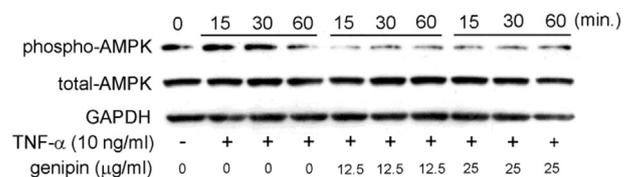
Next, we examined the role of AMPK pathway on MMP-1 and MMP-3 release from TNF- $\alpha$ -stimulated HPDLCs using compound C (AMPK inhibitor) though previous reports showed that AMPK is related to MMPs productions in some types of cells [18,19]. Fig. 5 showed that both MMP-1 and MMP-3 releases were inhibited by the compound C (AMPK inhibitor) treatment in a dose-dependent fashion.



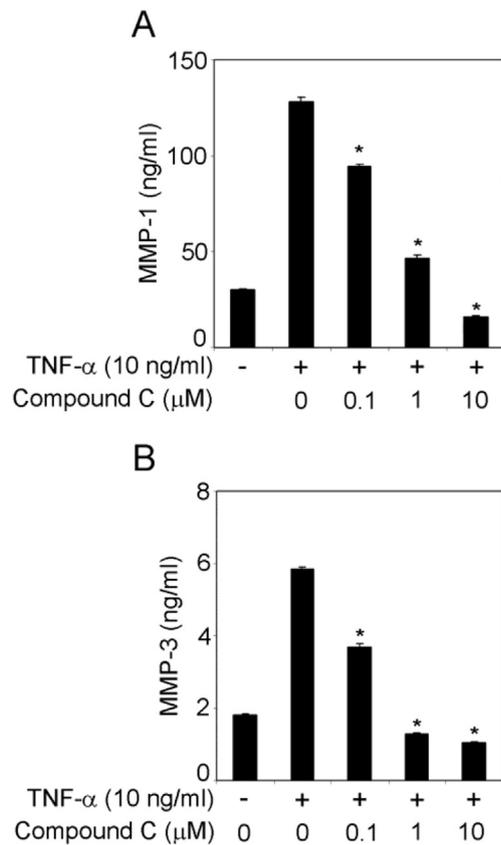
**Fig. 3.** Effects of signal transduction inhibitors on TNF- $\alpha$ -stimulated MMP-1 and MMP-3 releases from HPDLCs. The cells were pre-incubated with SB203580 (10  $\mu$ M), PD98059 (10  $\mu$ M), or SP600125 (10  $\mu$ M) for 1 h and then incubated with TNF- $\alpha$  (10 ng/ml). After 24 h incubation, the supernatants were collected, and MMP-1 (A) and MMP-3 (B) productions were measured by ELISA. The results are shown as the mean and SD of one representative experiment performed in triplicate. The error bars show the SD of the values. \*\* =  $P < 0.01$  or \* =  $P < 0.05$  significantly different from the TNF- $\alpha$ -stimulated HPDLCs without inhibitors.

### 3.6. Effects of the ERK inhibitor on AMPK activation in TNF- $\alpha$ -stimulated HPDLCs

Finally, we examined the effect on the ERK inhibitor on AMPK phosphorylation in TNF- $\alpha$ -stimulated HPDLCs because genipin could inhibit both ERK and AMPK activations and both signal transduction pathways are related to MMP-1 and MMP-3 productions in TNF- $\alpha$ -stimulated HPDLCs. Fig. 6 shows that the ERK



**Fig. 4.** Effects of genipin on the TNF- $\alpha$ -induced AMPK phosphorylation in HPDLCs. The cultured cells were pretreated with genipin (12.5 or 25  $\mu$ g/ml) for 60 min and then stimulated with 10 ng/ml TNF- $\alpha$  for 15, 30, or 60 min. The cell extracts were subjected to SDS-PAGE followed by Western blotting analysis with antibodies against phospho-specific AMPK, AMPK, or GAPDH. Each photograph is representative of the results in 3 separate experiments.

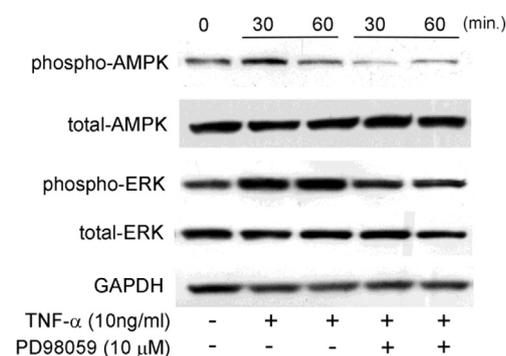


**Fig. 5.** Effects of the AMPK inhibitor on TNF- $\alpha$ -stimulated MMP-1 and MMP-3 release from HPDLCs. The cells were pre-incubated with Compound C (0.1, 1, or 10  $\mu$ M) for 1 h and then incubated with TNF- $\alpha$  (10 ng/ml). After 24 h incubation, the supernatants were collected, and MMP-1 (A) and MMP-3 (B) productions were measured by ELISA. The results are shown as the mean and SD of one representative experiment performed in triplicate. The error bars show the SD of the values. \* =  $P < 0.01$  significantly different from the TNF- $\alpha$ -stimulated HPDLCs without inhibitors.

inhibitor could decrease the AMPK phosphorylation in TNF- $\alpha$ -stimulated HPDLCs. This data shows that ERK is the upstream of AMPK in TNF- $\alpha$ -stimulated HPDLCs.

#### 4. Discussion

In this study, we demonstrated, for the first time to our knowledge, that genipin could suppress MMP-1 and MMP-3 productions



**Fig. 6.** Effects of the ERK inhibitor on the TNF- $\alpha$ -induced AMPK phosphorylation in HPDLCs. The cultured cells were pretreated with PD98059 (10  $\mu$ M) for 60 min and then stimulated with 10 ng/ml TNF- $\alpha$  for 30, or 60 min. The cell extracts were subjected to SDS-PAGE followed by Western blotting analysis with antibodies against phospho-specific AMPK, AMPK, phospho-specific ERK, ERK, or GAPDH. Each photograph is representative of the results in 3 separate experiments.

from TNF- $\alpha$ -stimulated HPDLCs. There is a report that demonstrated that genipin could modulate the MMP expression. Wang and the colleagues reported that genipin could inhibit MMP-2 activity in human hepatocellular carcinoma [19]. Their report and ours show that genipin could inhibit tissue destruction to inhibiting some kinds of MMPs in inflammatory tissues or cancer. They also reported that genipin could enhance TIMP-1 expression in human hepatocellular carcinoma [19]. We showed genipin did not change TIMP-1 expression in HPDLCs stimulated with TNF- $\alpha$ . We think that the effect of genipin on TIMP-1 expression is dependent on the type of cells.

Previous reports and ours show that effects of genipin on MAPKs activations are dependent on the type of cells and stimulants. We revealed that genipin could suppress ERK and JNK activations in TNF- $\alpha$ -stimulated HPDLCs in this report. We previously reported that genipin could inhibit the activation of ERK pathway though genipin did not modulate JNK phosphorylation in IL-1 $\beta$ -stimulated HPDLCs [15]. Jiang and the colleagues reported that genipin prevented ERK phosphorylation while left p38 MAPK and JNK unchanged in TNF- $\alpha$ -stimulated rat vascular smooth muscle cell [20]. Kitano and the colleagues reported that genipin could inhibit p38 MAPK phosphorylation though the phosphorylation of ERK was enhanced in porcine lens epithelial cells [21]. Further investigation should be necessary to clarify the effect of genipin on MAPKs activation in some kinds of cells.

We think that the roles of AMPK signaling cascade on MMPs expression are dependent on the kind of MMPs or the type of cells. We showed the activation of AMPK is important for MMP-1 and MMP-3 expression in TNF- $\alpha$ -stimulated HPDLCs in this report. Tong and the colleagues reported that the AMPK inhibitor suppressed MMP-3 production in adiponectin-stimulated human chondrocytes [17]. Dadson and the colleagues reported that MT1-MMP and MMP-2 activations in adiponectin-treated cardiac fibroblasts were inhibited by the AMPK inhibitor [18]. Their reports and ours showed that AMPK pathway positively regulated MMPs activation and production. However, Morizane and the colleagues reported that AMPK activation could suppress MMP-9 expression in mouse embryonic fibroblasts [22].

In this report, we demonstrated that the activation of ERK positively regulate AMPK phosphorylation in TNF- $\alpha$ -stimulated HPDLCs. We showed that the ERK inhibitor suppressed AMPK phosphorylation in TNF- $\alpha$ -stimulated HPDLCs. Cho and the colleagues previously reported that the ERK inhibitor down-regulated AMPK phosphorylation in cinnamaldehyde derivative-treated human SW620 colon cancer cells [23]. Their report supports our result in this report. We think genipin could directly modulate the activation of ERK or more upstream kinase activation. Further investigation should be necessary to clarify this question.

In summary, the current study demonstrates that genipin could suppress TNF- $\alpha$ -induced MMP-1 and MMP-3 productions in HPDLCs. In addition, we revealed that genipin inhibited TNF- $\alpha$ -induced ERK/AMPK pathway activation in HPDLCs. We think that the direct effects of genipin could inhibit MMP-1 and MMP-3 release from TNF- $\alpha$ -stimulated HPDLCs in order to inhibit the phosphorylation of signal transduction molecules. These data provide a new mechanism through which genipin could be used for the treatment of periodontal disease.

#### Conflict of interest

The authors confirm that there are no conflicts of interest.

#### Acknowledgments

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