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# The effects of *p*-hydroxycinnamaldehyde from *Alpinia galanga* extracts on human chondrocytes

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ABSTRACT

Osteoarthritis (OA) is the most common form of arthritis and affects millions of people worldwide. Patients have traditionally been treated with non-steroidal anti-inflammatory drugs (NSAIDs), but these are associated with significant side effects. Purification of the acetone extract of *Alpinia galanga* afforded *p*-hydroxycinnamaldehyde, as identified by nuclear magnetic resonance and mass spectrometry analyses. By exploiting the cartilage explant culture, *p*-hydroxycinnamaldehyde suppressed loss of uronic acid, resulting in release of hyaluronan (HA), sulfated glycosaminoglycans (s-GAGs) and matrix metalloproteinases (MMPs). *p*-Hydroxycinnamaldehyde and interleukin-1β (IL-1β), when incubated in primary human chondrocytes, also reduced release of HA, s-GAG and MMP-2. The results demonstrated: (a) that expression levels of the catabolic genes MMP-3 and MMP-13 were suppressed and (b) mRNA expression levels of anabolic genes of collagen II, SOX9 and aggrecan were increased. This study shows that *p*-hydroxycinnamaldehyde from *A. galanga* Linn. is a potential therapeutic agent for treatment of OA.



## 1. Introduction

Osteoarthritis (OA) is a degenerative process of the joints and is characterized by the progressive destruction and erosion of cartilage associated with an osteophytic response. In cartilage, this mechanism results from a homeostatic imbalance between matrix synthesis and matrix degradation. Cartilage tissue is believed to have a critical role in joint function as a shock absorber during loading and motion (van der Kraan and van den Berg, 2000). Chondrocytes in the cartilage constantly remodel the extracellular matrix (ECM) of the tissue throughout its life. The major load-bearing constituents of the ECM include type II collagen and an aggregation of a proteoglycan called aggrecan, which provides tensile and compressive stiffness to the tissue, respectively. The high ratio of degradation and biosynthesis of these ECM molecules are linked to pathogenesis and progression of OA, whereas the chondrocytes maintain a constant level of aggrecan tissue content. Anabolic growth factors such as insulin-like growth factor-1 and transforming growth factor-β promote synthesis and assembly of aggrecan (Luyten et al., 1988; Morales and Roberts, 1988); by contrast, catabolic agents such as interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), oncostatin M, fibronectin fragments and retinoic acid provoke the chondrocyte-mediated catabolic response when aggrecan is depleted (Sandy et al., 1991; Plass and Sandy, 1993; Malfait et al., 1994; Hui et al., 1996; Homandberg et al., 1996). Many studies have shown that IL-1β is important to induce and sustain the cartilage owing to its ability to both suppress the synthesis of specific ECM molecules and promote the imbalance between excessive cartilage (Badger et al., 1999; Shikhman et al., 2001). Matrix metalloproteinases (MMPs) are thought to be involved in the destruction of articular cartilage (Vincenti et al., 1994), so the highly expression of MMP-1/collagenase 1, MMP-3/stromelysin 1, MMP-9/gelatinase B and MMP-13/collagenase-3 would be expected to be detected in the pathologic synovium, synovial fluid and also cartilage samples. Thus, many forms of evidence have confirmed that MMPs have an important role in the pathogenesis of articular cartilage degradation (Mitchell et al., 1996; Murphy et al., 1987; Okada et al., 1992; Hembry et al., 1995 and Reboul et al., 1996).

In 2003, Matsuda et al., reported that Thai medicinal plant, *Alpinia galanga* Linn. (B.L. Burtt), has been categorized as an antimicrobial agent, and could also serve as carminative, stomachic, anti-rheumatic, anti-flatulent and anti-itching agents. Its anti-rheumatic action resulted in suppression of synthesis of prostaglandin through inhibition of cyclooxygenase-1 and cyclooxygenase-2 (Grzanna et al., 2005). In addition, the aqueous acetone extract from rhizomes of this plant has possible inhibitory effects on

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lipopolysaccharide (LPS)-induced nitric-oxide production (Morikawa et al., 2005). In 2001, highly pure extracts of *Zingiber officinale* and *A. galanga* were shown to significantly reduce symptoms of OA of the knee (Altman and Marcussen, 2001). Moreover, extraction of *A. galanga* (AG) and *Z. officinale* (ZO) could synergistically inhibit chemokine expression (TNF-α, MCP-1 and IP-10) (Phan et al., 2005). In 2006, Pothacharoen et al., reported that *A. galanga* extract inhibited degradation of cartilage matrix stimulated by IL-1β in the chondrosarcoma and synovial fibroblast; and it also increased levels of anabolic gene expression but decreased catabolic gene expression levels in both cell types.

This paper reports the chondroprotective effects of *A. galanga* extract, as demonstrated by using porcine cartilage explants as a screening model, a chondrocyte culture to identify the active chemical compound and its mode of action, and nuclear magnetic resonance (NMR) and mass spectrometry (MS) analyses to determine its chemical structure.

## 2. Results and discussion

### 2.1. Chondroprotective effects of A. galanga extracts in porcine cartilage explant

To investigate the inhibitory effects of *A. galanga* extracts (hexane, acetone, ethylacetate and methanol) on the degradation of the extracellular matrix in cartilage, porcine cartilage explants were co-treated with the pro-inflammatory cytokine, IL-1β (25 ng/ml), and various concentrations of the extracts (6.25, 12.5 and 25.0 μg/ml). After treatment for 3 days, the conditioned media were collected and analyzed. We used dye-binding assays and ELISA and gelatin zymography analyses to determine levels of s-GAGs released, HA released and MMP-2 activity, respectively. We then measured the uronic acid contents of the cartilage that were digested with papain. In the cartilage explant culture model, the results showed that IL-1β alone increased s-GAGs and HA release from the cartilage to media and, with co-culture of IL-1β and the individual of hexane, acetone, ethylacetate and methanol extracts, each extract significantly reduced the levels of s-GAGs release. By contrast, the release of HA from the explants treated with IL-1β was significantly reduced by the acetone and hexane fractions (Fig. 1). The hexane and acetone fractions inhibited reduction of uronic acid in cartilage tissue that was induced by IL-1β (Fig. 1), whereas the ethyl acetate and methanol extracts did not.

The activity of MMP-2 in the conditional media was analyzed using gelatin zymography. We found a high level of pro-MMP-2 production in cartilage treated with IL-1β. Furthermore, MMP-2 activity levels decreased under conditions of co-treatment between IL-1β and all four *A. galanga* extracts when compared with IL-1β treatment alone (Fig. 2). However, DMSO used to dissolve the samples did not affect s-GAGs release (data not shown).

We found that the acetone extracts had the strongest activity of the four fractions. It could inhibit up to 90% of IL-1β-induced s-GAGs release (at concentration 25.0 μg/ml) and up to 80% of HA release (at concentration 12.5 μg/ml) (Fig. 1).

### 2.2. The isolation of active compound in acetone extract of A. galanga

From Section 2.1, the acetone fraction had the highest chondroprotective activity. Column chromatography of this fraction results in many acetone sub-fractions being identified and screened using the method outlined in Section 2.1. MS and NMR spectroscopic analyses were used to identify *p*-hydroxycinnamaldehyde **1** or 3-(4-hydroxy-phenyl)-propenal (Fig. 3).

### 2.3. The effect of p-hydroxycinnamaldehyde *1* on porcine cartilage explant

By employing porcine cartilage explant to the isolated *p*-hydroxycinnamaldehyde **1**, it inhibited the effects of IL-1β on s-GAG, HA release, MMP-2 activity level, and the remaining of

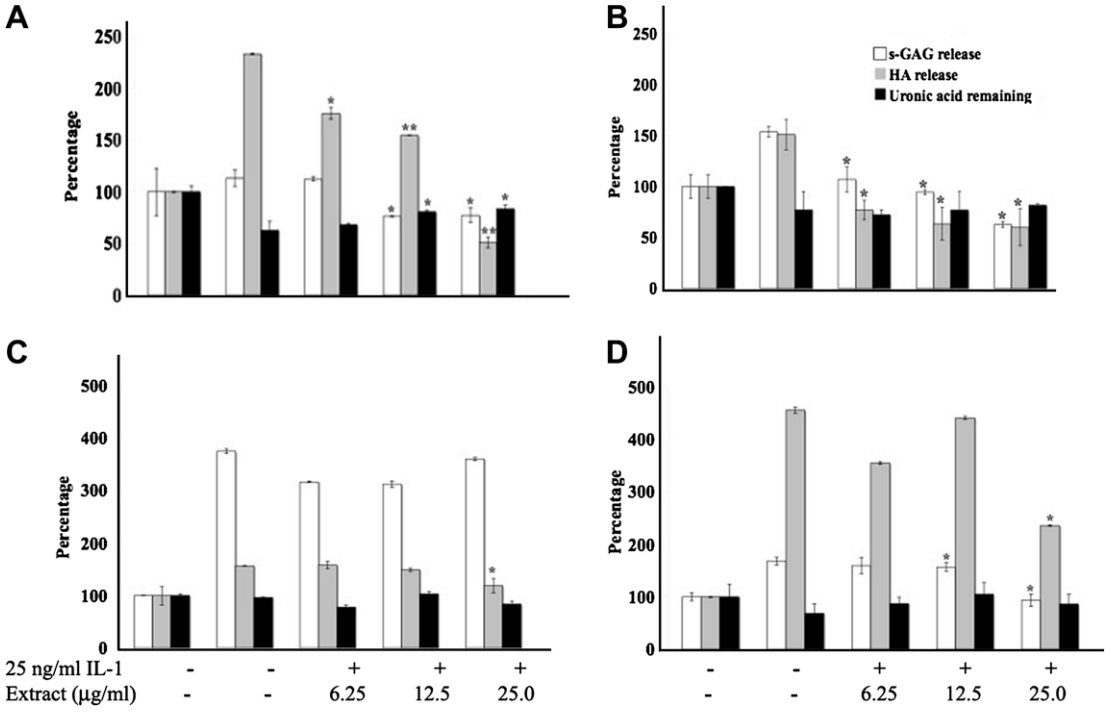


**Fig. 1.** *p*-Hydroxycinnamaldehyde **1** affects: release of s-GAG, HA from porcine cartilage tissues to the media and uronic acid remaining in the cartilage tissue. Porcine cartilage explants were cultured with IL-1β (25 ng/ml) in absence and presence of *A. galanga* hexane (A), acetone (B), EtOAc (C) and MeOH (D) extracts (at concentrations of 6.25–25.0 μg/ml) for 3 days. In the media, the s-GAG release was measured using a dye-binding assay, and HA release was measured by ELISA. Cartilage discs were digested with papain and then the uronic acid content was measured. * and ** denote a value that is significantly different ($p < 0.05$ and $p < 0.01$, respectively) from the IL-1 β control.

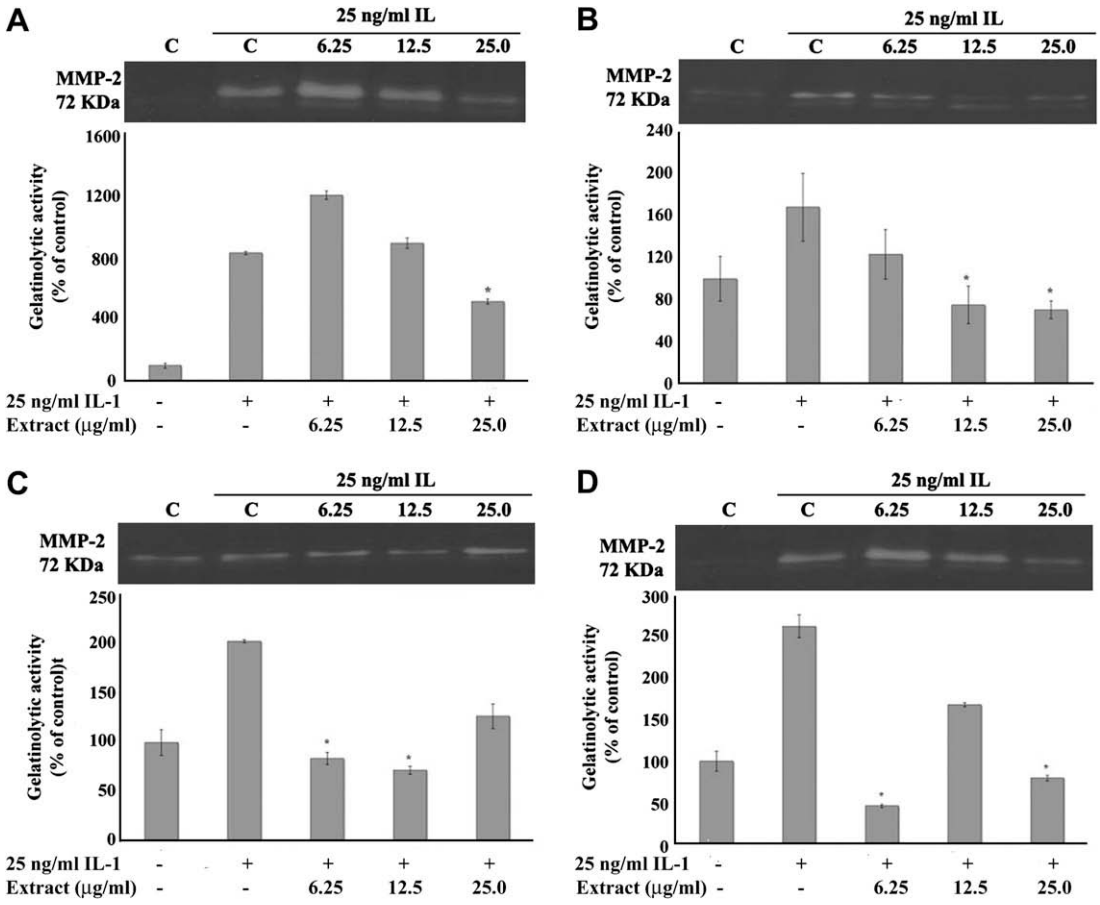


**Fig. 2.** Effects of *A. galanga* extracts on production of MMP-2. Porcine cartilage explants were cultured with IL-1β (25 ng/ml) in the absence and presence of *A. galanga* hexane (A), acetone (B), EtOAc (C) and MeOH (D) extracts (at concentrations of 6.25–25.0 μg/ml) for 3 days. Media were collected and were then analyzed by gelatin zymography as described in the text. Three experiments were highly reproducible. ∗ and ∗∗ denote a value that is significantly different ($p < 0.05$ and $p < 0.01$, respectively) from the IL-1β control.

uronic acid in cartilage tissues (see Fig. 4). We also found that *p*-hydroxycinnamaldehyde **1** maintained degradation of extracellular matrix biomolecules of cartilage tissues in the experiments which were not stimulated by IL-1β.

### 2.4. The effect of p-hydroxycinnamaldehyde **1** on human chondrocyte

We next determined the effect of *p*-hydroxycinnamaldehyde **1** using a primary human articular chondrocyte (HAC) monolayer culture to induce inflammation with IL-1β 10 ng/ml. As shown in Fig. 5 (A and B), IL-1β induced release of s-GAG and HA into the media. This induction was significantly suppressed by *p*-hydroxycinnamaldehyde **1**. As shown in Fig. 5C, MMP-2 activity increased in the presence of IL-1β, demonstrating that *p*-hydroxycinnamaldehyde **1** can reduce this effect.

### 2.5. The effect of p-hydroxycinnamaldehyde **1** on gene expression in human chondrocyte

Cartilage matrix maintains a balance between biosynthesis and degradation of ECM molecules, but IL-1β can disrupt this balance (Badger et al., 1999; Shikhman et al., 2001). Effects of *p*-hydroxycinnamaldehyde **1** in the primary HAC were investigated using RT-PCR. In response to 10 ng/ml of IL-1β, chondrocytes increased the expression levels of the catabolic genes MMP-1, MMP-3 and MMP-13 (Fig. 6). Furthermore, it was shown that *p*-hydroxycinnamaldehyde **1** could inhibit increases in MMP-3 and MM-13 gene expression; by contrast, it did not affect levels of MMP-1 gene expression.

IL-1β was shown to reduce expression levels of the anabolic genes collagen, SOX9 (the key transcription factor of collagen) and aggrecan core protein (the core protein of proteoglycan that is mainly found in cartilage), as shown in Fig. 7. *p*-Hydroxycinnamaldehyde **1** could also reverse inhibition of IL-1β on anabolic gene expression. Using IL-1β, it was then shown that the increased degradation and reduced synthesis resulting in *p*-hydroxycinnamaldehyde **1** had the ability to reverse these effects. The results established that for gene expression in the chondrocyte treated with IL-1β, *p*-hydroxycinnamaldehyde **1** had the ability to reduce catabolic gene expression and to induce anabolic gene expression; alternatively, it could inhibit the inflammatory effects of IL-1β.

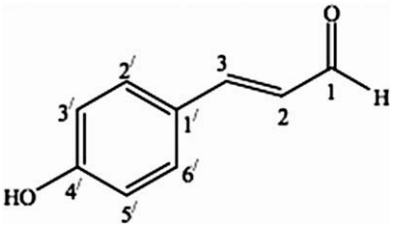


**Fig. 3.** *p*-Hydroxycinnamaldehyde **1**, 3-(4-hydroxy-phenyl)-propenal, the active compound of the acetone fraction of *A. galanga*.

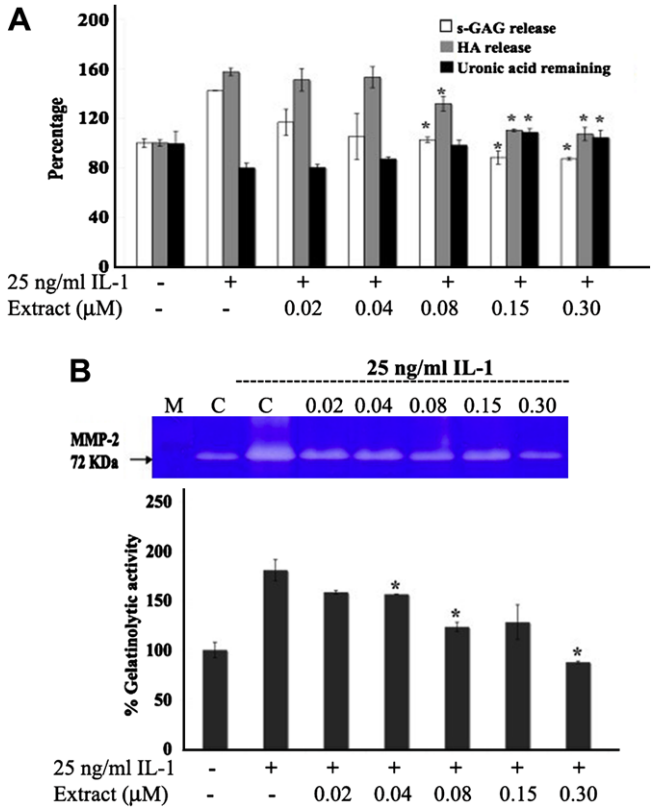


**Fig. 4.** *p*-Hydroxycinnamaldehyde **1** affects: release of s-GAG, HA from porcine cartilage tissues to the media; the uronic acid remaining in cartilage tissue (A), and production of MMP-2 (B). The porcine cartilage explants were cultured with IL-1β (25 ng/ml) in the absence and presence of *p*-hydroxycinnamaldehyde **1** for 3 days. In the media, s-GAG release was measured using a dye-binding assay, and HA was measured by ELISA. Cartilage discs were digested with papain, and then its uronic acid contents were measured. MMP-2 activities were at last analyzed in media by gelatin zymography. ∗ and ∗∗ denote a value that is significantly different ($p < 0.05$ and $p < 0.01$, respectively) from the IL-1β control.

## 3. Conclusion

In this study, we found that all four *A. galanga* extracts had chondroprotective effects, with the acetone extract having the greatest activity. The active compound *p*-hydroxycinnamaldehyde **1**, purified from the acetone fraction, inhibited the effects of IL-1β by reducing release of ECM molecules such as HA, s-GAGs and MMP-2. In addition, we demonstrated that *p*-hydroxycinnamaldehyde **1** also reversed effects of IL-1β on both catabolic and anabolic gene expression by reducing expression of the catabolic genes, MMP-3 and MMP-13, and inducing expression of anabolic genes, collagen, SOX9 and aggrecan core protein. This research established that *p*-hydroxycinnamaldehyde **1** is a good candidate for further *in vivo* studies of anti-arthritic and anti-inflammatory treatments.

## 4. Experimental

### 4.1. General experimental procedures

Siliga gel (Merck No. 7734, Mesh 70–230 ASTM) was purchased from Merck. Thin layer chromatography (TLC, siliga gel 60 $PF_{254}$, 20 × 20 cm, 0.2 mm) was used to examine separated fractions. The compounds were visualized by ultraviolet light (either at $\lambda_{max}$ 254 or $\lambda_{max}$ 366 nm). Collagenase, shark cartilage chondroitin sulfate C and gelatin were purchased from Sigma–Aldrich®, USA. The aurum total RNA purification kit was purchased from Bio-Rad Laboratories, Hercules, Ca, USA, and Revert Aid™ First Strand cDNA synthesis kit was purchased from MBI Fermentas, Germany.

### 4.2. Plant materials

Air-dried powder (5 kg) of *A. galanga* rhizomes, specimen BKF No. 102287, was obtained from the National Park, Wildlife and Plant Conservation Department, Ministry of Natural Resources and Environment, Bangkok, Thailand. Then was percolated with

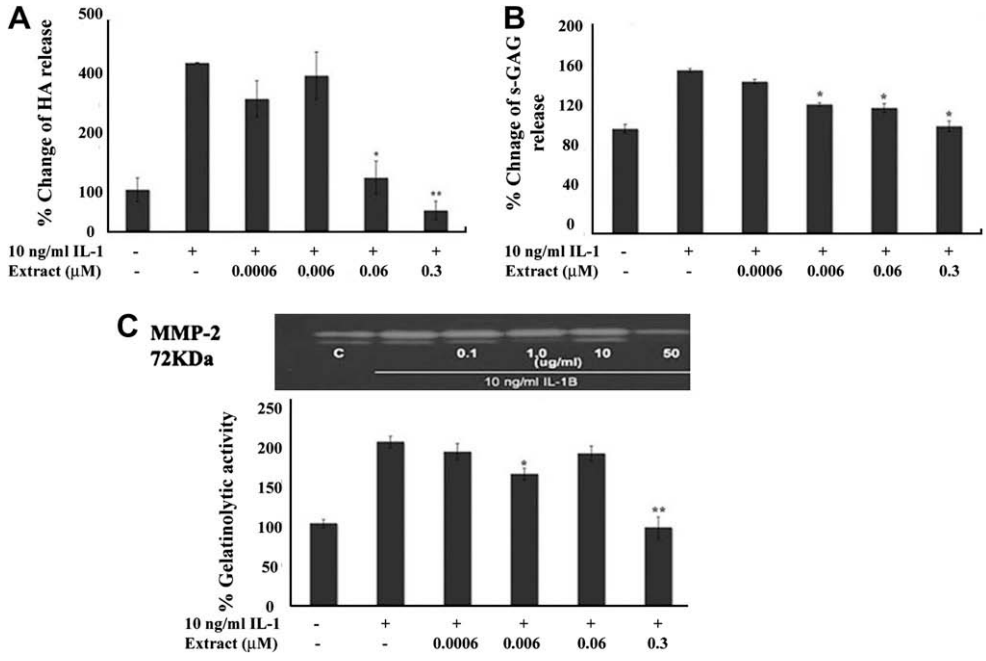


**Fig. 5.** Effects of *p*-hydroxycinnamaldehyde **1** on release of HA (A), s-GAG (B) and MMP-2 (C) from chondrocytes. Chondrocytes were co-treated with 10 ng/ml IL-1β and various concentrations of *p*-hydroxycinnamaldehyde **1** (0.1–50 μg/ml) for 24 h. The conditioned media were analyzed for HA, s-GAG and MMP-2 activity as described in the Section 4. ∗ and ∗∗ denote a value that is significantly different ($p < 0.05$ and $p < 0.01$, respectively) from the IL-1β control.

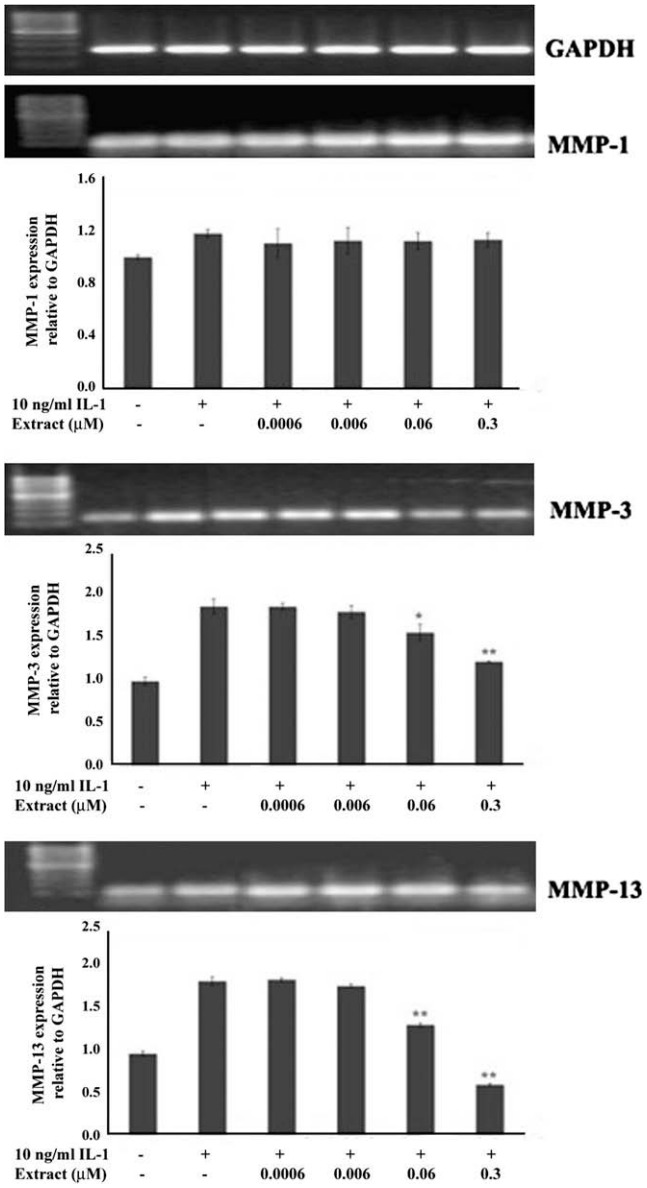


**Fig. 6.** Effect of *p*-hydroxycinnamaldehyde **1** on mRNA expression of proteinases (MMP-1, -3, -13). Confluent human chondrocytes in 25-cm$^3$ flasks were cultured with IL-1β (10 ng/ml) in the presence and absence of *p*-hydroxycinnamaldehyde **1** for 24 h. Cells were harvested and gene expressions were analyzed. MMP, matrix metalloproteinase. ∗ and ∗∗ denote a value that is significantly different ($p < 0.05$ and $p < 0.01$, respectively) from the IL-1β control.

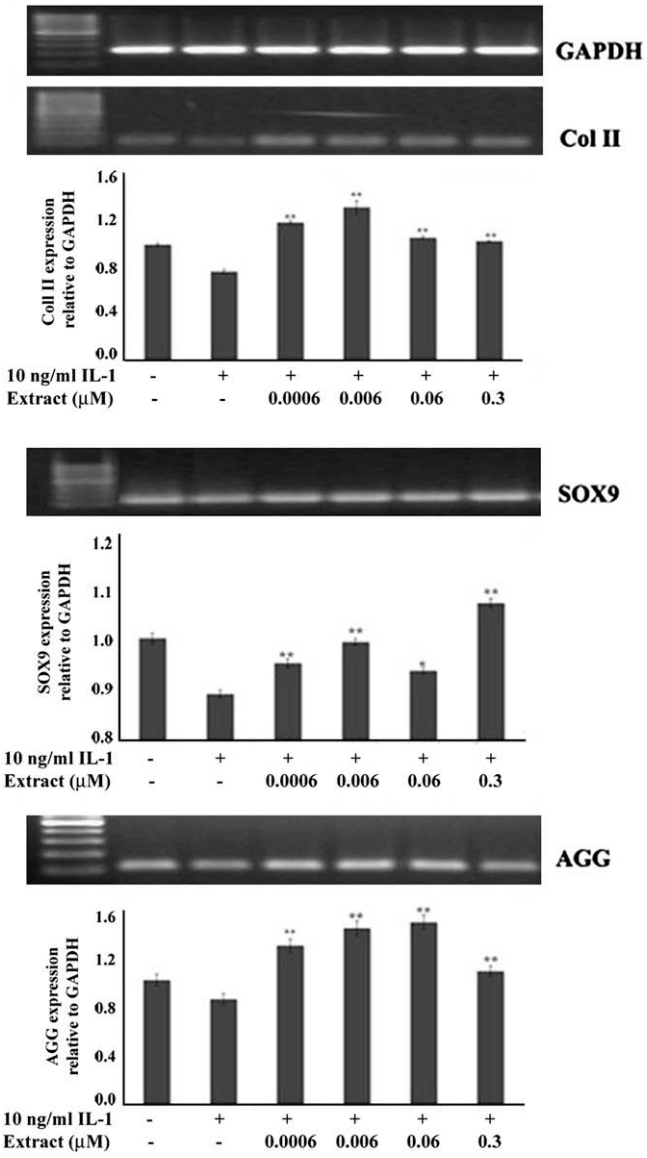


**Fig. 7.** Effects of *p*-hydroxycinnamaldehyde **1** on mRNA expression of cartilage genes (AGG, COL2 and SOX9). Confluent human chondrocytes in 25-cm$^3$ flasks were cultured with IL-1β (10 ng/ml) in the presence and absence of *p*-hydroxycinnamaldehyde **1** for 24 h. Cells were harvested and gene expressions were analyzed. AGG, aggrecan; COL2, collagen type II; SOX9, SRY-type HMG box. ∗ and ∗∗ denote a value that is significantly different ($p < 0.05$ and $p < 0.01$, respectively) from the IL-1β control.

hexane (17 l), with the process being conduced at room temperature with five repetitions taking up to 7 days. Then, the residue was subsequently percolated with EtOAc (19 l), acetone (17 l) and MeOH (20 l) under the same conditions, respectively. Finally, filtration and evaporation techniques were applied to produce crude hexane (72.84 g), EtOAc (89.35 g), acetone (52.92 g) and MeOH (115.38 g).

### 4.3. Isolation of active compound, p-hydroxycinnamaldehyde *1*

The active acetone extract (52.93 g) was subjected to silica gel CC (1.8 kg), eluted with a gradient of n-hexane and EtOAc. Fractions obtained from the CC were analyzed using thin-layer chromatography (TLC) with selected fractions combined, yielding a pale yellow crystal of *p*-hydroxycinnamaldehyde **1** and MS (HRFAB-MS) *m/z*, IR, UV, $^1$H NMR, $^{13}$C NMR, DEPT, DQF-COSY, HMQC and HMBC spectra, as well as elemental analysis are identical in all aspects to literature data (Kwon et al., 1996).

### 4.4. Preparation and treatment of cartilage explant

Metacarpophalangeal joints were dissected for articular cartilage from 20 to 24 week-old pigs. These were incubated in serum-free Dulbecco's modified Eagle's medium (DMEM) containing 200 units/ml of penicillin and 200 μg/ml of streptomycin with 5% $CO_2$ and at 37 °C. Explants were kept for 24 h, following which a recombinant human IL-1β (25 ng/ml) was used to induce cartilage degradation and then the explants were co-treated with IL-1β using various concentrations of each extract (6.25–25 μg/ml). The media were collected on the third day of treatment and stored at

−20 °C for further analyses. All experiments were performed in triplicate using tissue from one animal donor.

### 4.5. p-Hydroxycinnamaldehyde treatment with human chondrocyte

Primary chondrocytes were isolated from the non-inflammatory human cartilage that was collected ethically after arthroscopic diagnosis of flat-pad syndrome patients at the Maharaj Nakorn Chiang Mai Hospital. Cartilage was digested with trypsin at 4 °C for 12 h, collagenase (Sigma–Aldrich®, type IA) at 37 °C for 3 h. Then, cells were washed with phosphate-buffered saline (PBS) and grown at high density in a monolayer culture comprising 10% v/v fetal calf serum (FCS) DMEM. After the fourth cycle, human chondrocytes were maintained in serum-free DMEM for 24 h, prior to 24 h of co-treatment with 10 ng/ml IL-1β and various concentrations of *p*-hydroxycinnamaldehyde **1**.

### 4.6. Cytotoxicity detection

Cell death and cell lysis were quantified using a colorimetric assay, which measured levels of lactate dehydrogenase (LDH) activity (Decker and Lohnmann-Matthes, 1988); levels of LDH in the samples were compared with the cell culture media positive control (0.5 mM $H_20_2$, for 24 h).

### 4.7. Measurement of sulfated glycosaminoglycan concentration

The concentrations of sulfated glycosaminoglycan in conditional media were measured using dimethylmethylene blue (DMMB) (Farndale et al., 1986) and a standard of shark cartilage chondroitin sulfate C (Sigma–Aldrich®, USA). DMMB solution was added to the diluted sample, with standards and appropriate blank solution was also measured using the absorbance at 525-nm from a micro-plate reader spectrophotometer. Referring to Section 2.3, levels of ECM biomolecules released from cartilage due to induction by IL-1β were calculated by

$$\%\ \text{Change} = [(\text{medium from D3}) - (\text{medium from D0})]/(\text{medium from D0}) \times 100 \quad (1)$$

where D0 and D3 are media collected on the start day and the third day, respectively.

### 4.8. Detection of uronic acid

Concentrations of glucuronic acid (GlcUA) remaining in the explants were measured by a colorimetric assay using *m*-hydroxydiphenyl as a reagent (Blumenkrantz and Asboe-Hansen, 1973). Explants were digested by papain prior measurement. From Eq. (1), we derive:

$$\text{Percentage}\ (\%)\ \text{of remaining UA content} = [(\text{UA of control explant}) - (\text{UA of treatment explant})]/(\text{UA of control explant}) \times 100 \quad (2)$$

### 4.9. Measurement of hyaluronan concentration

HA concentrations were measured using the competitive inhibition-based enzyme-linked immunosorbent assay (ELISA) method reported by Kongtawelert and Ghosh (1990).

### 4.10. Gelatin zymography

Pro-MMP-2 in the conditioned medium was detected by gelatin zymography as previously described (Ito et al., 1995). Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using 10% acrylamide gel containing 0.1 mg/ml of gelatin (Sigma–Aldrich®, USA) at 4 °C under non-reducing conditions. After electrophoresis, SDS in the gel was removed by rinsing with 2.5% Triton-X 100, pH 7.5. The gel was incubated at 37 °C in the buffer (50 mM Tris–HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02% $NaN_3$) for 18 h and then stained with 0.1% Coomassie brilliant blue R250 (Bio-Rad Laboratories, Hercules, CA) in MeOH:-$H_2O$:AcOH (5:4:1, v/v), and destained with MeOH:$H_2O$:AcOH (5:4:1, v/v). Finally, we used a Scion image densitometer to analyze the gelatinolytic activity.

### 4.11. Gene-expression analysis

RNA was extracted from the monolayer cells using an Aurum total RNA purification kit (Bio-Rad Laboratories, Hercules, CA, USA). Using the RevertAid™ First Stand cDNA synthesis kit (MBI Fermentas, Germany), the net sum RNA (500 ng) of each sample was reverse transcribed into complementary DNA (cDNA). Primer and probe sets were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) and nucleotide sequences are shown in Table 1.

Amplified products were separated by electrophoresis on 2% (w/v) agarose gels, stained with ethidium bromide and then imaged using a Bio-Rad Gel-Doc fluorescent image analyzer. To allow semi-quantitative comparisons of mRNA levels, integrated densities were calculated by the Scion Image analysis software and divided by levels of the house-keeping gene GAPDH (glyceraldehydes-3-phosphate dehydrogenase) as previously described (Marchuk et al., 1998; Boykiw et al., 1998).

**Table 1**
Primers used for semi-quantitative RT-PCR.

| Gene | Annealing temperature (°C) | Product size (base pairs) | Sequences (5′–3′) | GenBank accession number |
|---|---|---|---|---|
| Aggrecan | 62 | 110 | Forward: ACTTCCGCTGGTCAGATGGA<br>Reverse: CAACACTGCCAACGTCCAGAT | NM_013227 |
| Collagen II | 65 | 106 | Forward: CAACACTGCCAACGTCCAGAT<br>Reverse: ATGATTAGTAACGATAGGCAAT | NM_000393 |
| SOX9 | 68 | 101 | Forward: ACACACAGCTCACTCGACCTTG<br>Reverse: GGAATTCTGGTTGGTCCTCTCTT | NM_000346 |
| MMP-1 | 68 | 84 | Forward: CTGTTCAGGGACAGAATGTGCT<br>Reverse: TCGATATGCTTCACAGTTCTAGGG | NM_002421 |
| MMP-3 | 65 | 138 | Forward: TTTTGGCCATCTCTTCCTTCA<br>Reverse: TGTGGATGCCTCTTGGGTATC | NM_002422 |
| MMP-13 | 65 | 96 | Forward: TCCTCTTCTTGAGCTGGACTCATT<br>Reverse: CGCTCTGCAAACTGGAGGTC | NM_002427 |
| GAPDH | 60 | 225 | Forward: GAAGGTGAAGGTCGGAGTC<br>Reverse: GAAGATGGTGATGGGATTTC | NM_002046 |

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