Plumbagin prevents osteoarthritis in human chondrocytes through Nrf-2 activation

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Abstract. Osteoarthritis (OA) is an inflammatory disorder dealing with the focal degradation of articular cartilage. Oxidative stress and inflammation are the major events in OA. The present study aimed at identifying the mechanism of the potent antioxidant, plumbagin, in protecting against hydrogen peroxide (H_2O_2)-induced chondrocyte oxidative stress and inflammatory signaling. Oxidative stress was determined by measuring reactive oxygen species, lipid peroxidation, non-enzymic (glutathione; GSH) and enzymic antioxidant activities (GSH, glutathione S-transferase, glutathione peroxidase, superoxide dismutase, catalase). Expression levels of nuclear factor (erythroid-derived 2)-like 2 (Nrf-2), heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO-1), nuclear factor-κB (NF-κB), cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) were determined by western blot analysis. Pro-inflammatory cytokine expression levels were assessed using ELISA. Results from reactive oxygen species generation, lipid peroxidation content and antioxidant enzyme activities demonstrated that plumbagin significantly inhibited oxidative stress status in H_2O_2-induced chondrocytes. In addition, plumbagin modulated transcription factors involved in redox and inflammation regulation, including NF-κB and Nrf-2, by nuclear expression. plumbagin enhanced antioxidant status by increasing the expression levels of Nrf-2 target genes, including HO-1 and NQO-1. An anti-inflammatory effect against chondrocyte-induced inflammation was demonstrated by downregulating COX-2, iNOS and pro-inflammatory cytokine expression levels (tumor necrosis factor-α, interleukin (IL)-6 and IL-8). The present study identified strong evidence for a protective role of plumbagin against H_2O_2-induced oxidative stress and inflammation in chondrocytes by modulating redox signaling transcription factors.

Introduction

Osteoarthritis (OA) is a chronic inflammatory disorder, its incidence increasing with obesity, poor lifestyle and ageing. Hydrogen peroxide (H_2O_2)-induced oxidative stress and loss of inflammatory homeostasis causes cartilage degeneration leading to OA and H_2O_2 is released during acute inflammatory responses (1,2). Oxidative damage and activation of cellular redox signaling mechanisms by H_2O_2 in chondrocytes is associated with the pathogenesis of OA (3). H_2O_2-induced oxidative stress causes proteoglycan degradation with structural and functional changes ultimately leading to apoptosis (2,4). Since oxidative stress serves a critical role in the development of OA, an effective preventative strategy may be stimulated through antioxidants and thereby redress redox imbalance.

Plumbagin (5-hydroxy-2-methyl-1, 4-aphthoquinone; $C_{11}H_8O_3$) occurs in various plant families including *plumbaginaceae, Droseraceae* and *Ebenceae*. It exhibits antioxidant properties and is involved in various biological activities against bacterial and fungal infection, inflammation and various types of cancer (5-9). The effect of plumbagin against osteosarcoma has been previously demonstrated through reactive oxygen species (ROS)-mediated activation of pro-apoptosis (10). Increasing evidence suggests that plumbagin also prevents migration of gastric and breast cancer by suppressing the chemokine receptor type 4 (11). Plumbagin acts as anti-inflammatory compound that suppresses lipopolysaccharide-induced endotoxemia by regulating nuclear factor-κB (NF-κB) and carrageenan-induced rat paw edema by modulating inflammatory mediators (12,13).

The present study evaluated the potential role of plumbagin against H_2O_2-induced oxidative stress and inflammation in primary culture of rat chondrocytes. The study evaluated various oxidative stress parameters, including ROS levels, antioxidant status and redox regulation of nuclear factor (erythroid-derived 2)-like 2 (Nrf-2), NF-κB and inflammatory cytokine levels. Therefore, plumbagin may be able to provide

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protection against osteoarthritis by modulating oxidative stress and inflammatory pathways.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, antibiotics, antimycolytic solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2',7'-dichlorofluorescin diacetate (DCF-DA) were obtained from Gibco BRL (Gaithersburg, MD, USA). Primary antibodies used at 1:1,000 dilutions [NF-κB (cat. no. 3031), cyclooxygenase-2 (COX-2) (cat. no. 12282), Nrf-2 (cat. no. ab31163), inducible NO synthase (iNOS) (cat. no. ab3523), NAD(P)H:quinone oxidoreductase 1 (cat. no. ab2346), heme oxygenase 1 (HO-1) (cat. no. ab13243)] and secondary antibodies used at 1:10,000 dilutions [(Anti-rabbit IgG, horseradish peroxidase-conjugated (HRP)-linked antibody (cat. no. 7074) and anti-mouse IgG, HRP-conjugated antibody (cat. no. 7076)] were used in the present study were purchased from CST Biological Reagents Co., Ltd., Shanghai, China.

Cell culture and cell viability. Primary rat chondrocytes were used in the present study. The isolation and cell culture were carried out as described previously (14). Cell viability was assessed by MTT assay. Cells ($1x10^4$) were seeded and allowed to attach overnight. Subsequently, cells were treated with different concentrations of H_2O_2 (0.2-1.0 μM) for 24 h. The half maximal inhibitory concentration (IC_{50}) was identified to be 0.8 μM. In order to determine the protective effect of plumbagin against H_2O_2-induced oxidative stress, cells were pre-treated with plumbagin and then with 0.8 μM H_2O_2 for 24 h at 37°C. Cells were treated with MTT for 5 h at 37°C and quantified at 570 nm (15).

Oxidative stress markers
Intracellular ROS generation. Cells were pre-treated with plumbagin and then with DCF-DA for 1 h at 37°C. To identify the protective effect of plumbagin against H_2O_2-induced oxidative stress, cells were washed and treated with H_2O_2 for 24 h. Cells were treated with plumbagin and H_2O_2 alone, and control cells without any treatment were maintained. Following complete treatment, cells were suspended in phosphate buffered saline and ROS levels were measured fluorimetrically (16).

Lipid Peroxidation. Following treatment, cells were analyzed for lipid peroxidation content using a Lipid Peroxidation (MDA) assay kit (cat. no. ab118970; Abcam, Cambridge, UK) in accordance with the manufacturer's protocol.

Antioxidant enzyme activities. Antioxidant assay kits were used for determining the specific activities. Superoxide dismutase activity (cat. no. 706002; Cayman Chemical Company, Ann Arbor, MI, USA), catalase (CAT) activity (cat. no. 707002; Cayman Chemical Company), Glutathione-S-Transferase (GST) activity (cat. no. 703302; Cayman Chemical Company), Glutathione Peroxidase (GPx) activity: (cat. no. 703102; Cayman Chemical Compnay) and glutathione (GSH) levels (cat. no. 703002; Cayman chemicals) were assessed.

Pro-inflammatory cytokine expression levels. Following treatment with plumbagin and H_2O_2, cell supernatants were analyzed for tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 levels using ELISA kits (Abcam). The levels of interleukins were expressed as pg/mg protein.

Immunoblot analysis. Cells were lysed using radioimmunoprecipitation assay buffer (cat. no. 89900; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for the isolation of whole cell protein extract. The nuclear extract was isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents, Thermofisher Scientific, (cat. no. 78833) for the determination of the expression levels of NF-κB and Nrf-2. The samples were run on 12% SDS-PAGE gels, and then transferred onto nitrocellulose membranes. The membranes were incubated with 5% non-fat milk for 2 h. Following washing, the blot was incubated with primary (1:1,000) and secondary antibodies (1:10,000). The bands were developed with enhanced chemiluminescence and the images were analyzed using ImageJ software (https://imagej.nih.gov/ij).

Statistical analysis. The results are expressed as mean ± standard deviation. One-way analysis of variance followed by Tukey's multiple comparison tests was performed for determining significance. SPSS software (version, 16.0; SPSS, Inc., Chicago, IL, USA) was used and statistical significance was set as ***$P<0.001$, when compared with the control, and ++$P<0.01$, +++$P<0.001$, when compared to the H_2O_2 group.

Results

Cell viability was increased by plumbagin treatment. In order to test H_2O_2-induced cell death, an MTT viability assay was performed. H_2O_2-induced dose-dependent cell viability was compared with that of the control cells. The IC_{50} value was found to be 0.8 μM. Next, the cells were pre-treated with different concentrations of plumbagin for 24 h and then treated with H_2O_2. Plumbagin dose-dependently improved the cell viability compared with H_2O_2-treated cells. However, treatment with plumbagin alone caused no cell death compared with that of the control cells (data not shown). plumbagin at 15 and 20 μM showed a protective effect and 15 μM was selected for further molecular studies, (Fig. 1A and B).

Plumbagin prevents H_2O_2-induced oxidative stress. H_2O_2 treatment of chondrocytes caused an increase in oxidative markers, including ROS and lipid peroxides, compared with control cells. Pre-treatment with plumbagin reduced these marker levels compared with those of H_2O_2 treated cells. Plumbagin treatment alone did not affect the oxidative stress marker levels (Fig. 2A and B). In addition, antioxidant enzyme activities including GSH, SOD, GST, GPx and CAT activities, were estimated for different groups. It was identified that H_2O_2 treatment reduced the antioxidant defense system by significantly reducing the levels compared with those of control cells. However, treatment with plumbagin increased the antioxidant enzyme activities to significant levels compared with those of H_2O_2-treated cells (Fig. 2C).

Plumbagin modulates NF-κB and Nrf-2 signaling. Next the H_2O_2-induced oxidative stress and inflammation in chondrocytes through NF-κB and Nrf-2 signaling was evaluated. H_2O_2
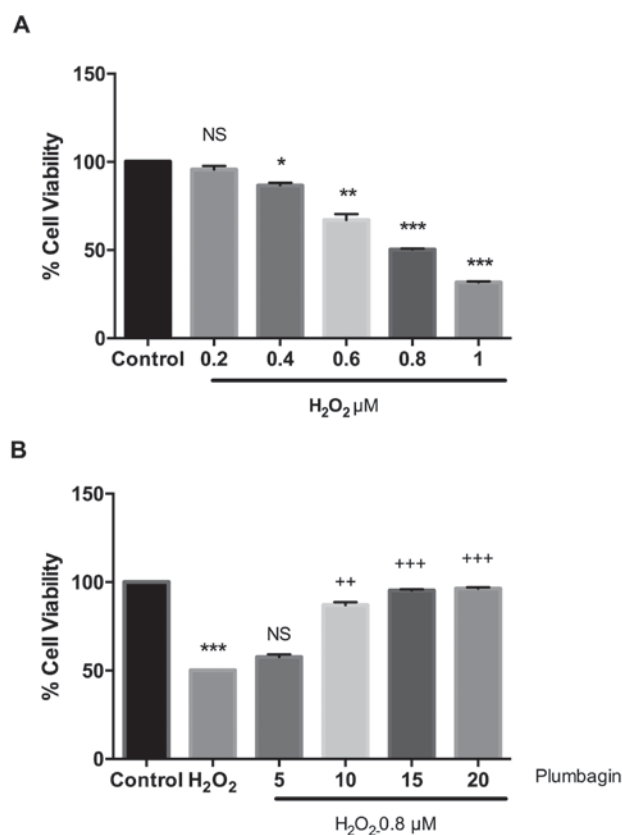
Figure 1. (A) H_2O_2 induces cell death in a dose-dependent manner. (B) Plumbagin prevents H_2O_2-induced cell death. Cell viability was measured using an MTT assay. [*]$P<0.05$, [**]$P<0.01$, [***]$P<0.001$, when compared with the control and [++]$P<0.01$, [+++]$P<0.001$, when compared to H_2O_2 group. The plumbagin and H_2O_2 group is compared with control; plumbagin + H_2O_2 is compared with H_2O_2 group. NS; non-significant vs. the control group (One-way analysis of variance followed by Tukey's multiple comparison). H_2O_2, hydrogen peroxide.
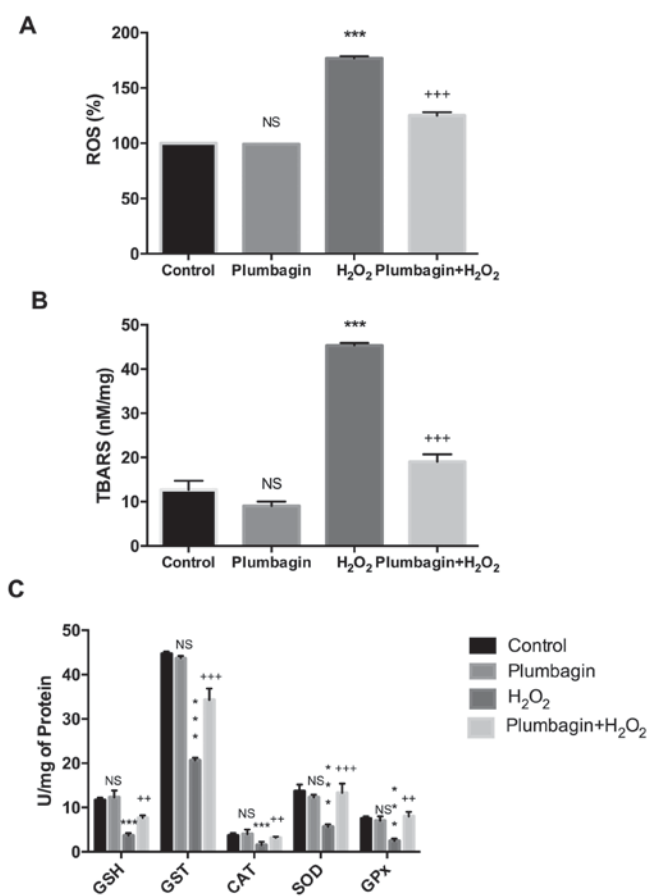

Figure 2. Plumbagin reduces H_2O_2-induced oxidative stress. (A) ROS expressed as the percentage of fluorescence. (B) TBARS formed/mg protein. (C) Anioxidant enzyme activities expressed as units/mg of protein. [***]$P<0.001$, when compared with the control and [+++]$P<0.001$, when compared to H_2O_2 group. Plumbagin and H_2O_2 group vs. control; plumbagin + H_2O_2 vs. H_2O_2 group (One-way analysis of variance followed by Tukey's multiple comparison). H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances.

treatment induced inflammatory signaling through increased NF-κB expression levels and the downstream targets (COX-2 and iNOS). Furthermore, Nrf-2 levels were significantly downregulated in chondrocytes treated with H_2O_2 compared with those of control cells. Nrf-2-induced expression levels of HO-1 and NQO-1 were significantly reduced. Plumbagin treatment followed by H_2O_2 treatment downregulated NF-κB and upregulated Nrf-2 levels (Fig. 3A and B).

Plumbagin reduces inflammatory cytokine expression. Pro-inflammatory cytokine expression, including TNF-α, IL-6 and IL-8, were evaluated by ELISA. H_2O_2 treatment increased the pro-inflammatory cytokine expression compared with that of control cells, while plumbagin pre-treatment significantly downregulated the cytokine expression levels (Fig. 4).

Discussion

The present study demonstrated certain important findings on plumbagin-mediated cytoprotection against OA in chrondrocytes by the regulation of redox signaling and inflammation. H_2O_2 treatment dose-dependently decreased chondrocyte viability, whereas plumbagin pre-treatment improved the cell viability of chondrocytes by up to 97%. Furthermore, H_2O_2 caused severe oxidative stress in chondrocytes by increasing

the ROS and lipid peroxide levels. Previous studies (17,18) have demonstrated the potential role of H_2O_2 in inducing oxidative stress with functional loss of chondrocytes and the development of OA. Plumbagin treatment reduced the oxidative stress markers perhaps due to its antioxidant potential. Plumbagin has been previously reported (19,20) as having strong antioxidant properties and thereby associated anti-mutagenic and anti-cancer effects.

The present study analyzed the detailed molecular mechanism of plumbagin's protection against H_2O_2-induced effects on chondrocytes. It identified that H_2O_2 treatment increased the oxidative stress by downregulating Nrf-2 signaling. Nrf-2 is the nuclear transcription factor activated under oxidative stress and translocates into the nucleus. Nrf-2 induces expression of antioxidant responsive element (ARE)-responsive genes including, NQO-1, GST, HO-1 at the transcriptional level (21). In the current study, H_2O_2-induced redox signaling is mediated through increased ROS levels and a decline in the expression levels of Nrf-2 and downstream proteins. This imbalance may be due to exacerbated oxidative stress, which is not sufficiently ameliorated by the endogenous antioxidant defence system inside the cells. However, treatment with plumbagin improved the antioxidant defence mechanism by inducing nuclear Nrf-2,
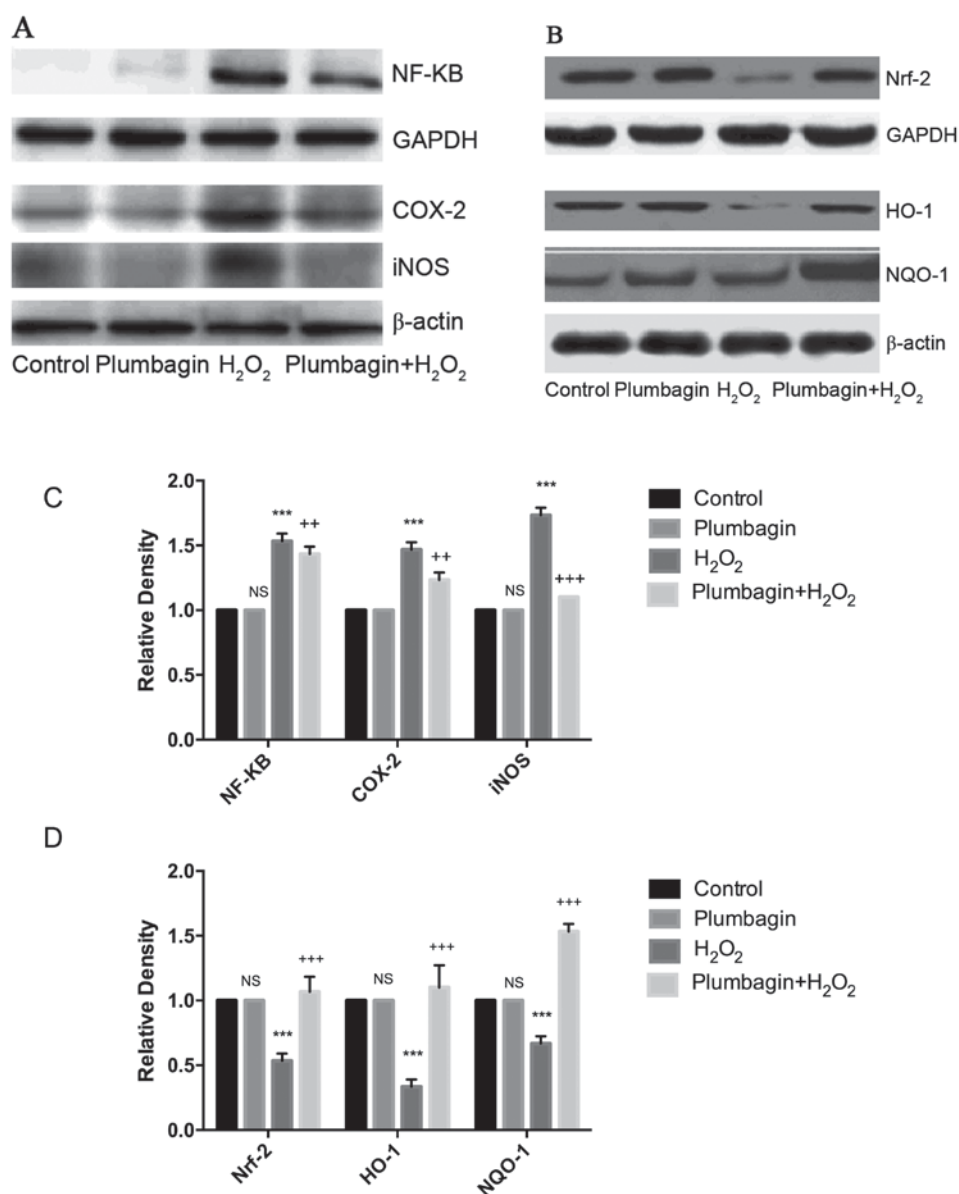
Figure 3. (A) Plumbagin downregulates H_2O_2-induced NF-κB, COX-2 and iNOS expressions in rat chondrocytes. (B) Plumbagin upregulates Nrf-2, HO-1 and NQO1 expressions in rat chondrocytes. (C and D) Densitometric analysis on the effect of plumbagin and H_2O_2 against NF-κB and Nrf-2 signaling proteins. ***P<0.001, when compared with the control. ++P<0.01, +++P<0.001, when compared to H_2O_2 group. Plumbagin and H_2O_2 group vs. control; plumbagin + H_2O_2 vs. H_2O_2 group (One-way analysis of variance followed by Tukey's multiple comparison). NF-κB, nuclear factor-κB; Nrf-2, nuclear factor (erythroid-derived 2)-like 2; H_2O_2, hydrogen peroxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; COX-2, cyclooxygenase-2; iNOS, inducible NO synthase; NS, non significance vs. control group; HO-1, heme oxygenase 1; NQO-1, NAD(P)H:quinone oxidoreductase 1.

HO-1 and NQO-1 expression levels. HO-1 is activated by oxidative stress and inflammatory cytokines, and produces biliverdin and bilirubin, which exert a cytoprotective effect by ameliorating ROS (22-24). Another important antioxidant enzyme is NQO-1 (also called DT-diaphorase). This serves a major role in preventing oxidative stress by reducing quinones to hydroquinones (25). A previous study by Son *et al* (26), reported the protective role of plumbagin by activating Nrf-2/ARE signaling against cerebral ischemia.

NF-κB is a redox sensitive transcription factor that is central to inflammation. NF-κB is held in the cytoplasm by inhibitor of κB (IκB) proteins, which thereby regulates its expression levels. Under oxidative stress, IκB undergoes proteosomal degradation resulting in nuclear localization of

NF-κB, which induces the expression levels of various inflammatory proteins, including COX-2, iNOS and inflammatory cytokines (27). In the present study, it was identified that H_2O_2 induces NF-κB activation and expression and further upregulated its downstream genes COX-2 and iNOS. The results of the present study are consistent with previous reports on H_2O_2-induced NF-κB activation, regulated through phosphorylation of IκB (28,29). H_2O_2 induces COX-2 expression through tyrosine kinase phosphorylation and is involved in wound repair in human umbilical vein endothelial cells (30). The H_2O_2 induced NF-κB levels and increased expression levels of inflammatory cytokines observed in the present study are consistent with the previous study of Lim *et al* (31), where the authors demonstrated the protective role of melatonin
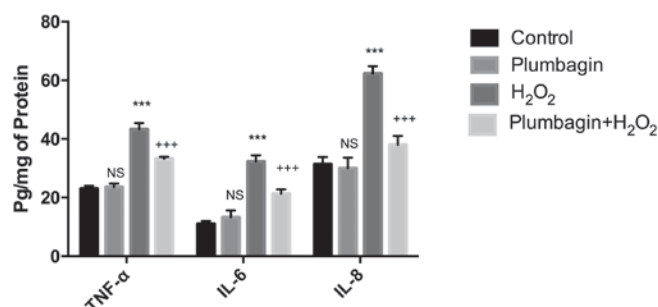
Figure 4. Plumbagin exerts an anti-inflammatory effect. Pro-inflammatory cytokines are expressed as pg/mg protein. ***$P<0.001$, when compared with the control. +++$P<0.001$, when compared to H_2O_2 group. Plumbagin and H_2O_2 group vs. control; plumbagin + H_2O_2 vs. H_2O_2 group. (One way ANOVA followed by Tukey's multiple comparison). H_2O_2, hydrogen peroxide; NS, non significance vs. control group; TFN-α, tumor necrosis factor-α; IL, interleukin.

against H_2O_2-induced inflammatory effects on the CHON-001 human chondrocyte cell line and a rabbit OA model. These H_2O_2-induced inflammatory effects were significantly downregulated by plumbagin preventing NF-κB expression levels and their downstream targets (COX-2, iNOS, TNF-α, IL-18 and IL-6). plumbagin suppressed osteoclastogenesis and osteolytic metastasis by downregulating receptor activator of NF-κB ligand-induced NF-κB activation (32). The immunomodulatory activity of plumbagin has been previously demonstrated by Checker et al (33) by suppressing Concanavalin A-induced NF-κB expression levels and various cytokine (IL-2, IL-4, IL-6 and interferon-γ) levels. Plumbagin also suppresses carrageenan induced edema by downregulating pro-inflammatory cytokines (13). Thus, H_2O_2-induced inflammation may be regulated by plumbagin modulating the NF-κB transcription factor and thereby regulating its downstream targets.

The findings of the present study are that plumbagin is a cytoprotective compound against H_2O_2-induced oxidative stress and inflammatory responses in chondrocyte cells. Additionally, plumbagin prevents oxidative stress by regulating Nrf-2 levels, induces the expression of antioxidant genes and improves the overall cellular antioxidant defense mechanism. In addition, plumbagin modulates NF-κB levels and suppresses inflammation by downregulating COX-2, iNOS and inflammatory cytokine expression levels. As a result, the present study provide evidence that plumbagin has the potential to protect against osteoarthritis by regulating redox signaling and inflammation.

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