Anti-osteoarthritis Effects of the Combination of *Boswellia serrata*, *Curcuma longa*, and *Terminalia chebula* Extracts in Interleukin-1β-stimulated Human Articular Chondrocytes

Hae Lim Kim[1,2], Daeun Min[2], Dong-Ryung Lee[2], Sung-Kwon Lee[2], Bong-Keun Choi[2], Seung Hwan Yang[1]*

1 : Department of Biotechnology, Chonnam National University, 2 : NUON Co., Ltd

In this study, extracts of *Boswellia serrata* gum resin, *Curcuma longa* rhizome, and *Terminalia chebula* fruit were combined in different ratios, and their anti-osteoarthritis effects were compared to determine which combination had the best synergistic effect. *B. serrata*, *C. longa*, and *T. chebula* extracts in a 2:1:2 ratio exhibited higher antioxidative activity in scavenging DPPH radicals than did the individual extracts alone or the other extract combinations. Additionally, the 2:1:2 combination significantly improved the levels of enzymatic antioxidants and antioxidant-related proteins. Moreover, this same combination ratio decreased the protein levels of matrix metalloproteinase (MMP) 3 and MMP13 in interleukin-1β-stimulated human articular chondrocytes (HCHs) and increased those of aggrecan and collagen type II alpha 1 chain (COL2A1). Analysis of the underlying mechanisms revealed that the 2:1:2 combination significantly inhibited the phosphorylation of nuclear factor kappa B (NF-κB) p65, extracellular regulated protein kinase (ERK), and p38 mitogen-activated protein kinase (MAPK). Therefore, the 2:1:2 combination of these three plant extracts has the best potential for use as an effective dietary supplement for improving joint health compared with the individual extracts and their other combination ratios.

keywords : *Boswellia serrata* extract, *Curcuma longa* extract, *Terminalia chebula* fruit extract, Osteoarthritis, Antioxidative activity, Cartilage degradation

Introduction

Osteoarthritis, a degenerative condition that causes physical disability in the elderly, occurs when the articular cartilage covering the bone surface is worn out or damaged.[1)] The symptoms of osteoarthritis include pain, morning stiffness, joint swelling, limited range of motion, decreased physical function, restriction of social activities, and/or compromised work capacity. The disease primarily affects the articular cartilage and subchondral bone of synovial joints and results in joint failure, leading to pain with weight-bearing activities, including walking and standing.[2)]

Articular cartilage, which acts as a support for joints, is a tissue composed mainly of an extracellular matrix (ECM) made up of collagen and aggrecan, and it is interspersed with specialized cells known as articular chondrocytes.[3)] An inflammatory response in joint tissue affects the migration and phenotype of the chondrocytes and promotes ECM degradation, all of which act as a major cause osteoarthritis.[4-6)] It has been reported that overexpressed proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), significantly upregulate the production of catabolic factors, such as matrix metalloproteases (MMPs) that degrade the ECM.[7-9)]

Boswellia serrata gum resin and *Curcuma longa* and the fruits of *Terminalia chebula* Retz contain powerful antioxidants that inhibit the production of reactive oxygen species (ROS).[10-12)] In India, *B. serrata* gum resin and extracts of *C. longa* and *T. chebula* are used as traditional Ayurvedic medicines to alleviate various inflammatory diseases.[13-15)]

In a previous study, we demonstrated that the herbal composition LI73014F2, which is a 2:1:2 mixture of *B. serrata* gum resin extract (BSE, designated as LI13121), *C. longa* extract (CLE, LI01106), and *T. chebula* extract (TCE, LI73000), could inhibit inflammatory factors, MMPs, and apoptosis-related factors in human articular chondrocytes (HCHs).[16)] Additionally, we confirmed that LI73014F2 inhibited

* Corresponding author

Seung Hwan Yang, Department of Biotechnology, Chonnam National University, Yeosu 59626, Korea

·E-mail : ymichigan@jnu.ac.kr ·Tel : +82-61-659-7306

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inflammation and MMPs activity in a rat model of monosodium iodoacetate-induced osteoarthritis.[17] However, we had not conducted studies to compare the antioxidative effects of the individual extracts alone or in various combination ratios in HCHs or their effects on MMPs expression in those cells.

In this study, BSE, CLE, and TCE were combined in various ratios to observe their synergetic effects against oxidative stress and inflammation. First, their antioxidative activities were compared using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. Then, IL-1β -stimulated-HCHs were treated with the individual extracts alone or their various combinations to evaluate their effects on several enzymatic antioxidants and antioxidant-related proteins. Furthermore, the cell expression levels of ECM degradation related MMPs and of the cartilage components collagen type II alpha 1 chain (COL2A1) and aggrecan were confirmed to select the best combination ratio of the three extracts. Finally, the combination with the best synergistic effects was compared with the individual extracts in terms of their influence on the NF-κB and MAPK signaling pathways.

Materials and Methods

1. Reagents

The cell culture reagents and chondrocyte growth media were purchased from PromoCell Bioscience Alive (Heidelberg, Germany). IL-1β was obtained from PeproTech, Inc. (Rocky Hill, NJ, USA). The primary antibodies against MMP3, MMP13, aggrecan, and COL2A1 were purchased from Abcam Inc. (Cambridge, CA, USA). Those against catalase, phospho-ERK, ERK, phospho-p38, p38, phospho-p65, p65, and β-actin were obtained from Cell Signaling Technology (Danvers, MA, USA). The primary antibodies against Nrf2 and SOD1 were obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG secondary antibodies were purchased from GenDEPOT (Barker, TX, USA). Triton X-100 and DAPI (4′,6-diamidino-2-phenylindole) solutions were obtained from Sigma Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2. Preparation of the individual extracts and combination formulations

Laila Nutraceuticals (Vijayawada, India) provided the individual BSE (LI13121), CLE (LI01106), and TCE (LI73000) compositions. These were then mixed in various ratios (Table 1). The individual extracts and their combinations were dissolved in DMSO as the treatment vehicle.

3. DPPH radical-scavenging assay

The DPPH radical-scavenging activities of the various extracts were evaluated to compare their antioxidative capabilities. All extracts (No. 1-7) were dissolved in 95% ethanol solution for this experiment and then diluted serially. In brief, 180 μL of 0.2 mM DPPH solution was dispensed into each well of a 96-well plate, following which 20 μL of the sample solution (diluted to various concentrations) was added. After 30 min of reaction, the absorbance of the mixture in each well was measured at 517 nm with a microplate reader (Tecan, Männedorf, Switzerland). For the control group, ethanol was added instead of the sample solution. The percentage DPPH radical-scavenging values were calculated using the following equation: % scavenging = [Absorbance of control − Absorbance of test sample/Absorbance of control] × 100. The IC_{50} value was calculated as the concentration of extract that resulted in a 50% decrease.

4. Cell culture

HCHs (PromoCell Bioscience Alive) were maintained in complete chondrocyte growth medium supplemented with 10% fetal calf serum in a humified incubator (37℃, 5% CO_2). The HCHs were seeded into the wells of a 6-well plate at a density of 1×10^5 cells/well. After 24h, the cells were treated with the various extracts (50 μg/mL), respectively, in the presence or absence of IL-1β (10 ng/mL).

5. Cell viability assay

HCHs were seeded into 96-well plates (1×10^3 cells/well) and treated with the various extracts (each at 50 μg/mL) in the presence or absence of IL-1β (10 ng/mL) for 24h. The MTT solution (5 mg/mL) was then added to each well. After 3h, the supernatant was removed and 100 μL of DMSO was added to dissolve the formazan crystals. Finally, the absorbance (570 nm) was measured using a microplate reader (Tecan, Mannedorf, Switzerland).

6. Safranin O staining

HCHs were seeded overnight in 24-well plates (1×10^4 cells/well) and then treated with IL-1β (10 ng/mL) alone or IL-1β with the respective extracts. The various media were replaced every 3 days with the same media containing IL-1β

and extracts. On day 7, the cells were fixed in 4% paraformaldehyde for 15 min at ambient temperature and then washed with phosphate-buffered saline (PBS). Thereafter, the cells were incubated in safranin O solution for 30 min at ambient temperature and then washed three times with PBS for 5 min each time to remove excess dye. The levels of red staining in the cells were observed under a microscope (×10 magnification; Nikon, Tokyo, Japan).

7. Immunofluorescence staining

HCHs were seeded in a 6-well plate and incubated overnight, following which they were pretreated with the various extracts for 1h. After the pre-incubation, the cells were treated with IL-1β (10 ng/mL) alone or IL-1β with the various extracts for 24h. Then, after washing with PBS, the cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 10 min, and then blocked with 1% bovine serum albumin for 1h at ambient temperature. The cells were then incubated overnight with the primary NF-κB p-p65 antibody (1:250 dilution) at 4℃. Then, after washing three times with PBS, the cells were incubated with the Alexa Fluor 488-conjugated secondary antibody (1:250) for 1h at 37℃. Finally, the cells were counterstained with DAPI solution and images were captured under a fluorescence microscope (×10 magnification; Nikon, Tokyo, Japan).

8. Western blot analysis

HCHs were lysed in CelLytic buffer (Sigma-Aldrich, St. Louis, MO, USA) and then centrifuged at 10,000× g for 15 min at 4℃. After centrifugation, the supernatant was collected and its protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Next, the proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, following which the protein bands were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was first blocked for 1h with 5% skim milk dissolved in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and then incubated overnight at 4℃ with the specific primary antibodies. Thereafter, the membrane was incubated with HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies for 1h at ambient temperature. Finally, detection of the protein bands was achieved using an enhanced chemiluminescence solution (GenDEPOT), and the band intensity was quantified using the LuminoGraph system (Atto, Tokyo, Japan). As control for normalization, β-actin was utilized. Bands on the membranes were quantified utilizing the ImageJ program (developed at the NIH).

9. Statistical analysis

All data are presented as the mean ± standard deviation. Groups were compared using the student's t-test and one-way analysis of variance, as applicable. Statistical analyses were performed using Origin 7.0 software (MicroCal Software, Northampton, MA, USA). Differences between groups were considered statistically significant at a P value of less than 0.05.

Table 1. Combination ratios of *Boswellia serrata* gum resin extract (BSE): *Curcuma longa* extract (CLE): *Terminalia chebula* extract (TCE) (No. 4-7), and individual extracts (No. 1-3).

No.	BSE	CLE	TCE
1	1	-	-
2	-	1	-
3	-	-	1
4	1	1	-
5	1	1	1
6	2	1	1
7	2	1	2

Results

1. DPPH radical-scavenging activities of the individual *B. serrata* gum resin, *C. longa*, and *T. chebula* extracts alone or in various combination ratios

The DPPH radical-scavenging activities of the individual extracts alone or in various combinations were determined in vitro. As shown in Fig. 1, the IC_{50} values in the DPPH radical-scavenging activity tests were 460.2 ± 6.2 μg/mL for BSE, 327.5 ± 1.2 μg/mL for CLE, 240.9 ± 2.0 μg/mL for TCE, 311.6 ± 7.6 μg/mL for the 1:1 (BSE:CLE ratio) combination, 233.5 ± 4.8 μg/mL for the 1:1:1 combination, 180.3 ± 0.6 μg/mL for the 2:1:1 combination, and 127.1 ± 6.3 μg/mL for the 2:1:2 combination (BSE:CLE:TCE ratio). Therefore, because it had the lowest IC_{50} value, the 2:1:2 combination was determined to possess the best DPPH radical-scavenging capacity among the various extracts tested.

2. Effects of the individual *B. serrata* gum resin, *C. longa*, and *T. chebula* extracts alone or in various combinations on human articular chondrocytes (HCHs) viability

The effects of the individual extracts alone or in various combinations on HCHs viability were evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. No cytotoxic activity was observed in the cells treated with 50 μg/mL of BSE, CLE, or TCE alone or

the 1:1, 1:1:1, 2:1:1, and 2:1:2 combinations at the same concentration each (as used in the previous study).[16] Moreover, concomitant treatment of the HCHs with IL-1β (10 ng/mL) and the various extracts also did not affect the cell viability (Fig. 2).

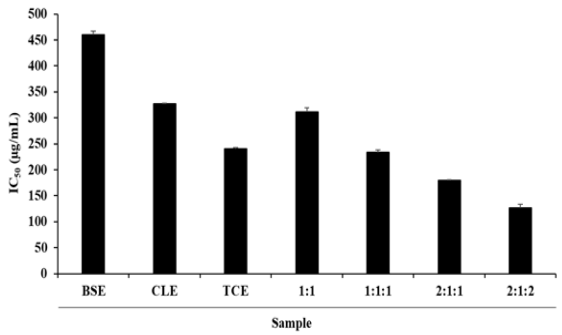

Fig. 1. DPPH radical-scavenging activities of the individual *B. serrata* gum resin, *C. longa,* and *T. chebula* extracts alone or in various combination ratios. All values are expressed as the mean ± standard deviation (n = 3).

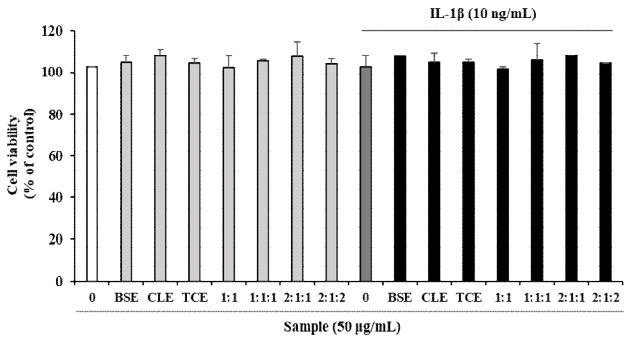

Fig. 2. Effects of individual *B. serrata* gum resin, *C. longa*, and *T. chebula* extracts alone or in various combinations on human articular chondrocytes (HCHs) viability. The cells were treated with or without IL-1 β and samples, following which their viability was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Data are presented as the mean ± standard deviation (n = 3).

3. Effects of the individual *B. serrata* gum resin, *C. longa*, and *T. chebula* extracts alone or in various combinations on antioxidant-related protein expression in IL-1β-stimulated HCHs

The expression levels of the antioxidative enzymes catalase and superoxide dismutase 1 (SOD1) and the antioxidant-related nuclear factor-erythroid factor 2-related factor 2 (Nrf2) in the HCHs were analyzed using the western blot assay. The catalase level had decreased by 7.46% ($P < 0.01$) in the IL-1β-treated control group. By contrast, no significant increase in catalase levels was observed in the IL-1β-treated cells co-treated with either BSE or CLE alone, whereas a 13.55% increase was obtained in the TCE-co-treated group ($P < 0.05$). Furthermore, the catalase levels in cells co-treated respectively with the 1:1, 1:1:1, and 2:1:1 mixtures of the three extracts had increased by 13.54-20.05% ($P < 0.01$), and the level in the 2:1:2 group had increased the most significantly by 32.60% ($P < 0.001$). The Nrf2 protein expression level was also significantly decreased in the IL-1β-alone control group ($P < 0.001$), whereas it had increased by 42.09% in the IL-1β + BSE group ($P < 0.05$), 49.55% in the IL-1β + TCE group, and 51.03-86.50% in the various IL-1β + mixed extracts groups ($P < 0.001$). Notably, the level of this protein was the highest in the IL-1β + 2:1:2 group, having increased by 101.51%. The increased level of SOD1 was significant in the IL-1β + 2:1:2 group only ($P < 0.01$) (Fig. 3).

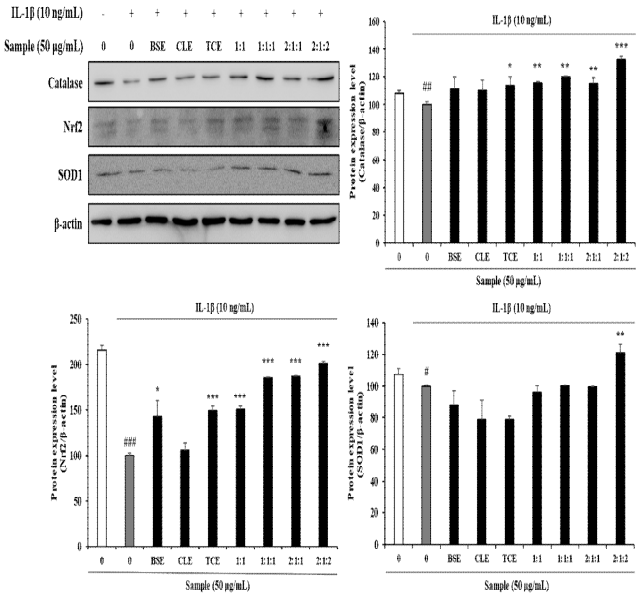

Fig. 3. Effects of the individual *B. serrata* gum resin, *C. longa*, and *T. chebula* extracts alone or in various combinations on antioxidant-related protein expression in IL-1β-stimulated HCHs. The cells were treated with IL-1β (10 ng/mL) alone or IL-1β plus the various extracts (50 μg/mL) for 24h. The levels of the target proteins were analyzed using the western blot assay and quantified using the ImageJ program. Data are presented as the mean ± standard deviation (n = 3). #P < 0.05, ##P < 0.01, and ###P < 0.001 compared with the vehicle control group. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the IL-1β-treated control group.

4. Effects of the individual *B. serrata* gum resin, *C. longa*, and *T. chebula* extracts alone or in various combinations on extracellular matrix degradation-related protein expression in IL-1β-stimulated HCHs

MMPs are key enzymes among the major catabolic enzymes of the chondrocyte matrix. Thus, we assessed the effects of the various extracts individually or in combination on MMP3 and MMP13 expression in the HCHs. Although the expression levels of MMP3 and MMP13 were significantly increased by the IL-1β treatment, the enzymes were inhibited by the various extracts, with the 2:1:2 combination

exhibiting the best inhibitory effects (Fig. 4A).

We also investigated the effects of the various extracts on the components of the ECM; namely, aggrecan and COL2A1. As shown in Fig. 4A, IL-1β significantly reduced the level of aggrecan protein in the cells, but the 2:1:2 extract combination inhibited this abnormal degradation of the protein the most effectively among the various extracts tested ($P < 0.001$). Moreover, although IL-1β also induced a significant reduction in the level of COL2A1 protein, cells treated with TCE alone or the 1:1:1 or 2:1:1 combination showed increases in the expression of this protein by 13.64%, 10.43%, and 13.37%, respectively ($P < 0.05$). However, among the various extract combinations, the 2:1:2 mixture increased the COL2A1 protein expression level the most significantly by 24.46% ($P < 0.001$). Additionally, safranin O staining of the cells co-treated with IL-1β and the 2:1:2 combination showed significantly higher redness than the cells in the control group (Fig. 4B), which was consistent with the western blot results in Fig. 4A.

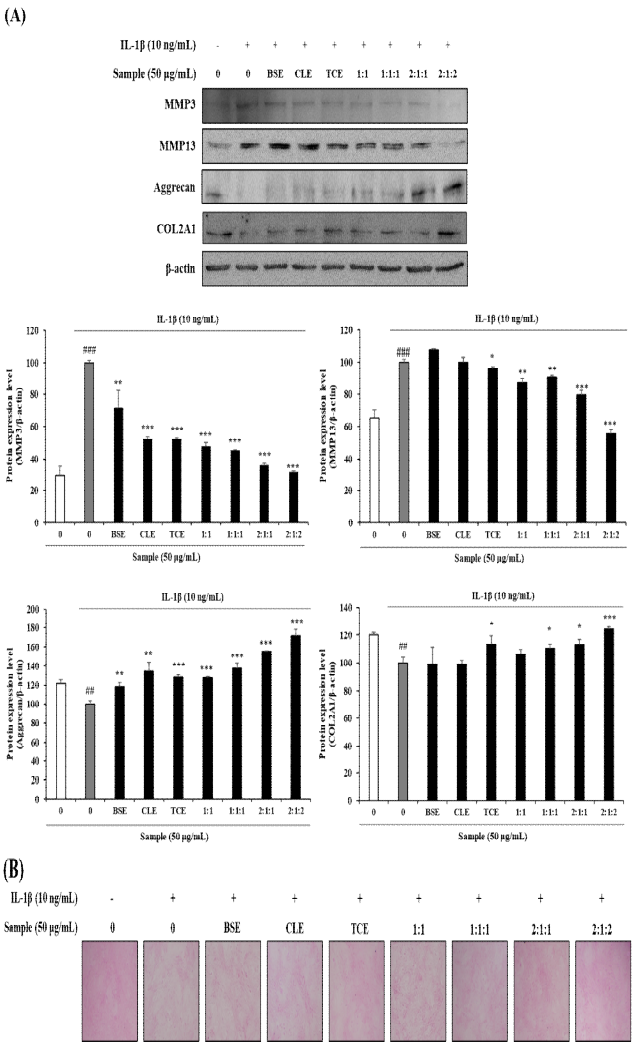

Fig. 4. Effects of the individual *B. serrata* gum resin, *C. longa*, and *T. chebula* extracts alone or in various combinations on extracellular matrix degradation-related protein expression in IL-1β-stimulated HCHs. The cells were treated with IL-1β (10 ng/mL) alone or IL-1β plus the various extracts (50 μg/mL). (A) MMP3, MMP13, aggrecan, and COL2A1 protein levels, as analyzed using the western blot assay. The data were quantified using the ImageJ program. Data are presented as the mean ± standard deviation (n = 3). ##P < 0.01, and ###P < 0.001 compared with the vehicle control group. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the IL-1β-treated control group. (B) Aggrecan expression level, as determined by safranin O staining.

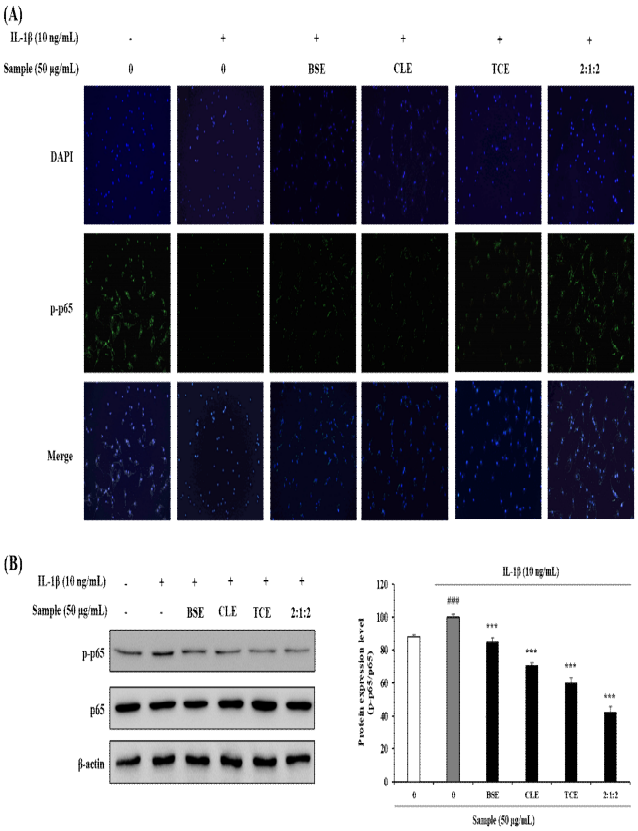

Fig. 5. Effects of the individual *B. serrata* gum resin, *C. longa*, and *T. chebula* extracts alone or the 2:1:2 combination on the NF-κB signaling pathway in IL-1β-stimulated HCHs. The cells were treated with IL-1β (10 ng/mL) alone or IL-1β plus the various extracts (50 μg/mL) for 15 min. The target protein levels were analyzed using (A) p-p65 immunofluorescence staining and (B) the western blot assay and quantified using the ImageJ program. Data are presented as the mean ± standard deviation (n = 3). ###P < 0.001 compared with the vehicle control group. ***P < 0.001 compared with the IL-1β-treated control group.

5. Effects of the individual *B. serrata* gum resin, *C. longa*, and *T. chebula* extracts alone or the 2:1:2 combination on the NF-κB signaling pathway in IL-1β-stimulated HCHs

Immunofluorescence staining and western blot assays were conducted to investigate the effects of the various extracts on the activation and inhibition of the NF-κB signaling pathway. The immunofluorescence staining was performed to confirm the inhibitory effects on signal translocation in the pathway (Fig. 5A). In HCHs stimulated with IL-1β, p-p65 signals were observed in the cell nuclei,

whereas they were not in cell nuclei of the untreated control group. However, in the presence of BSE, CLE, TCE, or the 2:1:2 combination, the levels of p-p65 entry into the nucleus had been inhibited. These findings were confirmed by the western blot results (Fig. 5B), where IL-1β also significantly enhanced the phosphorylation of p65 ($P < 0.001$), whereas the extracts alone or in combination reduced the IL-1β-induced effects significantly ($P < 0.001$), with the 2:1:2 combination showing the highest effect in inhibiting p-p65 nuclear translocation.

6. Effects of the individual *B. serrata* gum resin, *C. longa*, and *T. chebula* extracts alone or the 2:1:2 combination on the MAPK signaling pathway in IL-1β-stimulated HCHs

As shown in Fig. 6, IL-1β had triggered the phosphorylation of both p38 and ERK. However, treatment with BSE, CLE, or TCE inhibited the phosphorylation of both p38 ($P < 0.05$) and ERK significantly ($P < 0.001$). Especially, the 2:1:2 combination showing the strongest inhibitory effect in inhibiting the phosphorylation of p38 ($P < 0.01$) and ERK ($P < 0.001$).

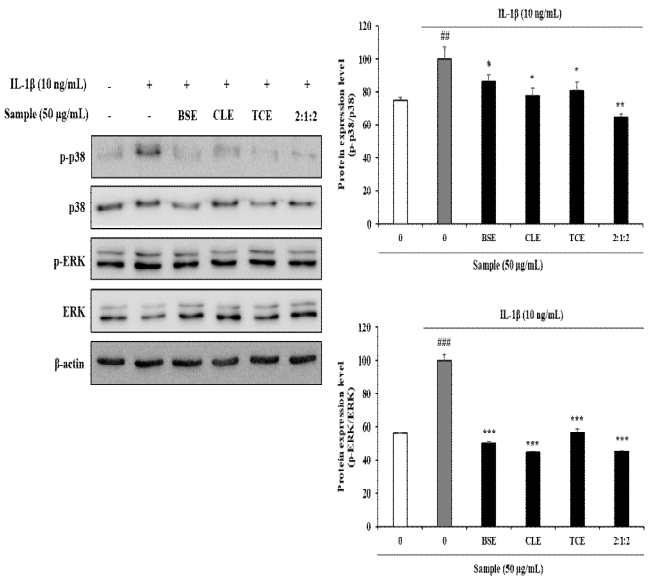

Fig. 6. Effects of the individual *B. serrata* gum resin, *C. longa,* and *T. chebula* extracts alone or the 2:1:2 combination on the MAPK signaling pathway in IL-1β-stimulated HCHs. The cells were treated with IL-1β (10 ng/mL) alone or IL-1β plus the various extracts (50 μg/mL) for 15 min. The target protein levels were analyzed using the western blot assay and quantified using the ImageJ program. Data are presented as the mean ± standard deviation (n = 3). ##P < 0.01 and ###P < 0.001 compared with the vehicle control group. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the IL-1β-treated control group.

Discussion

Osteoarthritis is the most common among all types of degenerative joint diseases and increases in prevalence with aging. Several studies have concluded that the progression of the disease is caused by oxidative stress due to ROS overproduction in the articular chondrocytes.[18-20] IL-1β is one of the most active cytokines during osteoarthritis development, stimulating the production of ROS and reducing that of SOD in the chondrocytes, thereby resulting in higher levels of cytotoxic peroxides. As a result, it also reduces collagen type II and aggrecan synthesis by the cells.[21]

The gum resin of *B. serrata* and *C. longa* and the fruits of *T. chebula* have been reported to show arthritis-relieving effects, which have been proven to be attributed to their powerful antioxidative activities.[22-24] In India, extracts from these three plant species have long been used as traditional Ayurvedic medicines to alleviate the symptoms of various inflammatory diseases.[25-27]

The combination of a curcumin complex from *C. longa* with boswellic acid from *B. serrata* was shown in a 12-week, randomized, double-blind, placebo-controlled clinical trial to reduce pain-related symptoms in patients with osteoarthritis.[2] Treatment with the combined extracts was shown to have a better therapeutic effect than that with the curcumin complex alone owing to the synergetic effects of curcumin and boswellic acid.[2] Moreover, in our previous study, the herbal composition LI73014F2 (the 2:1:2 mixture of BSE, CLE, and TCE) was more effective than the individual extracts alone in inhibiting IL-1β-induced prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) expression in HCHs, which is critical for alleviating the inflammatory effects of osteoarthritis.[16] However, studies comparing the antioxidative effects of individual BSE, CLE, and TCE alone and their various combinations as well as their effects on MMPs expression in HCHs have not been conducted.

In this study, the DPPH radical-scavenging activities of these extracts were compared, with the results showing the IC_{50} values of the 1:1:1, 2:1:1, and 2:1:2 combinations to be lower than those of the individual extracts, revealing their excellent anti-oxidative activities. In addition, 1:1 mixture of the *B. serrata* gum resin and *C. longa* showed a higher IC_{50} value than the *T. chebula* individual extract, indicating the excellent antioxidative capacity of *T. chebula* extract. Overall, the 2:1:2 extract combination showed the best effect among all the extracts assessed. Next, we evaluated the catalase, Nrf2, and SOD1 protein levels in the HCHs to further confirm the antioxidative effects of the extracts. Nrf2 is a transcription factor that modulates cellular defense responses by regulating the basic and inducible

expression of many cytoprotective proteins, such as catalase and SOD1.[28] The expression levels of the enzymatic antioxidants such as catalase and SOD1, and the antioxidant-related protein Nrf2 were significantly higher in the cells treated with the mixtures than in those treated with the individual extracts alone, with the 2:1:2 combination again showing the best effect.

Osteoarthritis is known to be exacerbated by an increase in the cartilage matrix-degrading enzymes in the ECM induced by an increase in inflammatory cytokines, such as tumor necrosis factor-alpha and IL-1β.[29-31] The persistent catabolism of articular cartilage causes joint pain, with IL-1β being a critical degradation-inducing factor in this pathological process.[32] The ECM is mostly an organization of COL2A1 and aggrecan molecules, which are created and retained by the articular chondrocytes and can be degraded by proteases,[33] especially MMPs secreted from these cells.[29-31]

In this study, the COL2A1 and aggrecan levels significantly decreased (by 16.65% and 18.65%, respectively; $P < 0.01$) in the IL-1β-treated HCHs. However, the levels were significantly elevated by the 2:1:2 extract combination (by 24.46% and 71.20%, respectively; $P < 0.001$). This increase was higher than that in the control group that was not treated with IL-1β. Therefore, it is possible that the 2:1:2 combination not only inhibits ECM degradation but also promotes collagen and aggrecan synthesis.

The increased activation of NF-κB and MAPK induced by cytokines such as IL-1β in articular chondrocytes indicates that these pathways play a key role in the progression of osteoarthritis.[34-37] IL-1β binds to receptors on the chondrocytes, promoting the phosphorylation of MAPK and NF-κB and thereby the activation of these pathways,[38] which results in upregulation of the expression of proinflammatory and catabolic proteases and the eventual destruction of the chondrocytes and ECM. Therefore, we investigated the relationship between the anti-osteoarthritis effects and inhibition of the NF-κB and MAPK pathways mediated by the various extracts. Once again, the 2:1:2 combination showed the best effects in significantly inhibiting the phosphorylation of p65, p38, and ERK and further inhibiting the nuclear translocation of p65 in IL-1β-stimulated HCHs. Taken together, these results indicate that the excellent anti-oxidative effect of the 2:1:2 combination and its inhibition of MMPs expression may be associated with its downregulation of the MAPK and NF-κB signaling pathways.

In conclusion, the synergetic effects of BSE, CLE, and TCE in the 2:1:2 mixture (i.e., herbal composition LI73014F2) provided this formulation with the highest antioxidative activity and inhibitory effect against ECM degradation among all the extracts tested. Therefore, herbal composition LI73014F2 is expected to be useful as an effective dietary supplement for aiding joint health.

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