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Cinnamic aldehyde, an anti-inflammatory component in Du-Huo-Ji-Sheng-Tang, ameliorates arthritis in II collagenase and monosodium iodoacetate induced osteoarthritis rat models

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ABSTRACT

Background and aim: Du-Huo-Ji-Sheng-Tang (DHJST) is a Chinese herbal formula used for arthralgia and arthritis treatment clinically. This study aims to evaluate the joint-protecting efficacy of DHJST and to identify the active constituents as the evaluation marker.
Experimental procedure: DHJST can be categorized into three recipes: Blood-tonifying-herbs Si-Wu-Tang (SWT), Wind-dampness-dispelling-herbs (WDH) and Qi-tonifying-herbs (TH). All formulas were used to explore the joint-protecting efficacies.
Results and conclusion: s: Firstly, DHJST could decrease the arthritis progression in the monosodium-iodoacetate-induced rat and cure arthritis in the type II collagenase-induced rat. Further, in lipopolysaccharide-stimulated RAW 264.7 cells, DHJST, TH and *Cinnamomum cassia* (CC), an ingredient in TH, were the most potent nitric oxide (NO) and prostaglandin E_2 (PGE_2) inhibitors. The major components, cinnamic aldehyde, showed the strongest NO and PGE_2 inhibition. Up-regulated inducible NO synthase (iNOS) and cyclooxygenase-2 were inhibited by DHJST, TH, CC, and cinnamic aldehyde. In interleukin-1β-stimulated primary chondrocytes, upregulated iNOS was inhibited by DHJST, TH, *Cinnamomum cassia*, and cinnamic aldehyde. Upregulated matrix metalloprotease-13 was only inhibited by DHJST and TH and *Eucommia ulmoides* (EU) extract. Results suggest that DHJST presented joint-protective and cure arthritis effects. TH presented equal joint-protective effects as DHJST. The major anti-inflammatory ingredient in TH was *Cinnamomum cassia* in TH. And cinnamic aldehyde was the potent anti-inflammatory active compound in *Cinnamomum cassia.* Therefore, this study may facilitate the modern use of DHJST with TH as a simplified version but equally effective anti-osteoarthritic agents with cinnamic aldehyde as a quality control marker of DHJST and TH in osteoarthritis prevention or treatment.

1. Introduction

From the perspective of traditional Chinese medicine (TCM), arthralgia is due to wind, cold, and dampness in the joints as a blockage in the smooth flow of qi and blood within the body. Du-

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List of abbreviations

ANOVA	a one-way analysis variance
CC	*Cinnamomum cassia*
CIA	type II collagenase-induced arthritis
COX-2	cyclooxygenase-2
DHJST	Du-Huo-Ji-Sheng-Tang
DMEM	Dulbecco's modified Eagle's medium
ELISA	enzyme-linked immunosorbent assay
EU	*Eucommia ulmoides*
FBS	fetal bovine serum
GU	*Glycyrrhiza uralensis*
H&E	hematoxylin and eosin
IC_{50}	50% inhibition
IL	interleukin
iNOS	inducible NO synthase
LPS	lipopolysaccharide
LSD	least significant different
MIA	monosodium iodoacetate
MMP	matrix metalloproteinase
NO	nitric oxide
OA	osteoarthritis
PG	*Panax ginseng*
PGE_2	prostaglandin E_2
PRCs	primary chondrocytes
RIPA	radioimmunoprecipitation assay
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SWT	Si-Wu-Tang
TCM	traditional Chinese medicine;
TH	Qi-tonifying-herbs
WDH	Wind-dampness-dispelling-herbs

Huo-Ji-Sheng-Tang (DHJST) has been widely and safely used for over 1000 years. This formula, originally recorded in *Bei-Ji-Qian-Jin-Yao-Fang* (in about 652 A.D.), relieves arthralgia due to wind, cold, and dampness by three pharmacological actions: nourishing the blood, invigorating Qi, and expelling and removing wind and dampness. Today, it is still one of the most commonly prescribed Chinese herbal formulas to treat osteoarthritis (OA).[1] In 2002, the prescription rate of DHJST was 26.6%, ranked highest among a total of 32,050 Chinese herbal formulae reimbursed by National Health Insurance for OA treatment in Taiwan.[2]

Goal of arthritis treatment is to reduce pain, improve mobility and prevent further damages to the joints. However, effective disease modifying drugs for osteoarthritis treatment are still limited. Because inflammation plays an important role in the development of arthritis, therefore, anti-inflammation is one of the most frequently treatment strategy used to treat arthralgia and arthritis.[3,4] *In vitro* and *in vivo* studies have demonstrated that when inflammation is activated, the activated NF-κB in the macrophage induce interleukin (IL)-1β release, which in turn induced caspase-mediated chondrocyte damage and arthropathy.[5–7] TCM is becoming a promising option for arthritis treatment, as TCM and its components could ameliorate symptoms or prevent progression of arthritis through multiple targets, including inhibition of pro-inflammatory cytokines and cartilage degradation regulators.[8,9] So far, how this complex formula works as an anti-arthritic formula remained inadequately characterized. Reported action mechanism of DHJST and its component on joint protection include by inhibiting hypoxia or inflammation related factors.[10–12] Interestingly, the blood tonic components in DHJST, *Angelica sinensis*, *Ligusticum chuanxiong*, *Paeonia lactiflora*, and *Rehmannia glutinosa*, is actually a famous formula known as Si-Wu-Tang (SWT), but anti-inflammatory effect has not been reported in SWT.[13] Therefore, we studied DHJST by grouping the constituent herbs into one of the following categories, blood nourishing, Qi invigorating, and wind/dampness expelling and removing, and compared their anti-inflammatory activities using *in vitro* and *in vivo* methods. Deciphering the active anti-inflammatory components of DHJST may modernize the cultivation, manufacture, and clinical applications of this popular formula.

2. Materials and methods

2.1. Preparation of extracts

DHJST is composed of the following 15 plant species, and the prescription is as shown in Table 1. The herbs were grouped into three compound recipes according to their use in clinical settings based on TCM theories: Recipe 1, Si-Wu-Tang (SWT): *Rehmannia glutinosa*, *Angelica sinensis, Paeonia lactiflora* and *Ligusticum chuanxiong*; Recipe 2, wind-dampness herbs (WDH): *Achyranthes bidentate, Angelica pubescens*, *Gentiana macrophylla*, *Taxillus chinensis*, *Saposhnikovia divaricate*, *Asarum heteropoides* and *Poria cocos*, and Recipe 3, tonifying herbs (TH): *Eucommia ulmoides* (EU), *Cinnamomum cassia* (CC), *Panax ginseng* (PG) and *Glycyrrhiza uralensis* (GU). Voucher specimens (DHJST-01 to DHJST-15) were deposited in the School of Pharmacy, College of Pharmacy, Taipei Medical University (Taipei, Taiwan). DHJST, SWT, WDH and TH decoction used in the experiment were extracted with 50% ethanol (10 times the weight of the material) and refluxed for 2 h at 65 °C, with this process being repeated twice. The twice-filtered solutions were combined and concentrated by rotary evaporation. The aquatic solution was freeze-dried to a powder.

2.2. HPLC analysis

The HPLC apparatus used was composed of an SCL-10Avp system controller, an LC-10ATvp liquid chromatographic pump, an SPD-M10A diode array detector, an SIL-10Avp auto-injector, a CTO-10A column oven, FCV-10Avp flow-channel selection valves (Shimadzu, Tokyo, Japan), and an ERC-3415 degasser (ERC, Altegolfsheim, Regensburg, Germany). The stationary phase was comprised of a Purospher® STAR RP-18e reverse-phase column (4 mm i.d. × 250 mm, 5 μm, Merck, USA), and the mobile phase system included 0.05% trifluoroacetic acid: CH_3CN gradient elution (5 min, 90:10; 15 min, 75:25; 25 min, 55:45; 35 min, 40:60; and 45 min, 0:100). The flow rate was 1 mL/min, and the column oven temperature was maintained at 40 °C. The ultraviolet wavelength was set to 280 nm for detecting the fingerprint chromatograms of the 50% ethanol extracts of DHJST, WDH, SWT, TH and CC.

2.3. Animals

Wistar Rats weighing 180–220 g were housed in a controlled environment at 21 °C with sufficient food and water and kept on an alternating 12 h dark and light cycle. Animal experiments were approved according to Ethical Regulations on Animal Research of Taipei Medical University (approval no: LAC-100-0043).

Table 1
Prescription of Du-Huo-Ji-Sheng-Tang and herbal traditional use in traditional Chinese medicine.

Traditional action	Name	Family	Scientific name	Part used	Dose (g)	Recipe
Blood-tonifying herb tonifies the blood in treatingblood deficiencies	地黃 Di Huang	Scrophulariaceae	*Rehmannia glutinosa* (Gaertn.) DC.	Root	2	SWT
	當歸 Dang Gui	Umbelliferae	*Angelica sinensis* (Oliv.) Diels	Root	2	SWT
	白芍 Shao Yao	Ranunculaceae	*Paeonia lactiflora* Pall.	Root	2	SWT
Blood-activating and stasis-resolving herb promotes blood flow to remove blood stasis	川芎 Chuan Xiong	Umbelliferae	*Ligusticum chuanxiong* Hort.	Rhizome	2	SWT
	牛膝 Niu Xi	Amaranthaceae	*Achyranthes bidentata* Blume	Root	2	WDH
Wind/dampness-dispelling herb dispels wind and dampness from the body	獨活 Du Huo	Umbelliferae	*Angelica pubescens* Maxim.	Root	3	WDH
	秦艽 Qin Jiao	Gentianaceae	*Gentiana macrophylla* Pall.	Root	2	WDH
	桑寄生 Sang Ji Sheng	Loranthaceae	*Taxillus chinensis* (DC.) Danser	Twig	2	WDH
Wind/cold-dispersing herb eliminates wind and cold in superficial layers of the body	防風 Fang Feng	Umbelliferae	*Saposhnikovia divaricata* (Turcz.) Schmidt	Root	2	WDH
	細辛 Xi Xin	Aristolochiaceae	*Asarum heterotropoides* Fr. Schmidt var. *mandshuricum* Kitag	Root	2	WDH
Dampness-draining diuretic herb increases urine excretion and water discharge	茯苓 Fu Ling	Polyporaceae	*Poria cocos* (Schw.) Wolf	Indian Bread	2	WDH
Yang-tonifying herb tonifies yang qi in treating yang deficiency	杜仲 Du Zhong	Eucommiaceae	*Eucommia ulmoides* Oliv.	Bark	2	TH
Interior-warming herb warms the interior and expels internal cold	肉桂 Rou Gui	Lauraceae	*Cinnamomum cassia* (L.) J.Presl	Bark	2	TH
Qi-tonifying herb tonifies the healthy qi in treating qi deficiencies	人 Ren Shen	Araliaceae	*Panax ginseng* C. A. Mey.	Root	2	TH
	甘草 Gan Cao	Leguminosae	*Glycyrrhiza uralensis* Fisch. ex DC.	Root and rhizome	2	TH

2.4. Monosodium iodoacetate-induced arthritis (MIA) in Wistar rats model

Monosodium iodoacetate-induced arthritis (MIA) model was used to induce osteoarthritis in rats through cartilage digestion, inflammatory cell proliferation, matrix degradation, and cartilage destruction. Male Wistar rats were used in the experiments. divided into following three groups: control, DHJST low dose (25 mg/kg) and high dose (50 mg/kg), and TH groups, with 8 rats per group. MIA injections of 50 μL of 80 mg/mL on day 0 and 40 mg/mL on day 6 were given into the left ankle in the control group and treatment groups. In addition, Treatment groups received medication (50 mg/kg) orally every day for 10 days since day 0. Control group received sham injection and served as the normal control (normal). Measurement of the efficacy of treatment included paw edema measurements using a plethysmometer (Ugo Basile, Comerio VA, Italy) on days 0, 1, and 3; hind-limb weight-bearing distribution ratio on days 0 and 5; and a hot-plate latent pain response test on days 0 and 10. This model was used to assay osteoarthritic protective effect of the treatment drug.

2.5. Type II collagenase-induced arthritis (CIA) in Wistar rats model

Type II collagenase was used to digest cartilage to produce osteoarthritis joint before treatment. The experimental rats were divided into control group and two treatment groups (each group with 8 rats). The left knee of a Wistar rat was injected with type II collagenase (4 mg/kg) under anesthesia with Zoletil and xylazine. Rats' weight-bearing ratio had significantly changed on day 14 after the type II collagenase injection suggesting OA knee was successfully established. After the MIA mode has been established, treatment groups received 25 or 50 mg/kg DHJST every day orally for 21 days. Measurement of the efficacy of anti-osteoarthritic effect of the treatment drug include hind-limb weight-bearing distribution ratio on treatment days 0, day 7 and day 21 and histological examination. The model was used to assay the cure effect of the treatment drug.

2.6. Assessment of change in hind limb weight distribution

Weight-bearing distributions of both hind limbs were measured, and the weight-bearing ratios of hind limbs were calculated. Measurements were observed by an incapacitance tester with a dual-channel weight averager (Linton Instrumentation, Norfolk, UK). The weight-bearing force measured by the hind limbs was averaged over a 3-s period. %weight distribution of left hind paw = weight on left hind limb/(weight on right hind limb + weight on left hind limb) X 100. The ratio of weight distribution for the MIA-treated group maintained significantly lower than that for the normal control group until day 21.

2.7. Hot-plate latent pain responses

The hot-plate latent pain response test was performed as previously described and was assessed on days 0 and 10.[14] Briefly, in the thermal hyperalgesia analysis, animals were individually placed on a hot-plate instrument (Ugo Basile, Comerio, Varese, Italy). A radiant heat source was applied underneath the glass floor. The time between the placement of the animal on the platform and licking of the paws was recorded as the hotplate latency. The vehicle (soybean oil), DHJST, and TH were orally administered every day at 1–2 h before the hot-plate test (8 animals/group) for 10 days.

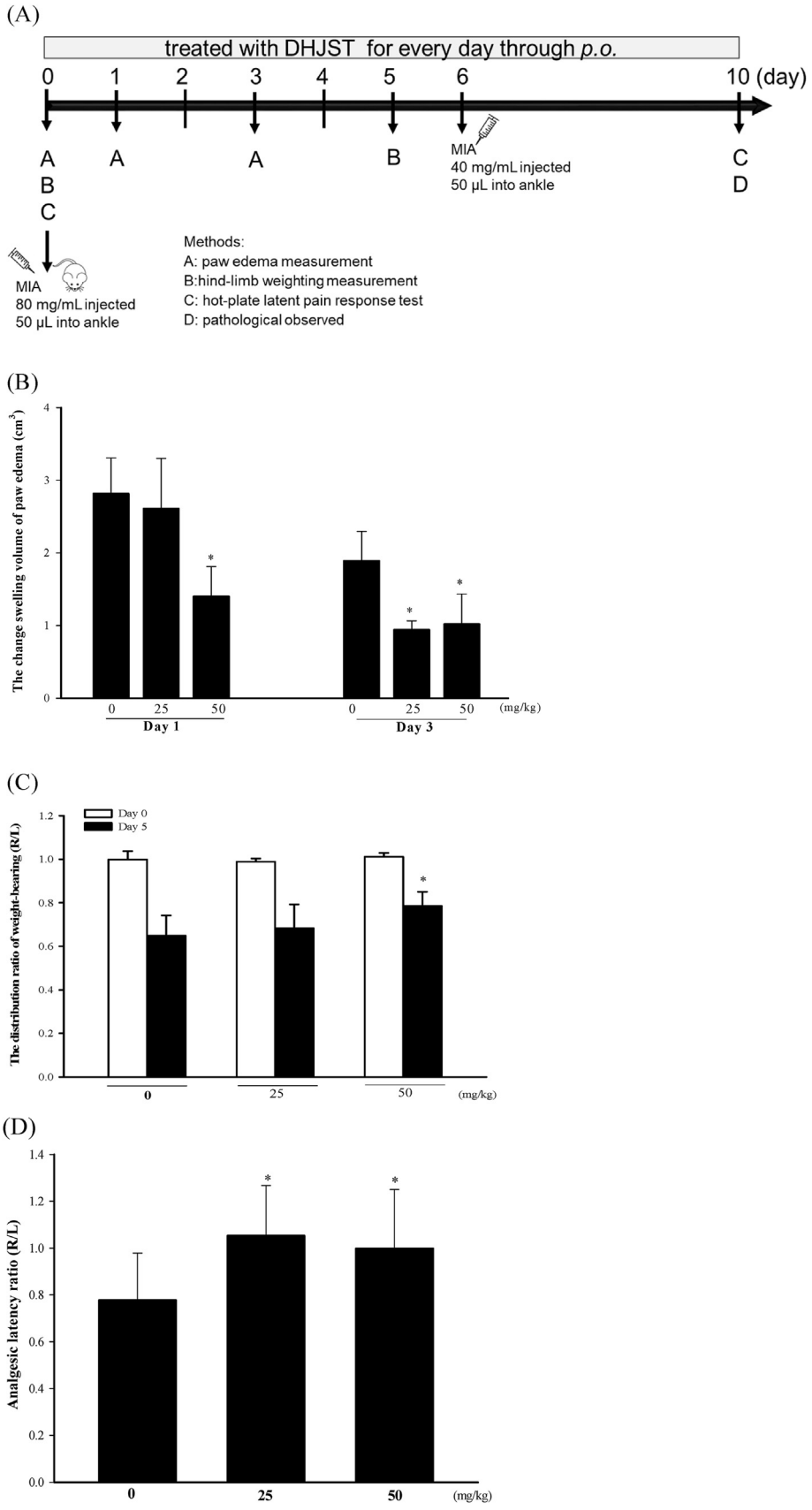

Fig. 1. Effects of DHJST on MIA-induced arthritis. Experimental flow chart (A). Effect of DHJST on MIA-induced paw edema swelling on days 1 and 3 (B). hind-limb weight-bearing on day 5 (C); hot-plate latent pain responses on day 10 (D). *$p < 0.05$, compared to the control. n = 3.

2.8. Histological examination

When the rats were sacrificed via anesthesia, joints from Type II collagenase and MIA-injected experimental animals were harvested and fixed in 10% (v/v) neutral buffered formalin for 24 h, paraffin-embedded, and cut into 5 μm thicknesses for histopathological assessments. Hematoxylin and eosin (H&E) staining was performed on paraffin-embedded sections to evaluate the severity of cell infiltration, and cartilage damage. After staining, cartilage damage in the joint tissue were investigated by histological examination under a light microscope. The severity of arthritis was categorized no detectable changes, mild, or severe.

2.9. Cell culture

The murine macrophage RAW 264.7 cell line was purchased from American Type Culture Collection (Rockville, MD, USA). Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL, Grand Island, NY, USA) in a humidified incubator containing 5% CO_2 at 37 °C.

2.10. Primary chondrocytes (PRCs) from rat cartilage

PRC culture was obtained as previously described.[15] Briefly, cartilage specimens cut into 1-mm^3 pieces were sequentially digested with pronase (10 mg/mL) and collagenase IV (5 mg/mL). A PRC monolayer culture was established in 60-mm Petri dishes at a concentration of 6×10^6 cells/mL in DMEM with 10% FBS, 100 μg/mL streptomycin, and 100 μg/mL penicillin. PRCs were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Experiments were performed with cells obtained from the 3rd passage.

2.11. NO and PGE_2 assays

Anti-inflammatory drugs often possess iNOS and COX-2 inhibitory actions, therefore, inhibition of their products, NO and PGE_2 is frequently used to identify anti-inflammatory activity in the investigational medicinal herbs. RAW 264.7 cells were treated with lipopolysaccharide (LPS) 500 ng/mL. After 18 h of incubation with or without samples, NO in the culture medium were assessed spectrophotometrically at 530 nm after the Griess reaction. The NO inhibition percentage was calculated using the following equation: NO inhibition (%) = [1 (T/C)] 100%; where T and C represent the mean optical density of LPS-stimulated RAW 264.7 cells with and without samples, respectively. PRCs were stimulated with IL-1β (10 ng/mL) for 6 h. PGE_2 concentrations were determined with an enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, NY, USA).

2.12. Western blot analyses of iNOS, COX-2, and matrix metalloproteinase (MMP)-13 expressions

Whole-cell lysates from cells treated with or without a sample for the scheduled hours were prepared by washing with PBS and lysing with radioimmunoprecipitation assay (RIPA) buffer. Equal amounts of protein from cell lysates were boiled for 5 min in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and visualized using a BCIP/NBT kit (Gibco BRL). GAPDH expression was used as an internal control to compare with iNOS, COX-2, and MMP-13 expressions.

2.13. Statistical analyses

Results are shown as the mean ± standard deviation of the mean (SD). Data were analyzed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Group differences were statistically assessed by a one-way analysis variance (ANOVA), followed by Fisher's least significant different (LSD) test for comparison of the means. p value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of DHJST on MIA-induced osteoarthritis

We first validated the joint-protective effect of DHJST with MIA-induced arthritic model. After MIA injection, paw edema swelling on days 1 and 3 (Fig. 1A), hind-limb weight-bearing on day 5 (Fig. 1B), and hot-plate latent pain responses on day 10 (Fig. 1C) were all beneficially and significantly attenuated by DHJST treatment as compared to the negative control group. These results supported that DHJST formulae possessed anti-inflammatory and joint-protective effects.

3.2. Effect of DHJST on weight bearing ratio and histopathology in type II collagenase-induced osteoarthritis (CIA) models

We further observed the joint-protective effect of DHJST on CIA model. As shown in Fig. 2, DHJST protected the joint from destruction as the treatment groups resumed more-equal weight-

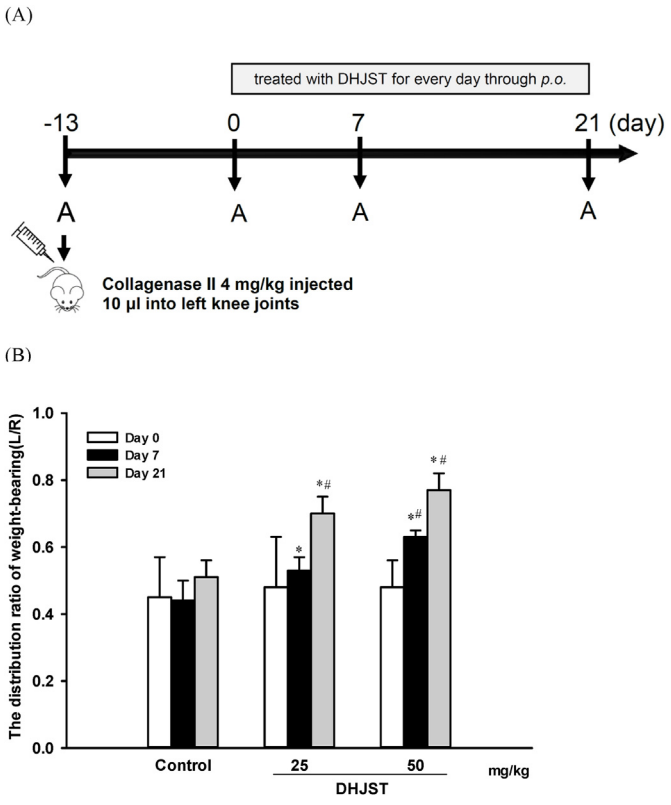

Fig. 2. Effect of DHJST on CIA-induced arthritis. Experimental flow chart (A). Changes in hind limb weight bearing in rats were measured (B). The weight distribution ratio was calculated as the left hind-limb (collagenase injection side) weight divided by the right hind-limb (control side) weight. Values are the mean ± SD. *p < 0.05, compared to the control; #p < 0.05, compared to day 0. n = 3.

bearing distributions of both hind limbs in a dose dependent manner. Histopathological assessments revealed low grade inflammatory cell infiltration and thinning of cartilage in the joint of control group, while joints from both low dose (25 mg/kg) and high dose (50 mg/kg) treated groups showed no detectable changes as compare to the normal group. These results confirm that DHJST has anti-arthritic effect. Effect of DHJST on pathological changes of joints from CIA rats was shown as in Fig. 3. As DHJST anti-arthritic effect was confirmed, the complete formula was used as positive control in the following experiments.

3.3. HPLC fingerprint profiles of DHJST, SWT, WDH and TH

Chromatographic profiles of DHJST and 3 recipes were analysis by HPLC. The results showed that DHJST, TH and *Cinnamomum cassia* samples contained the same major components. The three major peaks included 2-methylcinnamic acid (1), cinnamic acid (2), and cinnamic aldehyde (3), were identified by comparison to standard compounds (Fig. 4).

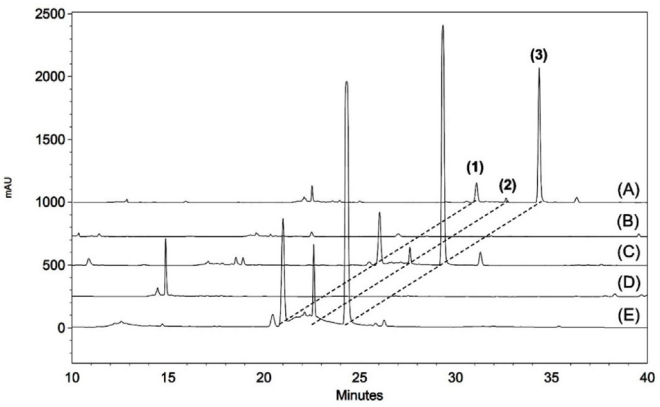

Fig. 4. HPLC profiles of DHJST (A), SWT (B); TH (C); WDH (D); Cinnamomum cassia (E). Abbreviations: Peak (1) 2-methylcinnamic acid; Peak (2) cinnamic acid; Peak (3) cinnamic aldehyde.

3.4. Effects of DHJST, SWT, WDH and TH and the constituent herbs on NO and PGE_2

Furthermore, the anti-inflammation effect of DHJST and 3 recipes were evaluated by LPS-stimulated RAW 264.7 cells. All of the tested samples showed no cytotoxicity. First, the NO inhibitory effect of DHJST (300 μg/mL) was compared with mixtures extracts of 3 recipes, WDH, SWT and TH (100 μg/mL each). These extracts showed no significant difference in NO inhibition (Fig. 5A). However, when each formula was examined individually, TH showed

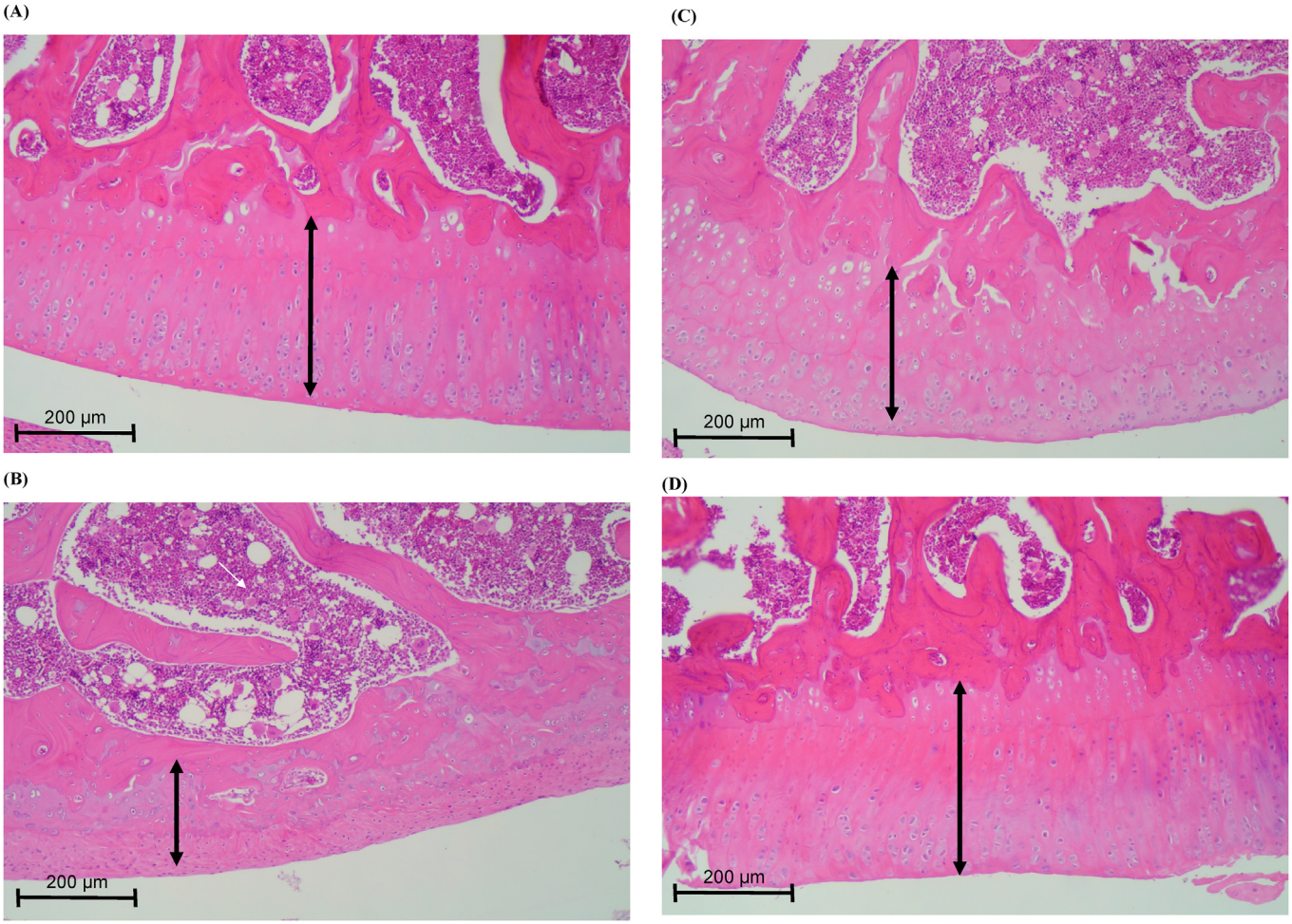

Fig. 3. Effect of DHJST on pathologic changes in CIA-induced arthritis model. Representative images were presented. (A), normal with normal cartilage thickness (double head arrow); (B), control with cartilage thinning (double head arrow); (C), DHJST treated (25 mg/kg/day) with cartilage thickness similar to the negative control (double head arrow); (D), DHJST treated (50 mg/kg/day) with cartilage thickness restored (double head arrow).

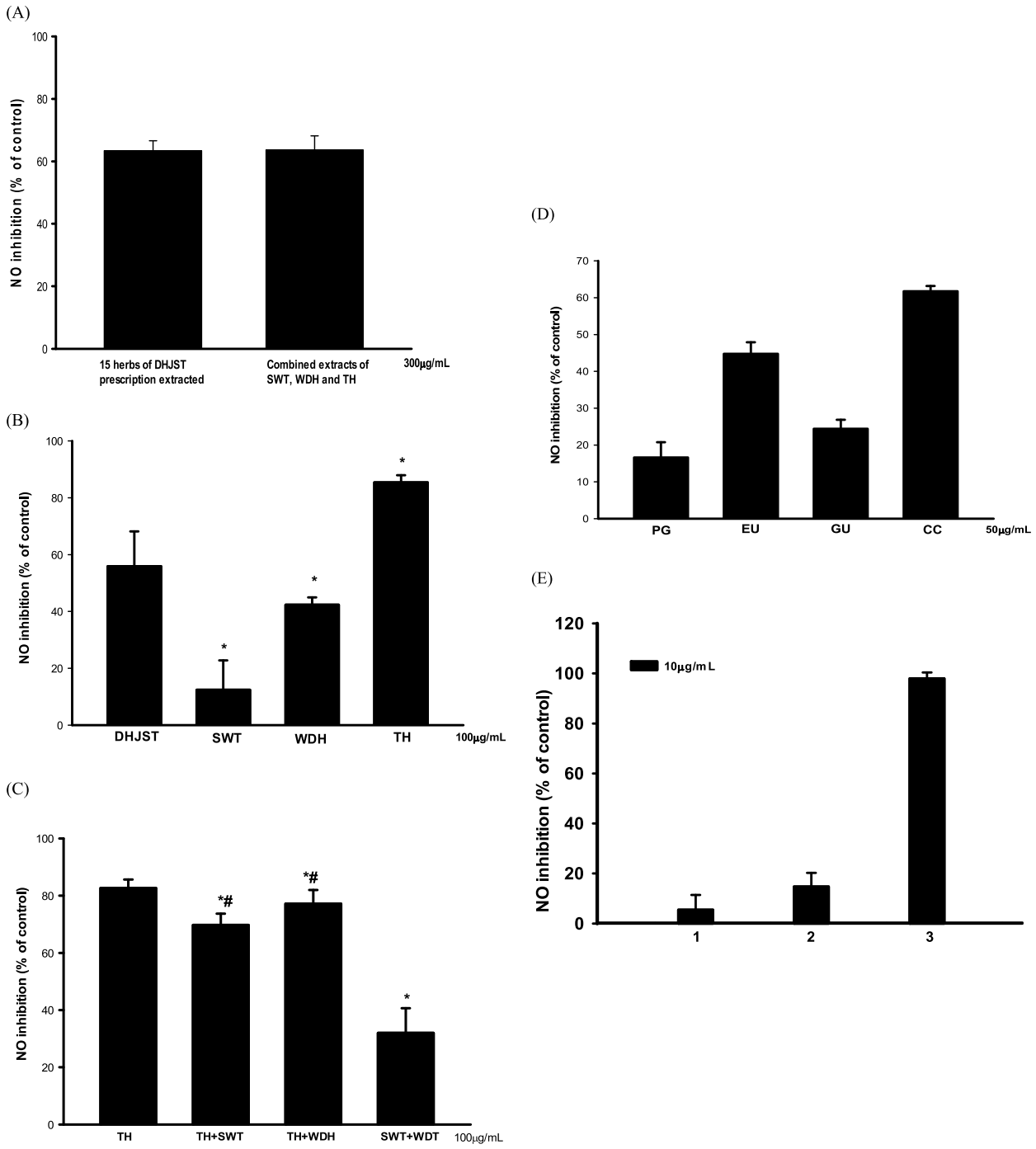

Fig. 5. Inhibitory Effect DHJST, SWT, WDH and TH on LPS-induced nitric oxide (NO) production in RAW 264.7 cells. (A), DHJST (300 μg/mL) and combined extracts of mixture of SWT, WDH and TH (100 μg/mL for each); (B), DHJST, SWT, WDH and TH. *$p < 0.05$, compared to DHJST; (C), TH (100 μg/mL), mixture of TH and SWT (50 μg/mL for each), TH and WDH (50 μg/mL for each), SWT and WDH (50 μg/mL for each) *$p < 0.05$, compared to TH; #$p < 0.05$, compared to SWT and WDH; (D), effect of TH ingredients on NO inhibition; (E), effect of three major compounds in *Cinnamomum cassia* on NO inhibition. Abbreviations: PG, *Panax ginsen*; EU, *Eucommia ulmoides*; GU, *Glycyrrhiza. Uralensis*; CC, *Cinnamomum cassia*; (1), 2-methylcinnamic acid; (2), cinnamic acid; (3), cinnamic aldehyde. Values are the mean ± SD. n = 3.

strongest NO inhibitory effects among all the tested formula (Fig. 5B). We also compared the NO inhibitory effect of two-combined recipes to TH alone. In order to understand the addition effects of 3 recipes, TH (50 μg/mL), SWT (50 μg/mL) or WDH (50 μg/mL) were added to each other. The results showed that TH could enhanced the NO inhibitory effect of combined extract (Fig. 5C), while combination of SWT and WDH showed no significant change on NO inhibitory activity. These results suggested that the NO inhibitory activity was related to herbs in TH. Further analyzing the NO inhibitory activity of the four constituent herbs of TH, extract of *Cinnamomum cassia* showed the most potent NO inhibitory activity, and the potency of EU was in the second place (Fig. 5D), suggesting that *Cinnamomum cassia* could be the major anti-inflammatory herb in TH. Among the three compounds, cinnamic aldehyde (3) showed the strongest NO inhibition (Fig. 5E). The concentration of DHJST, TH, *Eucommia ulmoides, Cinnamomum*

Table 2
The 50% inhibitory concentration (IC_{50}) values of tested samples on nitric oxide (NO) and prostaglandin E_2 (PGE_2) production from lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.

Tested sample	IC_{50} (μg/mL)					
	NO			PGE_2		
DHJST	123.94	±	1.45	131.73	±	5.42
TH	44.35	±	4.44	75.74	±	0.74
CC	18.45	±	3.03	9.49	±	0.00
EU	66.60	±	4.31	41.88	±	7.63
Cinnamic aldehyde	1.01	±	0.56	1.79	±	0.23

Data are presented as the mean (±S.D.) of at least three independent experiments, each performed in triplicate. DHJST: Du-Huo-Ji-Sheng-Tang, TH: Tonifying herbs, CC: *Cinnamomum cassia*, EU: *Eucommia ulmoides*.

cassia and cinnamic aldehyde (3) required for 50% inhibition (IC_{50}) of NO and PGE_2 induced by LPS is showed in Table 2. The above data supported the important anti-inflammatory role of TH in DHJST, and of *Cinnamomum cassia* in TH. Cinnamic aldehyde (3) was the active compound in them.

3.5. Effect of DHJST, TH, and component herbs and cinnamic aldehyde on macrophages stimulated with LPS

We then examined the modulatory effects of DHJST, TH, *Cinnamomum cassia*, and cinnamic aldehyde on iNOS and COX-2 expressions in LPS-stimulated RAW 264.7 cells (Fig. 6A–E). The upregulated iNOS and COX-2 were inhibited by DHJST, TH, *Cinnamomum cassia*, and cinnamic aldehyde, with cinnamic aldehyde showed the most potent inhibitory activities. EU did not decrease COX-2 expression, but decreased iNOS expression.

3.6. Effect of DHJST, TH, Cinnamomum cassia, Eucommia ulmoides and cinnamic aldehyde on chondrocytes stimulated with IL-1β

We also measured these extracts modulating effects on iNOS, and MMP-13 expressions and PGE_2 levels in IL-1β-stimulated PRCs. The upregulated iNOS were inhibited by all of the materials tested, but inhibitory activities on MMP-13 were only observed in DHJST, TH, and *Eucommia ulmoides* (Fig. 7A–E). Inhibitory activities of the 50 μg/mL of tested materials on PGE_2 levels in IL-1β-stimulated PRCs were shown in Fig. 7F.

3.7. Comparison of joint protective effects of DHJST and TH on MIA-induced arthritis

Lastly, we first validated the joint-protective effect of TH with MIA-induced arthritic model. After MIA injection, paw edema swelling on days 1 and 3 (Fig. 8A), hind-limb weight-bearing on day 5 (Fig. 8B), and hot-plate latent pain responses on day 10 (Fig. 8C) were all beneficially and significantly attenuated by DHJST or TH treatment. These results supported that TH formulae possessed anti-inflammatory and joint-protective effects as well as DHJST.

4. Discussion

In this study, we grouped the 15 constituent herbs of DHJST according to their clinical use in one of the following traditional anti-arthritis action mechanisms, blood nourishing, Qi invigorating, and wind/dampness expelling and removing, in order to illuminate the anti-inflammatory and cartilage protection components in such a complex formula. Our data first confirms that DHJST and TH is as effective as DHJST and not just ameliorates arthritic pain symptoms, but they also have chondroprotective effect as both agents

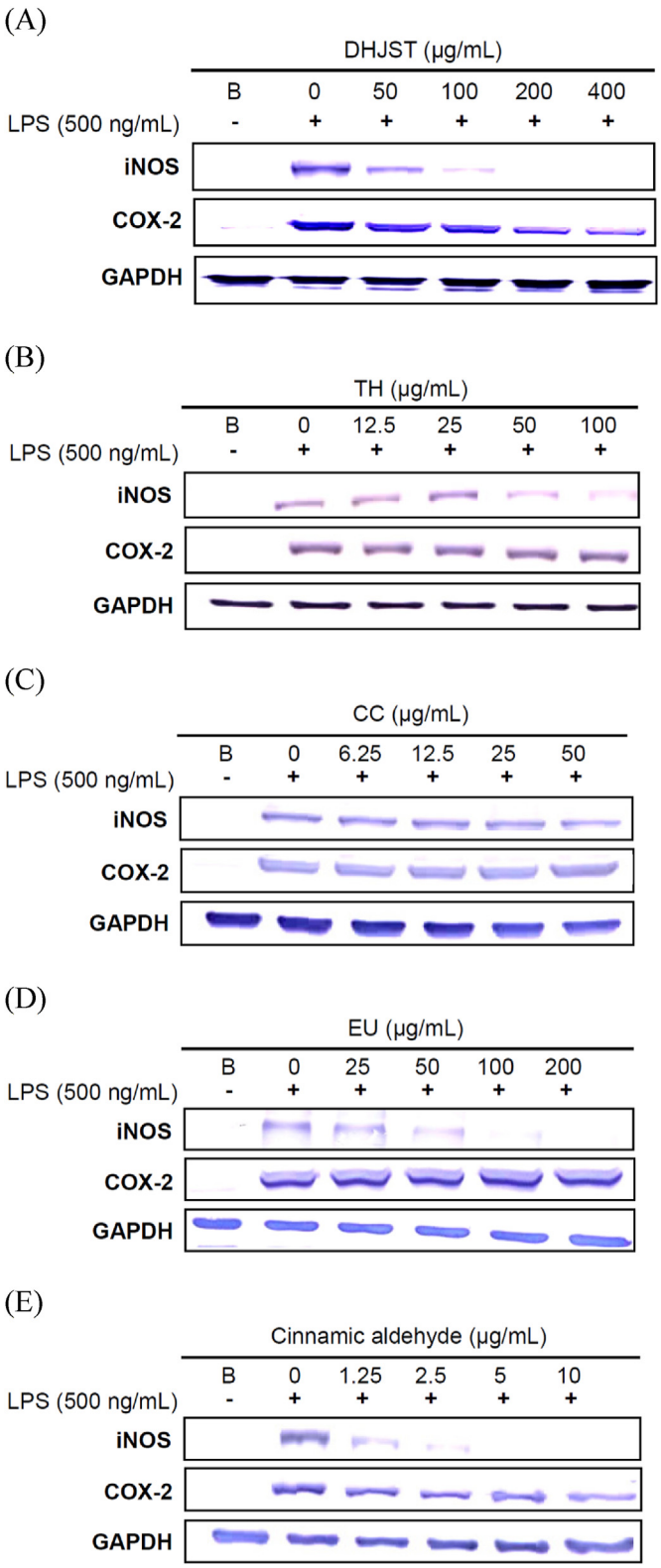

Fig. 6. Regulatory effect on lipopolysaccharide (LPS)-induced iNOS and COX-2 production in RAW 264.7 cells by DHJST (A), TH (B), *Cinnamomum cassia*; (C), *Eucommia ulmoides* (D), and cinnamic aldehyde (E). Abbreviations: CC, *Cinnamomum cassia*; EU, *Eucommia ulmoides*.

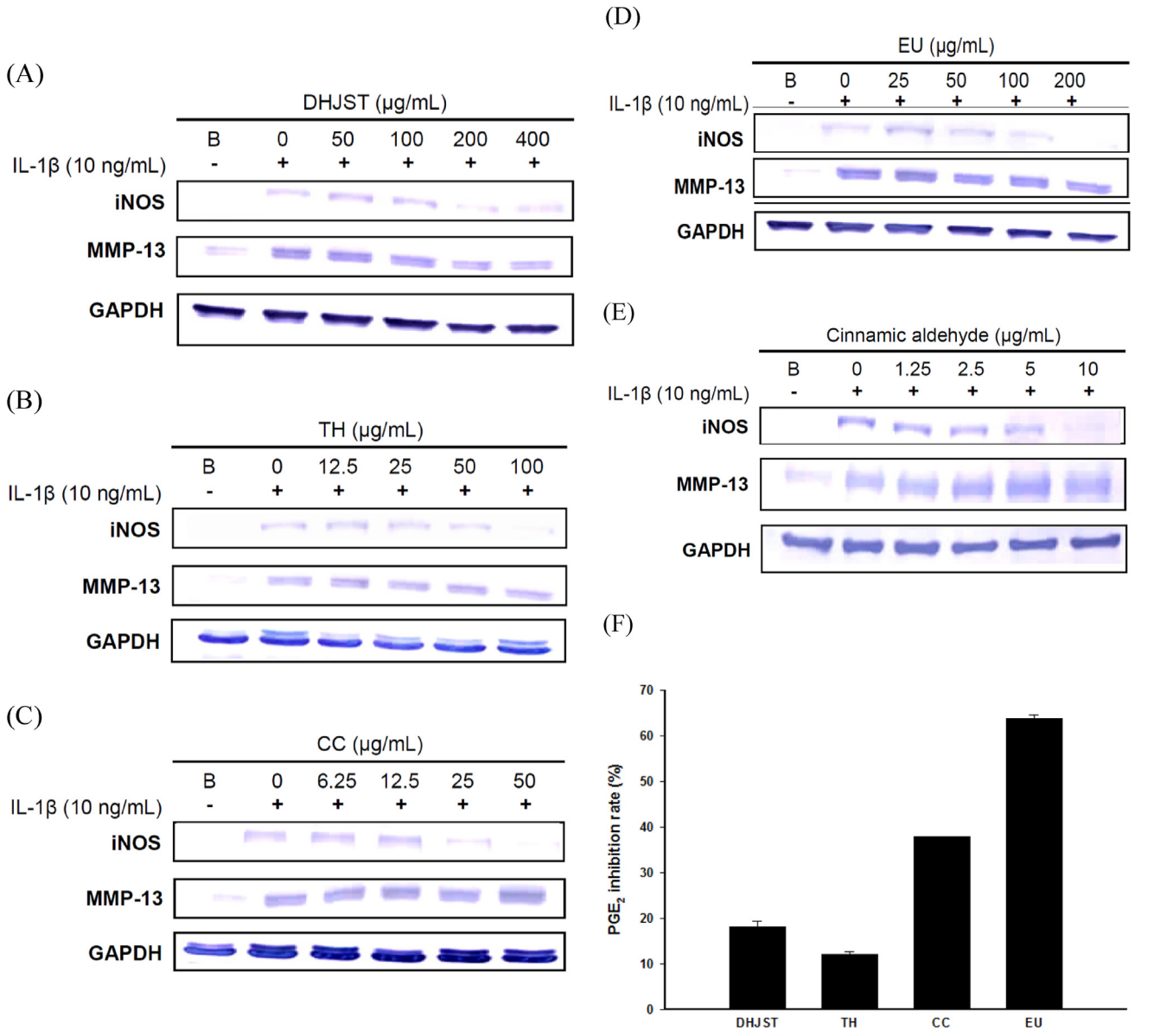

Fig. 7. Regulatory effect on IL-1β-induced iNOS and MMP-13 in PRCs by DHJST (A), TH (B), *Cinnamomum cassia* (C), *Eucommia ulmoides* (D), and cinnamic aldehyde (E). Effect of 50 μg/mL of DHJST, TH, *Cinnamomum cassia* and *Eucommia ulmoides* on PGE_2 level (F), data represented as the mean (±S.D.) of at least three independent experiments, each performed in triplicate. Abbreviations: CC, *Cinnamomum cassia*; EU, *Eucommia ulmoides*.

significantly improved weight-bearing ability, attenuated inflammatory cells infiltration and cartilage degradation in MIA and CIA joints. Our results also suggested that TH, the Qi invigorating group of herbs, contained the anti-inflammatory and joint protective components in DHJST. *Cinnamomum cassia* was the effective anti-inflammatory herb in TH, while cinnamic aldehyde was the major anti-inflammatory active compound in *Cinnamomum cassia*, as the IC_{50} values of tested samples on LPS-induced NO and PGE_2 productions decreased as the extract constituents reduced in complexity.

In TCM, *Cinnamomum cassia* is commonly used for treating colds, fevers, headaches, myalgia, arthralgia, and amenorrhea because of its anti-inflammatory and analgesic activities. Several compounds extracted from this herb showed iNOS and COX-2 suppressive effects.[16] The major anti-inflammatory compound identified in this experiment was cinnamic aldehyde. Through inhibition of NF-κB activation, cinnamic aldehyde was found to have anti-inflammatory and anti-oxidative activities on *Helicobacter pylori*-induced IL-8 secretion, and high glucose-induced damage in cultured dorsal root ganglion neurons.[17,18] Taken together, these results support our findings that cinnamic aldehyde could be used as an anti-inflammatory quality and quantity marker for DHJST.

Preclinical studies have provided sufficient evidences to support that iNOS, COX-2, MMP-13, and PGE_2 are all key targets for protecting joints from degradation during inflammation caused by inducers like IL-1β.[19,20] MMP-13 is over-expressed in the joints and articular cartilage in patients with OA, and is a new target of treatment. In fact, MMP-13 inhibitory activity in activated chondrocytes was reported in two TH constituent herbs, EU and *Panax ginseng*.[21–23] In our study, MMP-13 in IL-1β induced chondrocytes was inhibited by DHJST and TH extracts, and *Eucommia ulmoides*, but not by *Cinnamomum cassia*. We did not perform further study on *P. ginseng* as this herb showed only moderate anti-inflammatory activity on LPS induced macrophages. The results not only revealed

how multiple ingredients act on multiple targets to produce the beneficial effects of DHJST for arthritis treatment, but they also support the notion that the pharmacological effects of combinatory formula could be more than the sum of each individual component. Osteoarthritis is a chronic painful joint disease. Cytokines as TNF-β, IL-1α, IL-6 and IL-17 present in the OA joint can activate proinflammatory cytokines, there should have innervating nociceptors and leading to pain. For examples, TNF-β and IL-1α can stimulate TRPV1 expression and make joints feel hot and painful; IL-17 can increase TRPV4 expression and enhance the sensitivity to mechanical pain.[24] In this study, both DHJST and TH can inhibit the pain of osteoarthritic animal models. Therefore, in addition to DHJST and TH could reduce the production of PGE_2 in chondrocytes, there should be have other cytokine and chemokines targets, such as TNF-α, IL-1β and IL-17 et al. In the future, we will continue to explore whether DHJST and TH can reduce the expression of cytokines and chemokines in chondrocytes, synovial cells and other cells in the joints.

Du Huo (*A. pubescens*) is a widely used medicinal herb for arthritic treatment because of its anti-inflammatory, analgesic, and antioxidant properties through bioactive coumarin compounds.[25–28] Columbianectin, one of the coumarins obtainable from *A. pubescens*, has been reported to inhibit IL-1β, IL-6, IL-8, and TNF-α level in activated mast cells.[28–30] Du Huo is also a major herb in DHJST under the theory of TCM. However, because as a constituent in WDH group and the inhibitory activity of WDH on NO production by LSP induced RAW 264.7 cells was modest among the three compound recipes, Du Huo was not further explored in the current study either. However, due to the anti-inflammatory nature of Du Huo and columbianectin, investigation is worthwhile to illuminate whether this herb and its phytoconstituents are beneficial in the treatment of inflammatory arthritis in future clinical practice.

Drugs or therapies that could modify pathological structure and alleviate symptoms in the model animals may lead to the development of disease modifying drug for human arthritic disorders including osteoarthritis. Inflammatory reaction promotes cartilage destruction in animal models of arthritis such as the two models used in current study. CIA models have been extensively used in rheumatoid arthritis research. MIA model is frequently used as an acute osteoarthritis model because the chemical induce rapid cartilage degradation. Our results confirm the anti-osteoarthritic and anti-inflammatory effects of DHJST and the simplified formula TH by providing evidences from moderating inflammatory cytokine levels, weight-bearing distribution, and histopathological changes.

5. Conclusion

In our study, DHJST reduced paw swelling, weight bearing distribution, analgesic latency ratio of.collagenase type II- and MIA-induced arthritis in rats. The anti-inflammatory and joint protective mechanisms of DHJST were through inhibition of iNOS, COX-2, and MMP-13 expressions and PGE_2 production. Among the three simplified formulae we tested, TH, the Qi tonic, showed similar anti-inflammatory joint protective mechanism and effect as DHJST. A TCM prescription usually include at the same time the jun (or sovereign), chen (or minister), zuo (or assistant) and shi (or messenger) ingredient drugs. As *Cinnamomum cassia* showed the most potent anti-inflammatory effect among the 15 constituent herbs, it could be the sovereign medicine. *Eucommia ulmoides* could more inhibit MMP-13 expression than *Cinnamomum cassia*, therefore we suggested *Eucommia ulmoides* could be the minister medicine in DHJST or TH to help the action of *Cinnamomum cassia*. Cinnamic aldehyde is the active compound in *Cinnamomum cassia* and is suitable to be used as a quality control marker of DHJST. In the future, incorporation of the TCM network pharmacological approach with empirical evidence from traditional clinical experience and mechanistic studies will enhance the modern use of DHJST or the simplified version TH as effective anti-arthritic agents. Further toxicity and clinical studies are also warranted to establish more detailed data on the crude extracts and pure compounds.

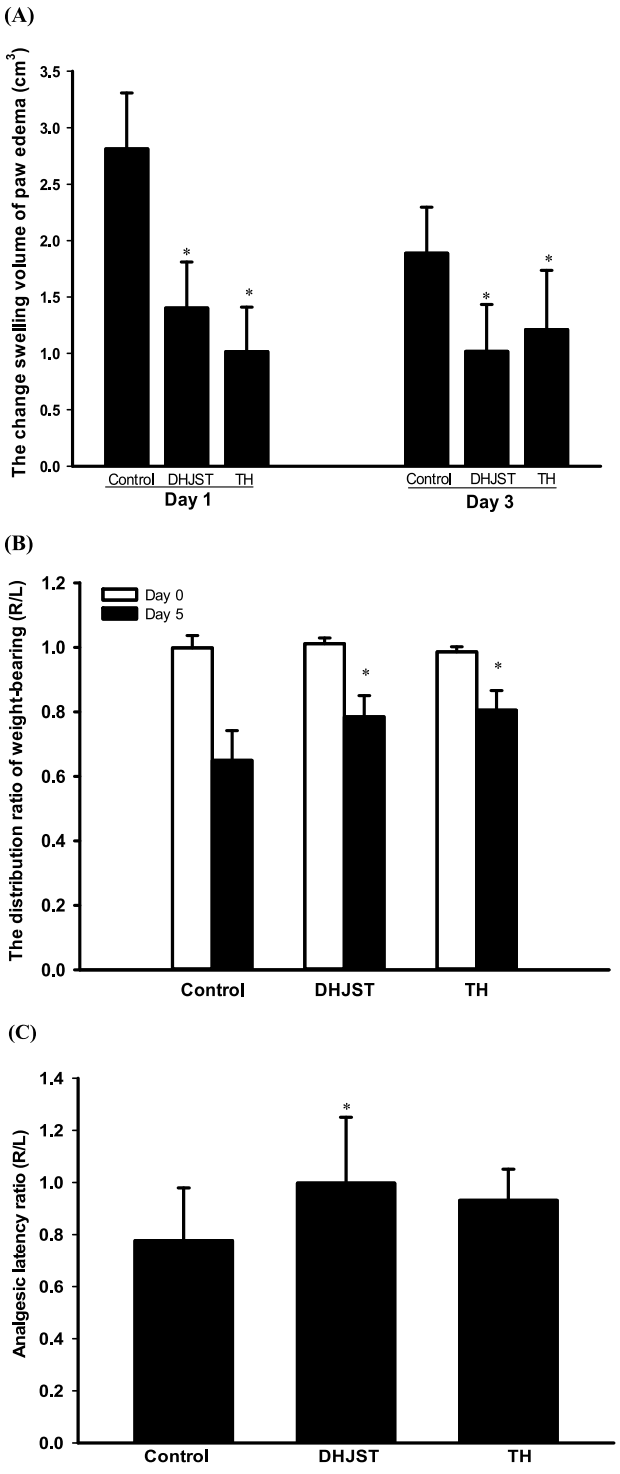

Fig. 8. Effects of DHJST and TH on MIA-induced paw edema swelling on days 1 and 3 (A). hind-limb weight-bearing on day 5 (B); hot-plate latent pain responses on day 10 (C). *$p < 0.05$, compared to the control. $n = 3$.

These results also can be used to guide future research into the effectiveness of DHJST with more convenient preparations in treating human arthritis with inflammatory symptoms.

Declaration of competing interest

The author reports no conflicts of interest in this report.

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