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Anti-Inflammatory Effects of the Chemical Compounds Obtained from *Celastrus hindsii* in RAW264.7 Cells

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Celastrus hindsii **(family Celastraceae) is located abundantly in the United States, China, and Vietnam, where it is utilized as a traditional herbal and traditional drug for the care of cancer. However, the antioxidant and anti-inflammatory effects of *Celastrus hindsii* extract are unknown. In our research, the antioxidant activity of *Celastrus hindsii* leaf extract was investigated, and then anti-inflammatory efficacy of *C. hindsii* extract in lipopolysaccharide (LPS)-induced RAW264.7 cells. First, our results revealed that *C. hindsii* extract showed powerful antioxidant capability. Moreover, the application of *C. hindsii* extract significantly reduced nitric oxide (NO) production without cytotoxic effects. Furthermore, *C. hindsii* extract reduced the generation of pro-inflammatory cytokines, like as interleukin (IL)-6, IL-1β, and TNF-α. Our results are the first to confirm the anti-inflammatory capability of *C. hindsii* extract in RAW264.7 cells.**

Keywords: **Anti-inflammatory, *Celastrus hindsii*, nitric oxide, proinflammatory cytokines, RAW264.7 macrophages**

Introduction

The inflammatory response is a complicated process mediated by diverse immune cells activation. Macrophages, type of immune cells, are essential for regulating the inflammatory reactions by releasing inflammatory mediators like as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and nitric oxide (NO) [1–3].

NO, a free radical, is generated from guanidino nitrogen of L-arginine and is oxidized by NO synthase [4]. NO plays a crucial role in the innate immune reactions to pathogens, like as viruses, and bacteria *etc* [5, 6]. Furthermore, although NO plays a crucial role for regulating the physiological functions, such as neurotoxicity and neurotransmission, abnormal NO generation can lead to the occurrence of inflammatory illnesses like as autoimmune disorders and rheumatoid arthritis [7–9]. Therefore, suppression of NO production is an important strategy for utilize as an anti-inflammatory drug.

Lipopolysaccharide (LPS) is an abundantly present in cell walls of gram-negative bacteria. It can upregulate the generation of inflammatory mediators in macrophages, resulting in tissue damage and an acute inflammatory response to pathogens. For this reason, LPS-stimulated RAW264.7 cells are a superior *in vitro* model for the investigation of potential inhibitors of inflammatory responses [10–12].

Celastrus hindsii (Celastracea family) is abundantly located in United States, China, and Vietnam [13, 14]. In Vietnam, *C. hindsii* exists in nature and is usually located in various provinces, like as Hoa Binh, Quang Ninh, Ninh Binh *etc*. *C. hindsii* is utilized as a traditional drug to care various illnesses and has anti-inflammatory, anticancer, and antitumor properties [13, 15]. This plant contains various bioactive compounds [13, 16] that exhibit antiviral [16], and anticancer activities [17, 18]. Especially, previous phytochemical studies of

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plants the genus *Celastrus* have found flavonoids, alkaloids, and terpenoids *etc* [13, 16].

However, the antioxidant activity and anti-inflammatory efficacy of *C. hindsii* extract are elucidated. This study confirmed whether *C. hindsii* extract suppressed the creation of pro-inflammatory cytokines in RAW264.7 cells treated with LPS.

Materials and Methods

Chemicals and reagents

C. hindsii leaves were collected from the Bac Giang Province, Vietnam, in June 2019. The leaves were lyophilized to obtain yield of 31% and kept at -20℃ until experimental use. 2,2'-azinobis-(3-ethyl-benzothiazoline)-sulfonic acid (ABTS) and 2,2-diphenyl-picryl-hydrazyl (DPPH) were offered from the Sigma-Aldrich (USA). LPS from *Escherichia coli* O111:B4 was obtained from Sigma Chemical (USA). RAW264.7 (TIB71) was offered from the American Type Culture Collection (USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin (10,000 U/ml) were purchased from Welgene (Korea).

Extraction from *C. hindsii* leaves

The compounds from the dried leaf powder were extracted using double-distilled water (ddH_2O), 95% (1:50 w/v) ethanol, methanol, or n-hexane in a rotary shaker at room temperature (RT) during 24 h. Then, each mixture was centrifuged (1500 ×*g*, 5 min), filtered utilizing Whatman No. 1 filter paper, evaporated at 37℃, and lyophilized to obtain the crude extracts named WCH (water extraction), 50ECH (extraction by 50% ethanol), 80ECH (extraction by 80% ethanol), 95ECH (extraction by 95% ethanol), MCH (methanol extraction), and n-HCH (n-hexane extraction). All the extracts were kept until further use at -20℃.

Determination of total phenolic (TP) and total flavonoid (TF) contents

Total phenolic content (TPC) and total flavonoid content (TFC) were confirmed as described in previous studies [19, 20]. In brief, 0.125 ml of Folin-Ciocalteu reagent was added to the sample solution followed by 1.25 ml of 7% Na_2CO_3. The solution mixture was adjusted to a volume of 3 ml with methanol, combined thoroughly, and reacted at RT in the dark for 30 min. Thereafter, the absorbance was confirmed at 765 nm utilizing a microplate reader (BioTek Instruments, USA). TPC was presented as milligrams of gallic acid equivalents per gram of extract or fraction (mg GAE/g of extract) following a standard curve.

To determine TFC, the sample solution was added with 0.6 ml of 5% $NaNO_2$ solution. Then, 0.5 ml of $Al(NO_3)_3$ (10% w/v) was mixed and 3.0 ml 4.3% NaOH was added followed by incubation for an additional 6 min before adding 70% EtOH. After incubation at RT in the dark for 15 min, the absorbance was confirmed at 430 nm. Rutin was utilized as a reference and the TFC was determined according to a standard curve and represented as milligrams of rutin equivalent per gram of extract or fraction (mg RE/g of extract).

Antioxidant activity of leaf extracts from *C. hindsii*

The cationic radical scavenging activity of the *C. hindsii* leaf extract was confirmed by making a mixed solution of ABTS (7.5 mM) and potassium persulfate (2.5 mM), keeping it in the dark during 15 h, then adding the ABTS solution to the *C. hindsii* leaf extract and reacting at RT during 90 min, followed by absorbance at 414 nm [21].

Free radical scavenging activity was confirmed by adding 0.2 mM DPPH solution to *C. hindsii* leaf extract and reacting at RT during 30 min, followed by absorbance at 517 nm [22].

Cell culture

RAW264.7 cells were maintained in DMEM (Welgene, Korea) supplemented with 10% inactivated FBS (Welgene), 10,000 U/ml penicillin-streptomycin (Welgene) at incubator (37℃, 5% CO_2).

Cell viability

Cell viability was measured using the EZ-Cytox assay kit (Daeil Lab Service Co., Korea). RAW264.7 cells (1×10^4 cells/well) were cultured in 96-well plates and incubated incubator (37℃, 5% CO_2) during overnight. Then, cells were induced with LPS (10 μg/ml) and various concentrations of 95ECH (0, 10, 30, 60, 120, 240, 480, or 960 μg/ml). Following incubation at 37℃ during 24 h, EZ-Cytox reagent (10 μl) was mixed to each well. After 24 h, absorbance were confirmed at 450 nm utiliz-

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ing a microplate reader (BioTek Instruments). The cell viability of the control, which is untreated cells, was considered 100%.

Determination of NO production

To determine NO production from the cell culture supernatants, the Griess assay was evaluated as described in a previous study [23]. RAW264.7 cells (1×10^5 cells/well) were cultured in 24-well plates and maintained in an incubator overnight. Cells were reacted without or with LPS (10 μg/ml) during 24 h in the presence of various concentrations of 95ECH (0, 10, 30, 60, or 120 μg/ml). 100 μl of Griess reagent (Sigma Chemical) combined with cell supernatant. Following 10 min of incubation, the absorbance was confirmed at 540 nm utilizing a microplate reader and NO concentration was calculated utilizing the standard curves of sodium nitrite ($NaNO_2$).

Enzyme-linked immunosorbent assay (ELISA)

To harvest the supernatant, centrifuge the cell culture medium at 4000 rpm at 4℃ during 10 min. The levels of IL-6, IL-1β, and TNF-α were confirmed utilizing commercially available ELISA kits (Neobiosciences, China) followed by the manufacturer's protocols. The levels of cytokines in the samples were confirmed from a standard curve generated utilizing a known concentration of recombinant IL-6, IL-1β, and TNF-α.

Real-time reverse-transcription polymerase chain reaction (RT-PCR)

RAW264.7 cells (1×10^6 cells/well) were cultured in 6-well plate and maintained during overnight in an incubator. Cells were induced with LPS (10 μg/ml) or untreated (Control) in the presence or absence of various concentrations of 95ECH (0, 10, 30, 60, or 120 μg/ml) during 24 h. LPS (10 μg/ml)-treated cells during 24 h served as negative control (sample "0 μg/ml"). Following 24 h of induction, total RNA was extracted from the cells utilizing the TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc., USA) followed by manufacturer's method. RNA (2 μg/μl) was used to obtain first-strand cDNA in a final reaction volume of 20 μl utilizing PrimeScript® 1st strand cDNA Synthesis kit (Takara Bio, Inc., Japan). qRT-PCR was conducted utilizing the SYBR® Select Master Mix (Applied Biosystems, USA) and analyzed on a 7300 Real Time PCR System (Applied Biosystems). The PCR condition was as follows: 95℃ (2 min), followed by 40 cycles at 95℃ (15 s) and 60℃ (30 s), with a final extension at 72℃ (20 s). The primers used are listed in Table 1. The relative expression levels were calculated utilizing the $2^{-\Delta\Delta Cq}$ method and normalized with that of β-actin, which was utilized as an internal control.

Table 1. Sequences of the primers utilized for real-time reverse-transcription PCR.

Gene		Primer sequence
IL-6	Forward	5′-CTTCTTGGGACTGATGCTGGTG-3'
	Reverse	3′-CGCTGGCTTTGTCTTTCTTGTTA-5'
IL-1β	Forward	5′-GTC ACA AGA AAC CAT GGC ACA T-3'
	Reverse	5′-GCC CAT CAG AGG CAA GGA-3'
TNF-α	Forward	5′-GTC ACA AGA AAC CAT GGC ACA T-3'
	Reverse	5′-GCC CAT CAG AGG CAA GGA-3'
β-actin	Forward	5′-CCT TCC TTC CTG GGC ATG GAG-3'
	Reverse	5′-CTC AGG AGG AGC AAT GAT CTT GAT-3'

Statistical analyses

The data are presented as the mean ± standard deviation (S.D.). Data were analyzed using SPSS 22.0 software using the Student's *t*-test for difference between two groups and using ANOVA (non-parametric) followed by the post-hoc Tukey's multiple comparison test for more than two groups. Results with either $p < 0.05$ or $p < 0.01$ were considered statistically significant.

Results and Discussion

Total phenolic (TP), total flavonoid (TF) contents, and antioxidant activity of *C. hindsii* leaf extracts

C. hindsii is utilized as a traditional herbal drug for treating cancer [13, 15]. This plant contains various bioactive compounds [13, 16] that exhibit antiviral [16], and anticancer activities [17, 18]. However, the antioxidant activity and anti-inflammatory effects of *C. hindsii* extract are unknown.

Table 2 shows the antioxidant activity of TPC and TFC, and antioxidant activity of *C. hindsii* leaf extracts. Among the extracts, 95ECH showed higher TPC (291.48 ± 0.145 mg GAE/g extract) than that seen with MCH, n-HCH, and WCH extracts with 93.48 ± 2.01, 15.48 ± 0.08, and 137.88 ± 0.712 mg GAE/g extract, respectively. The

Table 2. Total phenolic (TP) and total flavonoid (TF) contents and *in vitro* antioxidant activity of extracts from *C. hindsii* leaf.

Extracts	IC_{50} DPPH (μg/ml)	IC_{50} ABTS (μg/ml)	TPC (mg GAE/g extract)	TFC (mg RE/g extract)
WCH	163.01 ± 0.54^{C}	243.62 ± 0.54^{C}	137.88 ± 0.712^{ef}	88.09 ± 0.34^{e}
95ECH	89.05 ± 0.38^{b}	101.04 ± 0.65^{b}	291.48 ± 0.145^{f}	140.81 ± 0.481^{f}
MCH	-	-	93.48 ± 2.01^{e}	42.63 ± 0.176^{e}
n-HCH	50.50 ± 0.12^{a}	33.15 ± 0.12^{a}	15.48 ± 0.08^{d}	9.91 ± 0.258^{d}

The data reported as mean ± SD (n = 3); $^{a–d}$: Similar letters in a column indicated non-significantly different ($p < 0.05$); - = Not detected; TPC = total phenolic content; TFC = total flavonoid content.

highest content of TF was also detected in 95ECH (140.81 ± 0.481 mg RE/g extract). This indicated that 95% ethanol was a more efficient extractant than the others for obtaining *C. hindsii* extract. Hence, we used 95ECH for the subsequent experiments.

Furthermore, the results in Table 2 also revealed the antioxidant potential of *C. hindsii* as reflected by scavenging activities of both ABTS and DPPH radicals, represented by IC_{50} values. Of these samples, the maximum DPPH and ABTS activities were found in WCH, compared to 95ECH (89.05 ± 0.38; 101.04 ± 0.65, respectively) and n-HCH (50.50 ± 0.12; 33.15 ± 0.12, respectively), and they were not detected in the MCH. As the previous study [24], there was three main factors of phenolic content could effects on the scavenging activity of the plant extract includes 1) the different corresponding of the same levels phenolics to the same antioxidant properties; 2) the various methods which utilized to confirmed the antioxidant activity are basis of different response signals, they often give different results; and 3) extracts are complex combinations of many different chemicals with varying polarities, antioxidant and prooxidant capabilities, and can show synergistic effects when compared to isolated substances. Moreover, the different chemical structure could lead to the different results of the Folin-Ciocalteu assay. For these reasons, the radical scavenging activity of an extract cannot be predicted based on TPC. Especially, our result was similar to the previous study of Viet *et al.* [15]. Taken together, our results showed that *C. hindsii* extract obtained using water and ethanol possessed strong antioxidant capacity.

Effects of *C. hindsii* extract on RAW264.7 cell viability

To demonstrate the cell viability of *C. hindsii* extract on RAW264.7 cells, they were incubated with LPS (10 μg/ml) and various concentrations of *C. hindsii* extract (0, 10, 30, 60, 120, 240, 480, or 960 μg/ml) for 24 h. Our results demonstrated that 10 μg/ml of LPS had no effect on the cytotoxicity of RAW264.7 cells as revealed in Fig. 1. In particular, when cells were incubated with 10–120 μg/ml *C. hindsii* extract, there was no difference between cells treated with LPS (sample 0 μg/ml) and untreated cells (Control). The MTT assay showed that cell viability did not decrease when treated with *C. hindsii* extract (120 μg/ml). Therefore, *C. hindsii* extract were treated up to a concentration of 120 μg/ml in RAW264.7 cells depending on cell viability.

Effects of *C. hindsii* extract on NO generation in LPS-induced RAW264.7 cells

Nitrite oxide (NO) generation is supposed to be

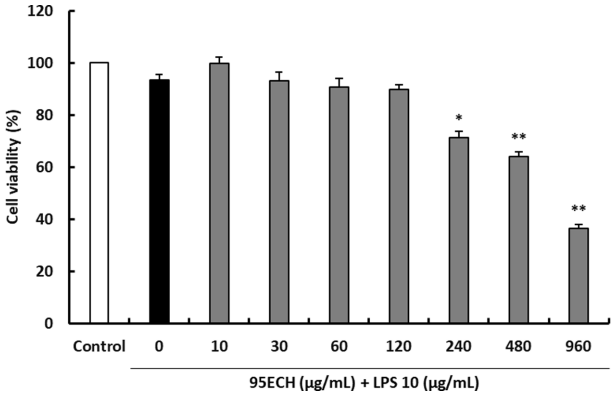

Fig. 1. Effect of *C. hindsii* extract on RAW264.7 cell viability. Cells were treated with various concentrations of *C. hindsii* extract (0, 10, 30, 60, 120, 240, 480, or 960 μg/ml) and LPS (10 μg/ml) for 24 h. Cell viability was confirmed utilizing the EZ-Cytox kit. The results are presented as the mean ± S.D. of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to only LPS treatment (sample 0 μg/ml).

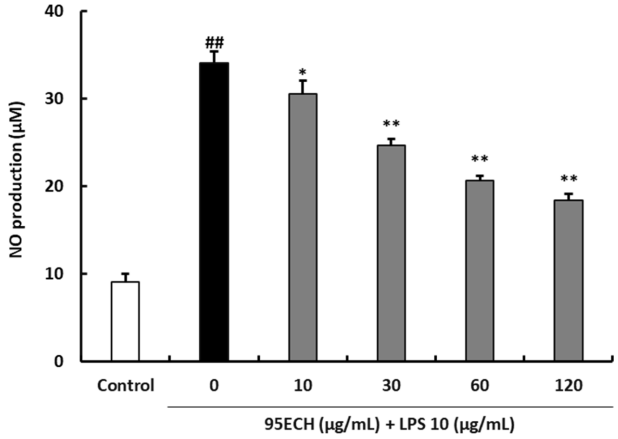

Fig. 2. Effect of *C. hindsii* extract on NO production. The levels of NO were measured in the culture medium utilizing Griess reagent. The results are presented as the mean ± S.D. of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to only LPS treatment (sample 0 μg/ml), $^{\#\#}p < 0.01$ as compared to the control.

involved in the body inflammatory activities throughout the iNOS expression [25]. The previous studies revealed the excessive NO synthesis was the main cause that led to some autoimmune diseases such as rheumatoid arthritis [9]. Therefore, NO synthesis suppression is an important strategy used for anti-inflammatory therapeutics. In our study, we examined the possibility of *C. hindsii* extract to inhibit LPS-induced NO production. Based on our findings, 10 μg/ml of LPS treatment increased NO production. By contrast, treatment with *C. hindsii* extract at 10, 30, 60, or 120 μg/ml decreased LPS-mediated NO production (Fig. 2).

Effects of *C. hindsii* extract on the secretion of inflammatory cytokines by LPS-induced RAW264.7 cells

In inflammation, macrophages are associated with anti-inflammatory functions by participating in the upregulated synthesis of TNF-α and related interleukin (IL)-6 and IL-1β in LPS stimulation. As a result, macrophages can self-tissue damage and inflammatory diseases, and even autoimmune disorders. To analyze the potential anti-inflammatory effects of *C. hindsii* extract, we first investigated whether *C. hindsii* extract influenced the secretion of pro-inflammatory cytokines by LPS-induced RAW264.7 cells. Expression levels of IL-6, IL-1β, and TNF-α were significantly increased in the culture medium of LPS-induced group (sample 0 μg/ml). However, *C. hindsii* extract suppressed the expression of IL-6, IL-1β, and TNF-α in LPS-treated RAW264.7 cells (Fig. 3).

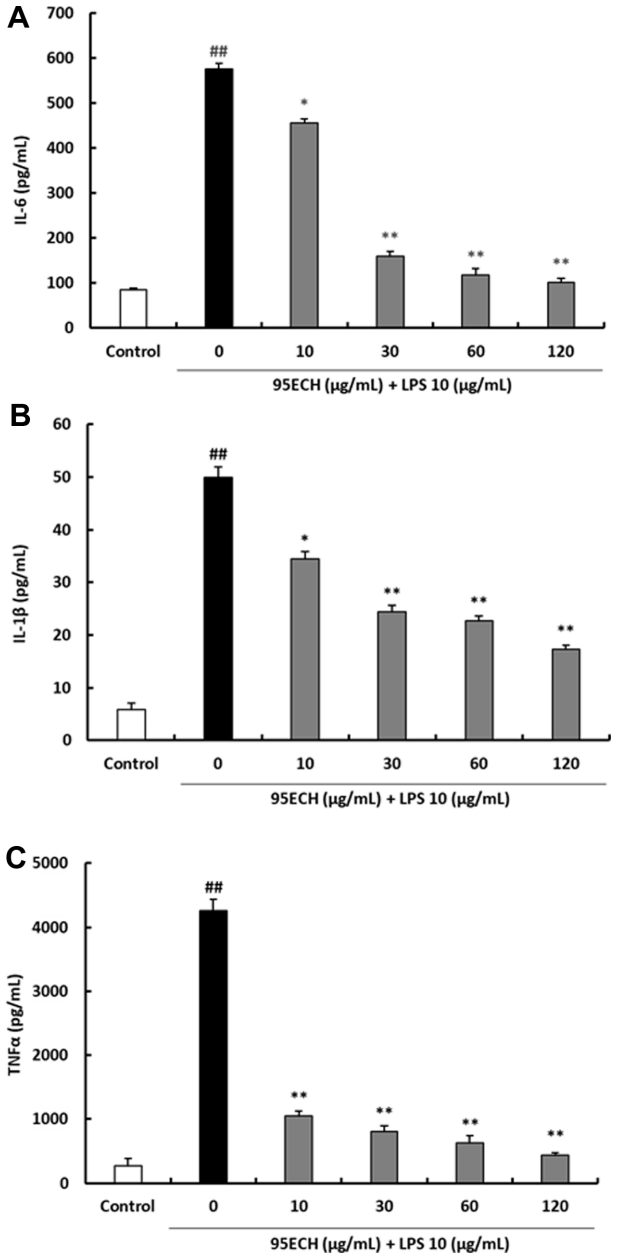

Fig. 3. Effects of *C. hindsii* extract on the secretion of IL-6 (A), IL-1β (B), and TNF-α (C) by RAW264.7 cells. Cells were treated with various concentrations of *C. hindsii* extract (0, 10, 30, 60, or 120 μg/ml) and LPS (10 μg/ml) during 24 h. Expression levels of IL-6, IL-1β and TNF-α were confirmed using commercially available ELISA kits. Each bar represents the mean ± S.D. ($n = 3$). Values with different letters represent statistically significant differences. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to only LPS treatment (sample 0 μg/ml); $^{\#\#}p < 0.01$ as compared to control.

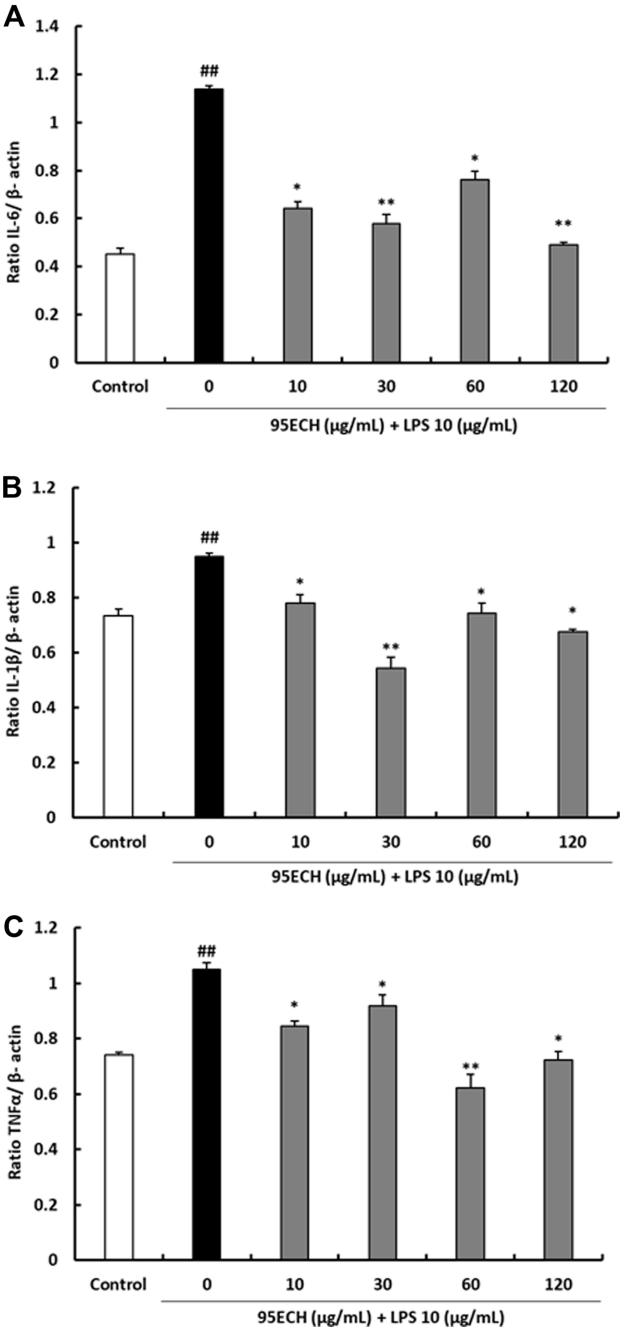

Fig. 4. Effects of *C. hindsii* extract on the mRNA expression levels of IL-6, IL-1β, and TNF-α in RAW264.7 cells. Cells were treated with various concentrations (0, 10, 30, 60, or 120 μg/ml) of *C. hindsii* extract and LPS (10 μg/ml) was added and incubated during 24 h. Control values were obtained in the absence of LPS. The values are presented as the mean ± S.D. of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to only LPS treatment (sample 0 μg/ml); $^{\#\#}p < 0.01$ as compared to control. Expression levels of β-actin were used to verify that the initial cDNA contents of the samples were similar.

Effects of *C. hindsii* extract on the mRNA levels of pro-inflammatory cytokines in LPS-induced RAW264.7 cells

Further, we investigated whether *C. hindsii* extract affected the mRNA expression of pro-inflammatory cytokines in LPS-treated RAW264.7 cells. Expression of IL-6, IL-1β, and TNF-α mRNA were evaluated using RT-PCR. The results showed that *C. hindsii* extract suppressed the mRNA expression of IL-6, IL-1β, and TNF-α in LPS-treated RAW264.7 cells (Fig. 4). Our results certainly demonstrated the anti-inflammatory activity of *C. hindsii* extract in upregulated inflammation in RAW264.7 cells treated wih LPS.

Phenolics and flavonoid molecules are thought to providing various of other health advantages. The antimicrobial and antioxidant activity of flavonoid and phenolic compounds in *C. hindsii* has been reported previously [13, 26, 27]. Moreover, followed by Kuo *et al* [28], four chemical compounds in *C. hindsii* leaves were found, which showed high potential antitumor activities. It possessed to inhibit the cell viability of HEPA-2B, HELA, COLO-205, and KB cells, as well as against HIV replication activity in H9 lymphocytes *in vitro* [28]. Interestingly, *C. hindsii* contains rosmarinic acid molecules, which are recognized to be natural antioxidants. These compounds were shown to have anti-inflammatory potential by lowering the concentrations of LDH and transaminases (ALT and AST) in liver ischaemia–reperfusion mice, as well as causing damage and considerably lowering multi-organ dysfunction markers (kidney, lung, and liver) in a thermal injury model by regulating NF-κB and MMP-9 [29]. However, the anti-inflammatory efficacy of *C. hindsii* extract in RAW264.7 cells are unknown, so in this study, we determined whether *C. hindsii* extract inhibited the generation of pro-inflammatory cytokines in RAW264.7 cells treated with LPS.

In this research our results showed that *C. hindsii* extract obtained using water and ethanol possessed strong antioxidant capacity. Moreover, we offer evidence that *C. hindsii* extract has anti-inflammatory effects. Herein, we first revealed the effect of *C. hindsii* extract on LPS-stimulated upregulation of pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) and NO generation. We have provided the anti-inflammatory effects of *C. hindsii* extract, which has excellent potential as novel therapeutic agent for human health. However, further research is required to elucidate the signaling of anti-

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inflammatory efficacy of *C. hindsii* extract in RAW264.7 cells as well as in an *in vivo* model of inflammation.

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Conflicts of Interest

The authors have no financial conflicts of interest to declare.

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