Anti-Inflammatory Effects of *Chrysanthemum indicum* Water Extract in RAW 264.7 Cell as a Whole Plant

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Purpose: *Chrysanthemum indicum* (CHI) has been used for edible and medical purposes for a long time in Korea. The purpose of this study was to evaluate the anti-inflammatory effects of CHI water extract in lipopolysaccharides (LPS)-induced RAW 264.7 macrophage cells. **Methods:** To investigate the anti-inflammatory effects on LPS-induced RAW 264.7 macrophage cells, CHI extract as a whole plant was used in this study. RAW 264.7 cells were treated with various concentrations of CHI extract (1, 10, and 100 μg/mL). After that Nitric Oxide (NO), inducible nitric oxide synthase (iNOS), interleukin (IL)-1β, cyclooxygenase (COX)-2 and prostaglandin E_2 (PGE_2) expression level were measured. **Results:** CHI extract significantly suppressed the LPS-induced NO production and decreased the level of iNOS, IL-1β, COX-2 messenger ribonucleic acid (mRNA) expression and also the down regulation of PGE_2 expression in a dose-dependent manner. **Conclusion:** The present study suggested that CHI extract can be substituted for anti-inflammatory drugs and provide a safe and effective non pharmacological therapeutic approach.

Key Words: Anti-inflammation; *Chrysanthemum indicum*; RAW 264.7 cell

INTRODUCTION

Inflammation is nonspecific response to tissue injury or microbial invasion, and its pathologic processes are complex. Inflammatory response is regulated by immune cells releasing different types of cytokines and inflammatory enzymes [1]. Macrophages are known to be critical immune cells in various inflammation responses [2]. Macrophages, which are activated by lipopolysaccharides (LPS), secrete various inflammatory cytokines and enzyme such as tumor necrosis factor (TNF)-α, Nitric Oxide (NO), inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, interleukin (IL)-1β and prostaglandin E_2 (PGE_2) [3]. RAW 264.7 macrophages are widely used in inflammation models to assess anti-inflammatory effect and usually treated with LPS to induce inflammatory response.

LPS are the components of the outer membrane of Gram-negative bacteria. They act as endotoxins, leading to tissue injury. Activated LPS increase the production of pro-inflammatory cytokines, as previously mentioned, from macrophages or monocytes [4]. NO, an endogenous free radical, plays an important role in host defense, immunity and modulation of inflammatory responses, and is produced by a family of NOS such as endothelial NOS (eNOS), neuronal NOS (nNOS) and iNOS. In particular, iNOS, which is induced by LPS, produces large amount of nitric oxide [5].

The overproduction of nitric oxide can promote tissue injury and contribute to the progression of diseases such as sepsis, ulcerative colitis, diabetes, rheumatoid arthritis, osteoarthritis, and other diseases [6,7]. Several studies have reported that the inhibition of nitric oxide production through the down-regulation of iNOS expression, has anti-inflammatory effects [8,9]. In addition, experimental animal models studies have also have shown that the reduced production of iNOS attenuates

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systemic lupus erythematosus (SLE), Crohn's disease and osteoarthritis [10-12]. COX is inflammatory enzyme to convert arachidonic acid to prostaglandins which are divided into two subtypes such as COX-1 and COX-2. Among them, COX-2 is related to inflammatory response. PGE_2, which is produced by COX-2, is the cause of pain and fever, and also well-known to promote angiogenesis in cancer conditions [13].

Currently nonsteroidal anti-inflammatory drugs (NSAIDs) are the most commonly used for the treatment of inflammatory diseases through the suppression of COX activity, but it is well known that they have gastrointestinal side effects such as indigestion, stomach upset or gastritis and other common side effects of NSAIDs include headache, raised liver enzymes, dizziness, hypertension [14]. In order to reduce those side effects, recent studies are being focused on searching new anti-inflammatory agents, which are based on biosynthesis using a herb, such as green tea, mushroom and compositae plant [2,15,16]. Among them, compositae plant is a flowering plant which spreads all over the world and has been used for edible and medical purposes for a long time in Korea. And there are several types of compositae plants such as Dendranthema zawadskii var. *latilobum*, Dandelion (*Taraxacum officinale*) and *Chrysanthemum indicum* (CHI) [17]. Recent studies have demonstrated that CHI has high anti-inflammatory and antioxidant effect through the down-regulation of IL, TNF-α expression. But most of the previous studies were used a specific part of a compositae plant such as stem, leaf and flower, which were extracted using an ethanol [8,16-19]. Few of existing studies, except one that analyzed the antioxidative effects of Dandelion [20] that is one kind of CHI, have attempted to examine the anti-inflammatory effect of CHI using water. Instead, most of them used ethanol to examine the anti-inflammatory effect of CHI focusing on a specific part of the plant such as root, stem, or leaves. Therefore, the present study was aimed to verify whether all components of CHI have anti-inflammatory effect, when they were extracted under the same conditions and the result of this study will provide an evidenced-base of safe and effective non-pharmacological therapeutic approach.

MATERIALS AND METHODS

1. Reagents

LPS and Dulbecco's modified Eagle medium (DMEM) were purchased from Sigma-Aldrich Corporation (St. Louis, USA). Primary antibodies for iNOS, COX-2, actin, and the peroxidase-conjugated second-

ary antibodies were purchased from Santa Cruz Biotechnology (Santa cruz, CA, USA).

2. Sample preparation

The dried flowers of CHI were extracted with distilled water (dH_2O) (100 g, dry weight, whole plant), and the extract was then concentrated under reduced pressure. The decoction was filtered through a 0.45 μm filter, lyophilized and stored at -20℃. The lyophilized powder was dissolved in 0.1% dimethyl sulfoxide (DMSO) and then diluted in phosphate buffered saline (PBS).

3. Cell culture

RAW 264.7 cells were obtained from Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were cultured in a 5% CO_2 incubator at 37℃ with DMEM supplemented with 10% fetal bovine serum (FBS) and 1% ampicillin and streptomycin. The entire reagents for cell culture were purchased from Life Technologies (Grand Island, NY, USA). Treatments with LPS and/or CHI extract were carried out under serum-free conditions.

4. NO assay and cell viability assay

In order to determine the NO production, RAW 264.7 cells were added to a 96-well plate at a density of 3×10^4 cells/well and cultured for 16 hours in DMEM medium. Cells were treated with various concentrations (1, 10, and100 μg/mL) of CHI extract and then incubated with LPS (100 ng/mL) for 24 hours. The supernatant (50 μL) was harvested and mixed with an equal volume of Griess reagent (Sigma, MO, USA) in a 96 well plate for 10 minutes at a room temperature. Finally, the absorbance values were measured at 570 nm using a microplate reader (Molecular Devices, CA, USA). At this time, sodium nitrite was used as a standard to calculate NO concentration. The 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used for the effect of CHI on cell viability. Cells were cultured in a 96-well plate at a density of 3×10^4 cells/well and treated with various concentrations (1, 10, and 100 μg/mL) of CHI extract for 24 hours. And then the supernatant was discarded and MTT solution 100 μL (5 μg/mL) was added to each well and incubated for 1 hour at 37℃ then the absorbance values were measured at 570 nm using a microplate reader.

5. Real time polymerase chain reaction (RT-PCR)

iNOS, COX-2, and IL-1β messenger ribonucleic acid (mRNA) expres-

sion levels were measured by real time PCR. RAW 264.7 cells were cultured in a 6-well plate for 24 hours then they were treated with both CHI extract (1, 10, and 100 μg/mL) and LPS (100 ng/mL) and cultured for another 24 hours. The harvested cells were washed with PBS, total RNA was isolated with Tri- reagent (Trizol) according to the manufacturer's instructions, and 1 μg of complementary deoxyribonucleic acid (cDNA) from the RNA samples were synthesized using Superscript II Reverse Transcriptase and oligo-dT (Invitrogen, CA, USA). The synthesized cDNA and SYBR green mixture reagent (Bio-rad, CA USA) were mixed and they were incubated at 94°C for 45 seconds, at 60°C for 30 seconds, and at 72°C for 30 seconds, respectively. This process was repeated 26 cycles to amplify them and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an reference gene. Forward and reverse primers were synthesized on known gene sequences RT-PCR was performed using a CFX 96 Real -Time PCR Detection System (Bio-Rad, CA, USA), according to the manufacturer's instructions. The primers used in this

Table 1. The Sequence of Primers used in the Real Time PCR

Gene	Primer sequences (5′→3′)
iNOS	CCG TCC ACA GTA TGT GAG GA (sense)
	GAA CTC CAA GGT GGC AGC A (anti-sense)
IL-1β	CCG TGG ACC TTC CAG GAT GAG (sense)
	ACC AGT TGG GGA ACT CTG CAG (anti-sense)
COX-2	GAA GGG ACA CCC TTT CAC AT (sense)
	ACA CTC TAT CAC TGGC ATC C (anti-sense)
GAPDH	TGA AGG TCG GAG TCA ACG GAT TTG GT (sense)
	CAT GTG GGC CAT GAG GTC CAC CAC (anti-sense)

PCR = Polymerase chain reaction; iNOS = Inducible nitric oxide synthase; IL-1β = Interleukin-1β; COX-2 = Cyclooxygenase-2; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.

experiment were shown in Table 1.

6. Measurement of PGE₂

The sandwich enzyme-linked immunosorbent assay (ELISA) kits used for PGE₂ were obtained from R&D Systems (Cayman Chemical, MI, USA). RAW 264.7 cells were added at 3×10^4 cells/well and incubated in an incubator in the same manner as NO measurement. And then the culture medium was discarded and serum free media was treated with both CHI extract and LPS (100 ng/mL), at the same time, under the condition of 5 % CO_2, 37°C and 24 hours. Media from culture medium was harvested and centrifuged at 12,000 rpm for 10 minutes to discard cell debris. PGE₂ level in the culture supernatants was measured by ELISA kit, according to manufacturer's direction.

7. Statistical analysis

Experiments were performed at least 3 times. All data were presented as mean ± SD and statistical significance was determined by Student's t-test. $p < .05$ was considered as statistically significant.

RESULT

1. Effects of CHI extract on LPS-induced NO production & cell viability

The effects of CHI extract on LPS-induced nitrite production in RAW 264.7 cells were measured for the amount of nitrite released into the culture medium. CHI extract significantly reduced the LPS-stimulated nitrite production, in a dose-dependent manner. Particularly, ni-
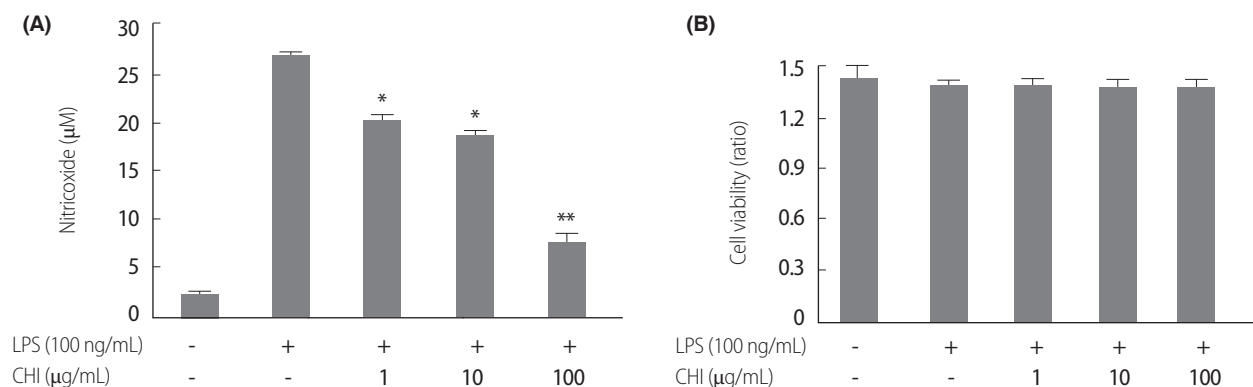
Figure 1. Effect of CHI extract on LPS-induced nitric oxide (A) and cell viability (B) in RAW 264.7 cell line. Cells were treated LPS and indicated concentration of CHI extract for 24 hours. CHI extract significantly reduced the LPS-stimulated nitrite production compared with LPS alone treated group in a dose-dependent manner. There was no cytotoxicity on RAW 264.7 cells with CHI extract up to 100 μg/mL.
*$p < .05$, **$p < .01$ compared to LPS stimulation. CHI = Chrysanthemum indicum; LPS = Lipopolysaccharides.
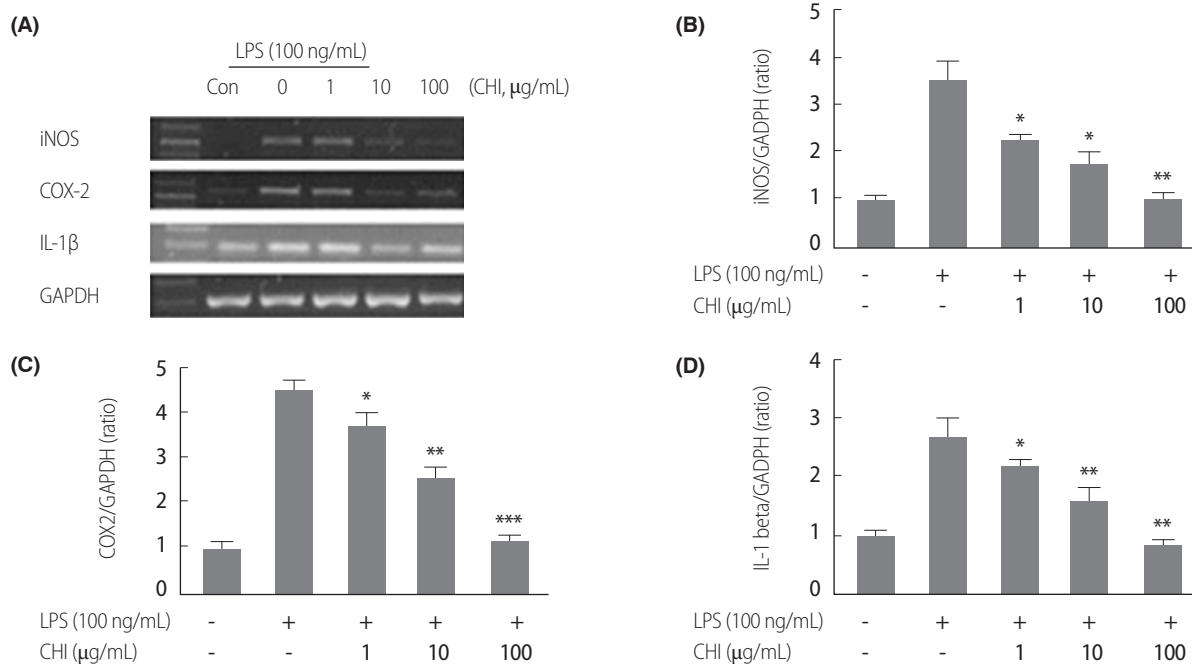
(A)

(B)

(C)

(D)

Figure 2. Effect of CHI extract on LPS-induced inflmmatory cytokien expression in RAW 264.7 cell line. The iNOS, COX-2 and IL-1β mRNA expression were analysed by real time PCR (A). All gene expressions were normalized using GAPDH as a reference gene. The iNOS (B), COX-2 (C) and IL-1β (D) mRNA expression were significantly decreased in CHI extract treated group compared with LPS treated group.

$*p < .05$, $**p < .01$, $***p < .001$ compared to LPS stimulation. CHI = *Chrysanthemum indicum*; LPS = Lipopolysaccharides; iNOS = Inducible nitric oxide synthase; COX-2 = Cyclooxygenase-2; IL-1β = Interleukin -1β; PCR = Polymerase chain reaction; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.
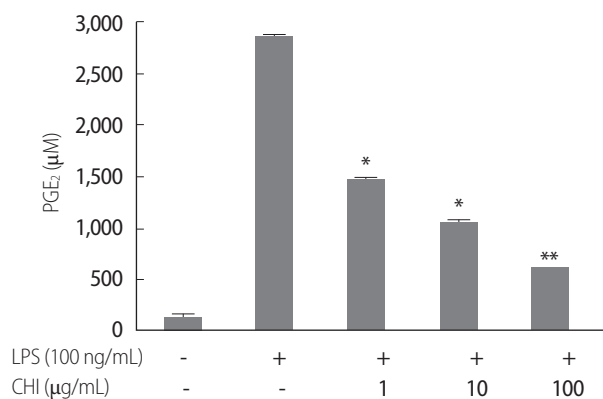

Figure 3. Effects of CHI extract on protein expression of PGE$_2$. Cells were treated with 1, 10, and 100 μg/mL of CHI extract and 100 ng/mL of LPS for 24 hours. Then 100 μL of the cell suspension were collected and PGE$_2$ protein were quantified by ELISA. PGE$_2$ protein expression on LPS-induced RAW 264.7 cells was dramatically decreased in CHI extract treated groups in a concentration dependent manner compared with LPS treated group.

$*p < .01$, $**p < .001$ compared to LPS stimulation. CHI = *Chrysanthemum indicum*; PGE$_2$ = Prostaglandin E$_2$; LPS = Lipopolysaccharides; ELISA = Enzyme-linked immunosorbent assay.

trite production was suppressed more than 70% at 100 μg/mL CHI extract, compared with only LPS treated group (Figure 1A). The cytotoxic effect of CHI extract on LPS-induced RAW 264.7 cells was assessed using the MTT assay. There was no cytotoxicity on RAW 264.7 cells with CHI extract up to 100 μg/mL and cell viability was not significantly altered by doses of CHI extract (Figure 1B).

2. Effects of CHI extract on mRNA expression levels of iNOS, COX-2, and IL-1β

To verify the inhibiting mechanism of CHI on NO production, this study used real time PCR to measure mRNA expression levels of iNOS. In addition, it also confirmed mRNA expression levels of COX-2 and IL-1β, which are believed to cause inflammation. As a result, it was found that CHI inhibits iNOS mRNA expression levels, depending on concentration. In particular, the experimental group treated with 100 μg/mL of CHI showed statistically significant difference in the inhibitive effect on inflammatory response from the control group. As for its effect on COX-2, CHI also reduced the mRNA expression of COX-2 1.5 times at 100 μg/mL than by LPS, which increased the expression 4.5 times. In indicates that CHI extract also have anti-inflammatory effect on COX-2 mRNA expression, and the decrease of IL-1β by CHI extract was also

observed (Figure 2).

3. Effects of CHI extract on protein expression of PGE₂

PGE_2 is produced through COX-2 activation. To verify whether CHI extract has anti-inflammatory effect on PGE_2 as it does on COX-2 expression in Figure 2. Sandwich ELISA was used to measure the expression levels of PGE_2 protein. As expected, PGE_2 protein expression on LPS-induced RAW 264.7 cells was dramatically decreased in CHI extract treated groups in a concentration dependent manner. In particular, the effect was significant even at 1 μg/mL, which reconfirms the anti-inflammatory effect of CHI (Figure 3).

DISCUSSION

This study, we tried to find out the effects of non-pharmacological agent on the expression level of inflammatory mediators and to provide an evidence-based of non-pharmacological therapeutic approach. In order to investigate the anti-inflammatory effects on LPS-induced RAW 264.7 macrophage cells, CHI extract as a whole plant was used in this study. Recent studies reported that CHI extract significantly inhibited the expression of pro-inflammatory cytokines but most of the previous studies were used a specific part of CHI such as roots, stem, leaf and flower, which was extracted using an ethanol [18,19,21]. There was no study using whole plant extracts of chrysanthemum with water. For this reasons, present study would find out anti-inflammatory effects of CHI, which was extracted using water and used a whole plant, including roots, stems, leaves, and flowers.

Inflammation is a complex response regulated by a variety of immune cells through the releasing of different types of cytokines and inflammatory enzymes such as of TNF-α, NO, iNOS, COX-2, IL-1β, and PGE_2 [1]. In inflammatory response macrophages play a key role by regulating inflammatory mediators such as NO, PGE_2 and pro-inflammatory cytokines and also as host-defense mechanism [22]. RAW 264.7 macrophage is widely used in inflammation models to assess anti-inflammatory effect. In this study, therefore RAW 264.7 macrophage cells were treated with LPS to induce inflammatory response. It is well known that the overproduction of inflammatory mediators is the cause of cancer or inflammatory diseases. Therefore, such anti-inflammatory medicine as steroids or NSAIDs has been used in order to inhibit the activity of anti-inflammatory mediators. Furthermore, NSAIDs drugs are used a long term in general and high doses are required to treat chronic inflammation disease so there are many side effects such as indigestion, stomach upset, gastritis, headache and dizziness. Therefore, many studies have made efforts to develop natural plant components that can substitute these drugs to reduce side effects [2,15]. NO is produced by L-arginin and plays an important role in modulating inflammatory responses, also is involved in the activation of vasodilation, neurotransmitter system and immunity. The excessive amounts of NO are the cause of serious tissue injury. Particularly, iNOS, which is induced by LPS, produces large amount of NO [5].

In this study, CHI extract inhibited mRNA expression of iNOS and IL-1β as well as NO production. These results demonstrates that CHI extract suppresses iNOS expression and down regulation of iNOS and IL-1β enzymatic activity as a result of reduced NO production.

Several previous studies reported that compositae plant such as *Stevia rebaudiana* and dandelion has anti-inflammatory activity which acts via the suppression of NO and iNOS production [20,23]. A previous study which analyzed the anti-inflammatory effects of the isolated compounds from the flowers of CHI. The extract of CHI was rich in phenolic compounds and this compounds decreased iNOS and COX-2 protein expression in a dose dependent manner [18]. COX-2 is related to biosynthesis of the PGE_2 in inflammatory responses. Therefore, medicines that are currently used for the treatment of inflammation through the down regulation of PGE_2 take advantage of the inhibition of COX-2 production and activity [13]. The results of the present study demonstrated that CHI extract suppressed PGE_2 protein expression on LPS-induced RAW 264.7 cells significantly. This result is associated with the down-regulation of COX-2 mRNA. In several studies using natural plants such as green tea, Mori Folium and phloretin from apple wood, it was reported that they have anti-inflammatory effects through inhibiting of COX-2 and PGE_2 expression [9,15,24]. In addition, the study that used ethanol to extract CHI by 25, 50, 100, and 200 μg/mL, respectively, to analyze its anti-inflammatory effect reported that the CHI extract reduced the activation of such anti-inflammatory cytokines as NO, COX-2, TNF-α, and PGE_2 depending on concentration [19]. However the present study used a lower concentration (1, 10, and 100 μg/mL) than in the previous studies and also confirmed the concentration-dependent effect of CHI extract on anti-inflammation response.

As a result of this study, it is demonstrated that CHI water extract has anti-inflammatory effect as the ethanol extract, also this study observed that the whole pant of CHI has the same effect as specific parts of the

plant. Therefore, the present study proposed the scientific ground that CHI water extract is a safer and more effective non-pharmacologic therapeutic approach to the treatment of inflammation. Therefore, it is believed that this result can be used as the basic data for nursing education for patients with inflammation disease. Furthermore, the mechanism of anti-inflammatory effects of CHI still remains to be defined through more in vivo study. In conclusion, CHI water extract acts as an anti-inflammatory agent in inflammatory conditions through the inhibition of NO production and down regulation of inflammatory mediators, iNOS, IL-1β, COX-2, and PGE$_2$. This present study suggested that CHI water extract can be substituted for anti-inflammatory drugs and provide safer and more effective non pharmacological therapeutic approach.

CONCLUSION

This study was aimed to evaluate whether all components extract of CHI have anti-inflammatory effect, when they were extracted under the same conditions. RAW 264.7 cells were treated with various concentrations of CHI extract (1, 10, and 100 μg/mL), and mRNA expression and protein levels of inflammatory mediators were confirmed. CHI extract significantly inhibited the levels of NO, iNOS, IL-1β, COX-2, and PGE$_2$ in a dose-dependent manner. According to these result, CHI extract can substitute anti-inflammatory drugs and provide safer and more effective non pharmacological therapeutic approach. However, this study analyzed only the effects of several pro-inflammatory cytokines. Therefore, further studies are necessary to be conducted to confirm down signaling pathways associated with these cytokines.

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