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Inhibitory Effects of Camellia Japonica on Cell Inflammation and Acute Rat Reflux Esophagitis

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Research

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Abstract

Background

Excessive and continuous inflammation may be the main cause of various immune system diseases. Reflux esophagitis (RE) is a common gastroesophageal reflux disease (GERD). It is an inflammation of the esophagus caused by the reflux of gastric contents to the esophagus due to the dysfunction of the lower esophageal sphincter. *Camellia japonica* has high medicinal value and has long been used as a traditional herbal hemostatic medicine in China and Korea.

Materials and methods

Dried *C. japonica* buds were extracted with 75% ethanol. DPPH and ABTS radical scavenging assay were evaluated according to previous method. The ROS production and anti-inflammatory effects of *C. japonica* buds ethanol extract (CJE) were evaluated on LPS-induced RAW 264.7 macrophage cells. The protective effects of CJE on RE were conducted in a RE induced Sprague Dawley rat model.

Results

CJE eliminated over 50% of DPPH and ABTS radical at concentration of 100 and 200 μg/mL, respectively. CJE alleviated changes in cell morphology, reduced production of ROS, NO and IL-1β. Also, down-regulated expression levels of iNOS, TNF-α, phosphorylated NF-κB, IκBα, and JNK/p38/MAPK. CJE reduced esophageal tissue damage ratio (40.3%) and attenuation of histological changes. In addition, CJE down-regulated the expression levels of TNF-α, IL-1β, COX-2 and phosphorylation levels of NF-κB and IκBα in esophageal tissue .

Conclusions

CJE possesses good anti-oxidation and anti-inflammatory activity, and can improve RE in rats caused by acid reflux. Therefore, CJE may be a natural medicine source with good anti-oxidation and anti-inflammatory effects, and a candidate phytomedicine source for treating RE

Background

Inflammation is a protective response that occurs when the host is stimulated by certain factors, including pathogens, radiation and physical forces. However, excessive and continuous inflammation may be the main cause of various immune system diseases including diabetes, rheumatoid arthritis, neurodegenerative diseases, and even cancer [1-2]. Macrophages are the main cells that maintain stability of the host and react to infection by enhancing the immune response. They play an important ution of inflammation [3]. Lipopolysaccharide (LPS)-induced

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cellular inflammation models have been used for evaluating the anti-inflammatory effects of active substances such as plant extracts and compounds.

Reflux esophagitis (RE), a common gastroesophageal reflux disease (GERD), is inflammation of the esophagus caused by reflux of gastric contents to the esophagus due to dysfunction of the lower esophageal sphincter [4]. RE is showing a continuous upward trend worldwide. As stomach contents are mainly composed of gastric acid, frequent reflux can cause ulceration of the esophageal epithelial tissue and even esophageal cancer [5]. Currently, the most effective drugs are mainly gastric acid secretion inhibitors (such as omeprazole and ranitidine); although these drugs have good therapeutic efficacy, long-term use may cause side effects like intestinal infection [6]. Therefore, it is necessary to discover natural pharmaceutical materials with limited side effects for treating RE.

Camellia japonica L. (Theaceae), also called Japanese camellia, is native to China and Japan [7]. As an ornamental plant, *C. japonica* is often planted in courtyards and roadsides. *C. japonica* has high medicinal value and has long been used as a traditional herbal hemostatic medicine in China and Korea [8]. Many previous studies have demonstrated the various biological activities of extracts from parts of *C. japonica*. The leaf extract of was shown to promote anti-oxidative protein expression and suppress apoptosis in human corneal epithelial cells [9]. Antioxidant properties of the flower ethanol extract were also investigated in human HaCaT cells, and the anti-atherogenic effects of the fruit extract were confirmed in rats fed a high-fat diet [10-11].

To determine the physiological activity of *C. japonica* buds ethanol extract (CJE), we measured its antioxidant and anti-inflammatory activity at the cellular level. We used a rat RE model to explore its effect on RE. Our results show that CJE possesses excellent antioxidant and anti-inflammatory activity and improves RE in rats. Therefore, CJE is a potential natural medicine for treatment of inflammatory diseases.

Materials And Methods

Reagents and antibodies

LPS, HEPE, ranitidine hydrochloride, protease inhibitor cocktail, DCFH-DA and DMSO were purchased from Sigma (St. Louis, MO, USA). The cell viability, proliferation, and cytotoxicity assay kits were purchased from DoGenBio Co., Ltd (Guro-gu, Seoul, South Korea). DMEM, FBS, and penicillin and streptomycin were purchased from Welgene (Namcheon-ro, South Korea). Griess reagent was purchased from Promega (Fithchburg, WI, USA). DAPI (Sigma-Aldrich, St.Louis, MO, USA). DC^{TM} Protein assay reagent, polyvinylidene fluoride membranes, and bovine serum albumin standard were purchased from Bio-Rad Laboratories (Hercules, CA, USA). The phenylmethylsulfonyl fluoride, and ethylenediaminetetraacetic acid were purchased from Corporation Cleveland (OH, USA). iNOS, COX-2, IL-1β, TNF-α, β-actin antibodies, m-IgGκ BP-HRP secondary antibody and Luminol reagent were purchased from Santa Cruz Biotechnology (Delaware Ave, CA, USA). The p-IκBα, IκBα, p-NF-κB p65, NF-κB p65, p-

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SAPK/JNK, SAPK/JNK, p-p38 MAPK, p38 MAPK, p-p44/42 MAPK (Erk1/2), p44/42 MAPK (Erk1/2), and Lamin B1 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

CJE preparation

Buds from *C. japonica* plants were collected in the mountain area of Jeju, South Korea, and authenticated by the Korean Institute of Oriental Medicine (KIOM). Dried buds were suspended in 10 volumes of 75% ethanol and extracted three times for two hours each in a circulation distillation unit at 50°C. The ethanol extracts were filtered through advance filter paper, freeze-dried, pulverized, and stored at -80°C until use.

DPPH and ABTS radical scavenging assay

The DPPH radical scavenging assay was conducted using the method of Liyana-Pathirana C and Shahidi F [12]. For this, DPPH (0.2 mM) in methanol and different concentrations of CJE (25, 50, 100 μg/mL) were mixed in a 1:1 ratio. The mixture was incubated at 37℃ for 30 min, and then the absorbance at 517 nm was measured using a microplate reader (Multiscan spectrum, Thermo Scientific).

The ABTS radical scavenging assay was conducted using the method of Jing et al [13]. Here, ABTS (0.7 mM) and 2.4 mM potassium persulfate ($K_2S_2O_8$) solution were mixed in a 2:1 ratio and reacted at room temperature for 16 h in the dark. The absorbance of the mixture at 734 nm was measured using a microplate reader, and the mixture was used when the value was in the range of 0.70±0.02. ABTS and different concentrations of CJE (50, 100, 200 μg/mL) were mixed in a 1:9 ratio. The absorbance at 734 nm was measured using a microplate reader (Multiscan spectrum, Thermo Scientific).

Cell culture

RAW264.7 cells were obtained from the American Type Culture Collection and cultured in medium containing DMEM, 10% FBS, 100 units/ml penicillin, and 100 μg/mL streptomycin. Cells were incubated in a 5% CO_2 and 95% air environment at 37°C. The cells were grown to logarithmic phase for the experiments.

Determination of intracellular reactive oxygen species (ROS)

To measure intracellular ROS content, RAW264.7 cells were plated at a concentration 3×10^5 cells/mL. CJE at concentrations of 12.5, 25, and 50 μg/mL was added and incubated for 1 h, and LPS (1 μg/mL) was added for co-treatment for another 8 h. Then DCFH-DA (10 uM) solution was added to cells. After 30 min, the fluorescence intensity was measured by multimode microplate reader (Perkin Elmer, Gyeonggi-do, South Korea) at excitation wavelength 485 nm and emission wavelength 535 nm.

Cell viability and cell morphology observation and quantitation assay

To determine cell viability, RAW264.7 cells were seeded at a concentration of 5×10^5 cells/mL in a 96-well plate. Cells were pre-treated with CJE at concentrations of 50, 100, and 200 μg/mL and incubated for 1 h.

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Then, LPS (1 μg/mL) was added to the plate, and the cells were further incubated for 18 h. Cell viability was measured with the proliferation and cytotoxicity assay kit. Cell morphology was observed by inverted microscope (Nikon, Melville, NY USA) and quantified by the Image J program. The calculation formula was as follows: Roundness factor (RNF) = $\frac{4A}{\pi \times d_{max}^2}$ (A: cell area, d_{max}: maximum diameter).

Nitric oxide assay

To determine the production of nitrite in culture media, RAW264.7 cells were seeded at a concentration of 5×10^5 cells/mL in a 96-well plate. Cells were pre-treated with CJE at concentrations of 50, 100, and 200 μg/mL and incubated for 1 h. Then, LPS (1 μg/mL) was added, and the plate was further incubated for 18 h. Production of nitrite was measured in the Griess reagent according to the manufacturer`s instructions. Nitrite concentration was determined at 540 nm using $NaNO_2$ as a standard curve. Each treatment was carried out in three replicates.

IL-1β ELISA kit assay

Concentration of IL-1β in culture media was measured using human IL-1β/IL-1F2 Immunoassay kit (R&D Systems, MN, USA) according to the product datasheets.

Immunofluorescence assay

RAW264.7 cells were plated on a square glass coverslip at a concentration of 3×10^5 cells/mL in a 6-well plate. Cells were pretreated with CJE (200 μg/mL) for 1 h, and LPS (l μg/mL) was added for another 30 min. Cells were harvested for fixing, permeating, and blocking as described in our previous study. Cells were incubated with the primary antibody of p65 (1:200) overnight at 4 ℃ and then incubated with the second antibody (m-IgGk BP-FITC 1:1000) for 2 h at room temperature. After staining the nuclei with DAPI, images were collected using super resolution confocal laser scanning microscope (magnification, 63 ×).

Preparation of RAW264.7 cells protein extracts

RAW264.7 cells were plated at a concentration of 1×10^6 cells/mL in a 6-well plate. The normal control group cells were treated with medium, and the LPS-treated group cells were stimulated by LPS (1 μg/mL) only. The co-treatment group cells were pre-treated with different concentrations of CJE (100 or 200 μg/mL) for 1 h, and then LPS (1 μg/mL) was added. All cells were incubated for 30 min or 18 h at 37°C in a 5% CO_2 and 95% air incubator. The cells were washed with ice-cold phosphate-buffered saline (PBS) three times and then lysed with 100 μL of whole protein cell lysis buffer [5 M NaCl, 0.5 M EDTA, 1 M Tris (PH 8.0), 10% NP-40, 10% sodium deoxycholate, and 10% SDS]. The tubes were placed on ice for 15 min, vortexed every 5 min, and then centrifuged at 13000 rpm for 15 min. The supernatant containing the protein was collected and stored at -80°C until analysis.

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Rat maintenance and surgery of acute reflux esophagitis

Seven-week-old male Sprague-Dawley rats (180 ± 20 g) were used according to the animal welfare recommendations of the Institutional Animal Care and Use Committee of Chonbuk National University Laboratory Animal Center in South Korea. Rats were kept in standard rat cages and supplied food and water *ad libitum* in a facility maintaining a 12 h light/dark cycle and a controlled 21°C to 25°C temperature and 35% to 60% humidity environment. Rats were acclimated for one week under these conditions and subsequently divided into four groups (n = 8) randomly: normal group, RE control group, medication group, and positive control group. Rats were fasted for 18 h before surgery. Two h before surgery, the normal group rats did not receive oral administration, and the RE control group rats were orally administered saline; the medication group rats were orally administered CJE at a dose of 200 mg/kg body weight, and the positive control group rats were orally administered ranitidine at 40 mg/kg body weight. The rats were anesthetized nasally. A 2 cm incision was made in the rats' abdomens to expose the stomach, and both the junction of the stomach and forestomach and the pylorus were ligated using a 3-0 silk thread. Care was taken to ensure that the vagus nerve remained intact [14, 15]. All rats were sacrificed 4.5 h after the surgery. The stomach and esophagus were removed to determine the ratio of esophageal injury. The esophageal tissue was stored at -80°C until analysis.

Esophageal damage ratio

The esophageal and gastric tissue was placed on paper and digitally photographed after esophageal mucus was washed away with 0.9% saline. The esophageal injury was analyzed using Image J software. The esophageal lesion index was calculated as follows: lesion index (%) = [area of esophageal damage (mm^2)/total area of esophagus (mm^2)] × 100.

Esophageal histological study

A 4-5 mm section of esophageal tissue was removed, placed in 4% NBF fixative, and fixed with paraffin. Slices were 5 μm thick and stained with hematoxylin/eosin. Images were acquired with a Leica microscope at 10 X magnification.

Preparation of cytoplasmic and nuclear protein fractions from esophageal tissue

The nuclear and cytoplasmic proteins of esophageal tissue were extracted according to our previous method [16]. In brief, esophageal tissues were homogenized in ice-cold lysis buffer containing 10 mM HEPES, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and protease inhibitor mixture solution. Samples were stained on ice for 30 min. After centrifugation at 13000 rpm for 2 min at 4°C, the supernatant with the cytoplasmic proteins was collected. The remaining pellet was re-suspended with extraction buffer containing 50 mM HEPES, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1% (v/v) glycerol, 1 M DTT, and protease inhibitor mixture solution. Following incubation on ice for 30 min, the sample was centrifuged at 13000 rpm for 10 min at 4°C. The supernatant with the nuclear protein fraction was collected and stored at -80°C until analysis.

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Western blot analysis

Equal amounts of quantitative proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with different percentages and transferred to PVDF membranes. The membranes were blocked with 5% skim milk for 2 h at room temperature, washed with ice-cold PBS, and then incubated with primary antibodies (1:1000) overnight at 4 °C. Finally, the membranes were treated with HRP-conjugated secondary antibodies (1:10000) for 1.5 h with gentle shaking at room temperature. Bands were visualized using a Luminol reagent, a 1:1 ratio of solutions A and B, and Bio-Rad image software (NY, USA).

Statistical analysis

All data are expressed as mean ± standard deviation. One-way ANOVA followed by LSD multiple comparisons analyses were performed using SPSS 12.0K for Windows. A *P*-value < 0.05 indicated a significant difference.

Results

Effect of CJE on DPPH and ABTS free radical scavenging activity

DPPH and ABTS free radical scavenging assays are widely used to evaluate the anti-oxidative activity of medicinal plant extracts. Ascorbic acid is a natural antioxidant commonly used as a reference material. As shown in Figures 1A and B, CJE eliminated 50% of DPPH radicals at a concentration of 50 μg/mL and more than 70% of DPPH radicals at a concentration of 100 μg/mL, similar to ascorbic acid (20 μg/mL). CJE eliminated 60% of ABTS radicals at a concentration of 200 μg/mL, also similar to ascorbic acid (40 μg/mL).

Effect of CJE on production of intracellular reactive oxygen species (ROS) in LPS-induced RAW264.7 cell

ROS play an important role in cell signaling and maintaining homeostasis. However, excessive ROS caused by stimulation can induce tissue and cell structure damage. As shown in Figure 1C, LPS stimulation increased the production of intracellular ROS compared with normal cells, while CJE treatment significantly inhibited the amount of ROS production dose-dependently.

Effect of CJE on RAW264.7 cell viability and morphological change

As shown in Figure 2A, no cytotoxicity was observed until a concentration of 200 μg/mL of CJE. As shown in Figure 2B, cell morphological changes like shape change were observed in the LPS-induced group, and co-treatment with CJE reduced the morphological changes. The quantitative result of cell morphology is shown in Figure 2C. The roundness factor (RNF) is an important parameter for evaluating cell shape. The closer is the RNF value to 1, the closer is the cell shape to a circle [17].

Effect of CJE on NO production, IL-1β production, iNOS and TNF-α expression levels in LPS-stimulated RAW264.7 cells

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Nitric oxide (NO) is an important signaling molecule that transmits intracellular and intercellular signals and functions as a biological mediator, similar to neurotransmitters in the neuronal system, to protect the host against invading foreign pathogens [18]. Excessive production of NO is regulated by iNOS, which induces inflammatory responses in macrophages. IL-1β and TNF-α, important pro-inflammatory cytokines that are central regulators of inflammation, are secreted by several types of cells, such as macrophages and monocytes [19]. Cells were incubated with CJE at different concentrations and then stimulated with LPS (1 μg/mL) for another 18 h. As shown in Figure 3A, LPS caused an increase in NO production, while CJE pre-treatment significantly reduced NO production dose-dependently. The inhibition rate of NO production was greater than 50% at a CJE concentration of 200 μg/mL. LPS also increased the concentration of IL-1β, and inhibition of IL-1β production was close to 50% with a CJE concentration of 100 μg/mL (Fig. 3C). Furthermore, pretreatment with CJE at concentrations of 100 and 200 μg/mL clearly down-regulated the expression levels of iNOS (Fig. 3B) and TNF-α (Fig. 3D) induced by LPS.

Effect of CJE on the phosphorylation of NF-κB and IκBα and NF-κB nuclear translocation in LPS-stimulated RAW264.7 cells

Nuclear factor (NF)-κB is an important regulator of inflammatory responses. The activity of NF-κB is regulated mainly by inhibitory proteins of the IκB family. Activated NF-κB translocate into the nucleus to further regulate the expression of inflammatory cytokines [20]. Cells were incubated with CJE at concentrations of 100 and 200 μg/mL and then stimulated with LPS (1 μg/mL) for another 30 min. As shown in Figure 4B, LPS stimulation induced nuclear transfer of NF-κB, though CJE (200 μg/mL) significantly inhibited this condition. In addition, phosphorylation of NF-κB and IκBα increased wiht LPS treatment but CJE pretreatment significantly suppressed their phosphorylation at a concentration of 200 μg/mL (Fig. 4A).

Effect of CJE on the phosphorylation of JNK/p-38/MAPK in LPS-induced RAW264.7 cells

MAPK cascade pathways are involved in regulating the protein expression of some pro-inflammatory genes [21, 22]. Cells were incubated with CJE at concentrations of 100 and 200 μg/mL for 1 h and then stimulated with LPS (1 μg/mL) for another 30 min. As shown in Figure 5, LPS activated JNK and p-38 expression; in contrast, CJE significantly decreased the expression levels of phosphorylated JNK and p-38 at a concentration of 200 μg/mL.

CJE suppressed esophageal damage in RE rats

After ligation surgery, rat esophagi were stimulated by repeated reflux of gastric acid, which led to tissue damage and inflammation. Unblemished, intact esophageal tissue was observed in the normal group, while large-scale black-red hemorrhagic lesions and erosion of esophageal tissue were observed in the RE control group (Figure 6A). Oral administration of CJE (200 mg/kg) reduced esophageal tissue damage by 50% (Figure 6B). Oral administration of the positive control ranitidine (40 mg/kg) reduced esophageal tissue damage by 70% compared with the RE control group.

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CJE attenuated histological changes in RE rats

Intact mucosa, submucosa, and muscularis externa tissue were observed in the esophagi of normal group rats (Figure 6C). In contrast, serious histological changes including complete exfoliation of the epithelial layer and sparse tissue structure in the submucosal and muscularis externa layer were observed in the esophagis of the RE control group rats. However, the degree of esophageal tissue histological changes in the medication group (oral administration of CJE 200 mg/kg) and positive control group (oral administration of ranitidine 40 mg/kg) was significantly improved.

CJE inhibited protein expression of COX-2, TNF-α, and IL-1β in the esophagus

Increased expression of COX-2, TNF-α, and IL-1β induced by repeated reflux of gastric acid was observed in the RE control group, while oral administration of CJE markedly inhibited the expression of these proteins, similar to the positive control group (ranitidine) (Figure 7).

CJE suppressed activation of NF-κB in the esophagus

To investigate whether NF-κB was activated in the rat RE model, we evaluated the protein expression of phosphorylated NF-κB and the inhibitory protein IκBα. NF-κB and IκBα phosphorylation was markedly increased in the RE control group, indicating the activation of NF-κB and IκBα by RE (Figure 8). In contrast, the phosphorylation levels of NF-κB and IκBα were significantly reduced by oral administration of CJE, similar to the effect of the standard drug ranitidine.

Discussion

Oxidative stress refers to a state of imbalance between oxidative and anti-oxidative effects in the body. It is caused by excessive generation of ROS and is an important factor leading to aging and disease [23]. Certain plant extracts have been shown to protect biological systems from oxidative stress caused by ROS by eliminating free radicals [24]. Research has shown that under LPS stimulation, peroxiredoxin 2 (PRDX-2) is released to further induce TNF-α production [23]. In the present study, we explored the anti-oxidant and anti-inflammatory effects of CJE on LPS-induced RAW 264.7 cells. The results indicate that CJE has strong anti-oxidant and anti-inflammatory effects. CJE eliminated DPPH and ABTS free radicals and inhibited production of ROS in LPS-induced RAW 264.7 cells.

The macrophage RAW264.7 inflammatory model induced by LPS is usually used to demonstrate the physiological activities of plant extracts or active substances, such as antioxidant and anti-inflammatory activity. Inflammatory proteins (iNOS and COX-2) and cytokines (TNF-α and IL-1β) are closely related to inflammation. The degree of inflammatory response can be judged by the expression levels of inflammation-related proteins and the secretion levels of inflammatory factors in cells [2]. CJE inhibited production of NO and IL-1β in culture medium and protein expression levels of iNOS and TNF-α by regulating the activation levels of NF-κB and IκBα. Furthermore, MAPK (JNK/ERK/p38) signaling pathways also play a key role in inflammatory responses in cells **[25]**. Activated MAPKs regulate the gene

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expression of cytokines, including TNF-α and IL-1β, and inflammatory proteins such as iNOS and COX-2 [26]. Our results revealed that LPS activated the JNK and p38 signaling pathways, and CJE primarily inhibited JNK and p38 phosphorylation. These results indicate that inhibition of the NF-κB JNK/p38MAPK signaling pathway activation may contribute to the anti-inflammatory activity of CJE.

RE is an inflammatory disease, the incidence of which has shown a significant increase from 2.5 to 7.8% in the past 10 years in some Asian countries, like as China and Korea [27]. RE patients endure different levels of distress such as heartburn, reflux, and sleep disorders [28]. Plant extracts are rich in active substances that have various physiological activities, including anti-oxidation and anti-inflammation [29]. Some studies have shown that plant extracts can reduce the extent of esophageal damage in RE rats [30, 31]. Most research on RE mainly uses the rat RE model induced by surgery. By ligating the pylorus to prevent gastric contents from entering the duodenum and by ligating the forestomach to reduce stomach volume, gastric acid is more likely to reflux into the esophagus [32]. Gastric acid reflux leads to esophageal damage including inflammatory infiltration of esophageal cells and shedding of mucosal epithelial tissue [33]. In addition, the expression levels of inflammatory proteins and pro-inflammatory factors in the esophageal tissue vary depending on degree of esophageal injury and inflammation [34]. According to our results, gastric reflux induced severe esophageal tissue damage such as esophageal mucosal tissue exfoliation and muscular layer tissue inflammation. However, treatment with CJE alleviated the degree of injury. The NF-κB signaling pathway has long been recognized as a classical pro-inflammatory pathway due to the role of activated NF-κB in the expression of inflammation-related mediators and cytokines [35]. In the cytoplasm, the inhibitory protein IκBα inactivates NF-κB transcription factor by masking its nuclear localization signal. When cellular inflammation occurs, activated NF-κB enters the nucleus to bind to DNA and induce transcription of target genes such as iNOS, COX-2, TNF-α and IL-1β. In this study, CJE treatment significantly reduced the expression levels of COX-2, TNF-α, and IL-1β and, also inhibited the phosphorylation levels of NF-κB and IκBα in esophagitis tissue. These results suggest that regulation of NF-κB activity may be an important factor in the treatment of RE.

Conclusions

CJE possesses anti-oxidant and anti-inflammatory activity, and can improve RE caused by acid reflux. Therefore, CJE is a good candidate phytomedicine for treatment of RE.

List Of Abbreviations

RE: Reflux esophagitis, GERD: gastroesophageal reflux disease, CJE: *C. japonica* buds ethanol extract, DPPH: 1,1-diphenyl-2-picrylhydrazyl, ABTS: 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, ROS: reactive oxygen species, NO: nitric oxide, IL-1β: interleukin-1β, iNOS: inducible nitric oxide synthase, TNF-α: tumor necrosis factor-α, NF-κB: nuclear factor κ-light-chain-enhancer of activated B cells, IκBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-α , JNK: c-Jun N-terminal kinase, p38/MAPK: p38 mitogen-activated protein kinases, LPS: Lipopolysaccharide, HEPE: 4-(2-hydroxyethyl)-1-

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7-Dichlorofluorescin diacetate, DMSO: dimethyl sulphoxide,

DMEM: Dulbecco's modified eagle medium, FBS: fetal bovine serum, DAPI: 4`,6-diamidino-2-phenylindole, EDTA: ethylene diamine tetraacetic acid, SDS: sodium dodecyl sulfate.

Declarations

Ethics approval and consent to participate

Seven-week-old male Sprague-Dawley rats were used according to the animal welfare recommendations of the Institutional Animal Care and Use Committee of Chonbuk National University Laboratory Animal Center in South Korea (IACUC; CBNU 2017-00079, date of approval, August 5, 2018).

Consent for publication

Not applicable

Availability of data and materials

The datasets used in this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors` contributions

Hyeon hwa Nam and Li Nan are the co-first author, they made the same contribution in this study.

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Figures

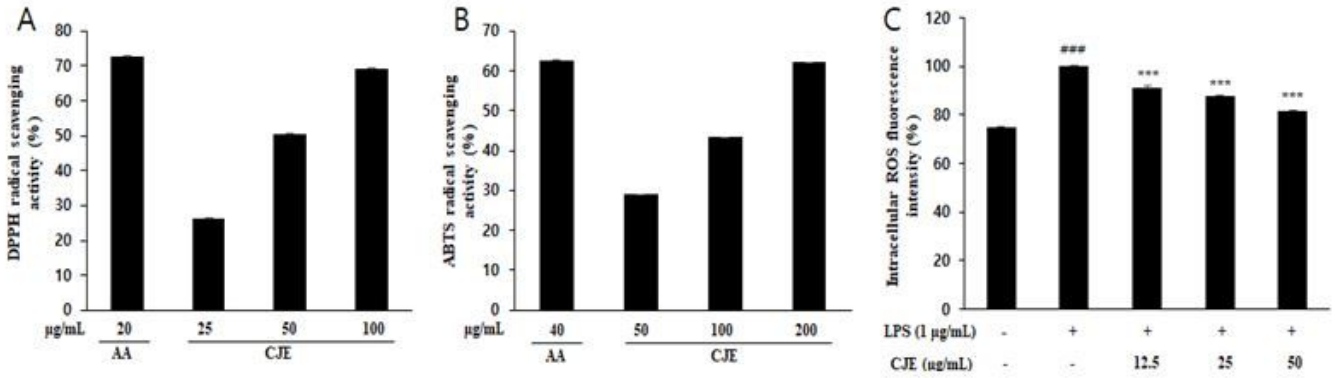
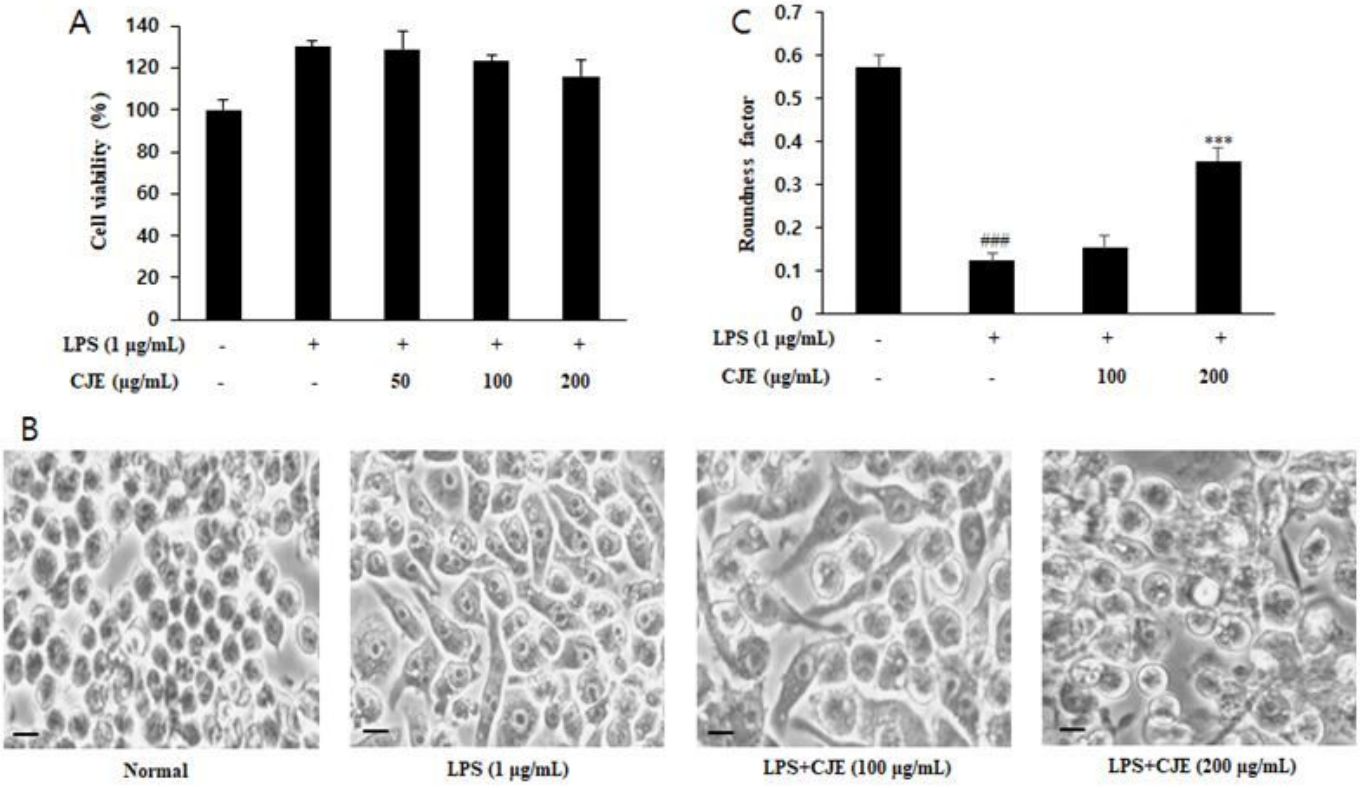


Figure 1

DPPH (A) and ABTS (B) radical scavenging activity of CJE was measured using DPPH and ABTS radical scavenging assay. AA, ascorbic acid was used as a reference material. Fluorescence intensity of intracellular ROS (C) in LPS-induced RAW264.7 cell was measured by DCFH-DA fluorescent probe. ###P<0.001 vs. normal cells; ***P<0.001 vs. LPS-induced cells. Results are the mean ± SD from three independent experiments.



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Figure 2

Effect of CJE on cell viability (A) in LPS-induced RAW264.7 cell was checked using proliferation and cytotoxicity assay kit. Cell morphology (B) was observed by inverted microscope and cell morphology quantitative calculation was using Image J program. ###P<0.001 vs. normal cells; ***P<0.001 vs. LPS-induced cells. Results are the mean ± SD from three independent experiments.

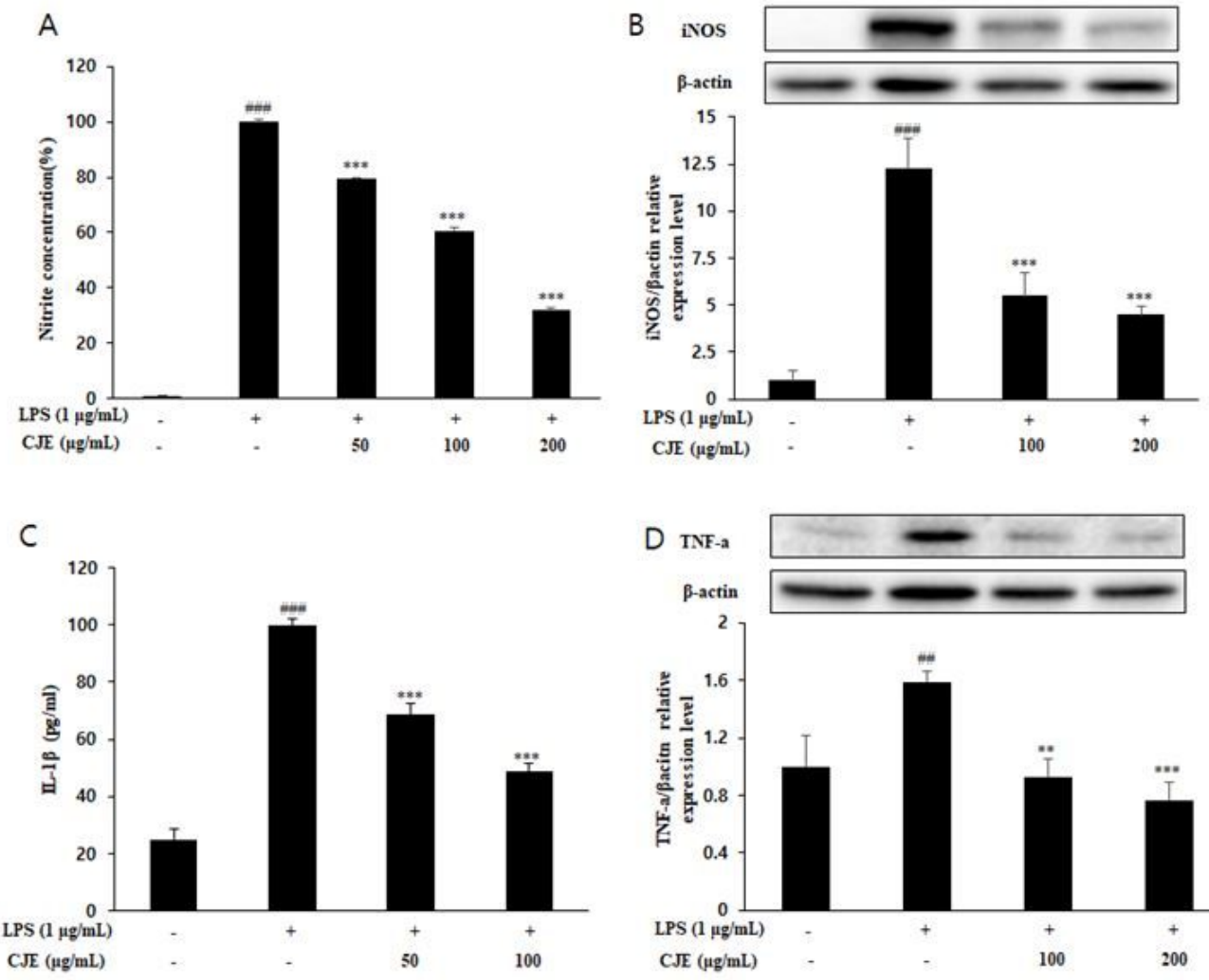


Figure 3

Effect of CJE on nitrite production (A), expression level of iNOS (B), IL-1β production (C) and expression level of TNF-α in LPS induced RAW264.7 cells. Nitrite concentration was measured by the griess reagent, IL-1β production was measured using human IL-1β/IL-1F2 Immunoassay kit. Expression level of iNOS and TNF-α was measured by western blotting. ###P<0.001, ##P<0.01 vs. normal cells; ***P<0.001, **P<0.01 vs. LPS-induced cells. Results are the mean ± SD from three independent experiments.

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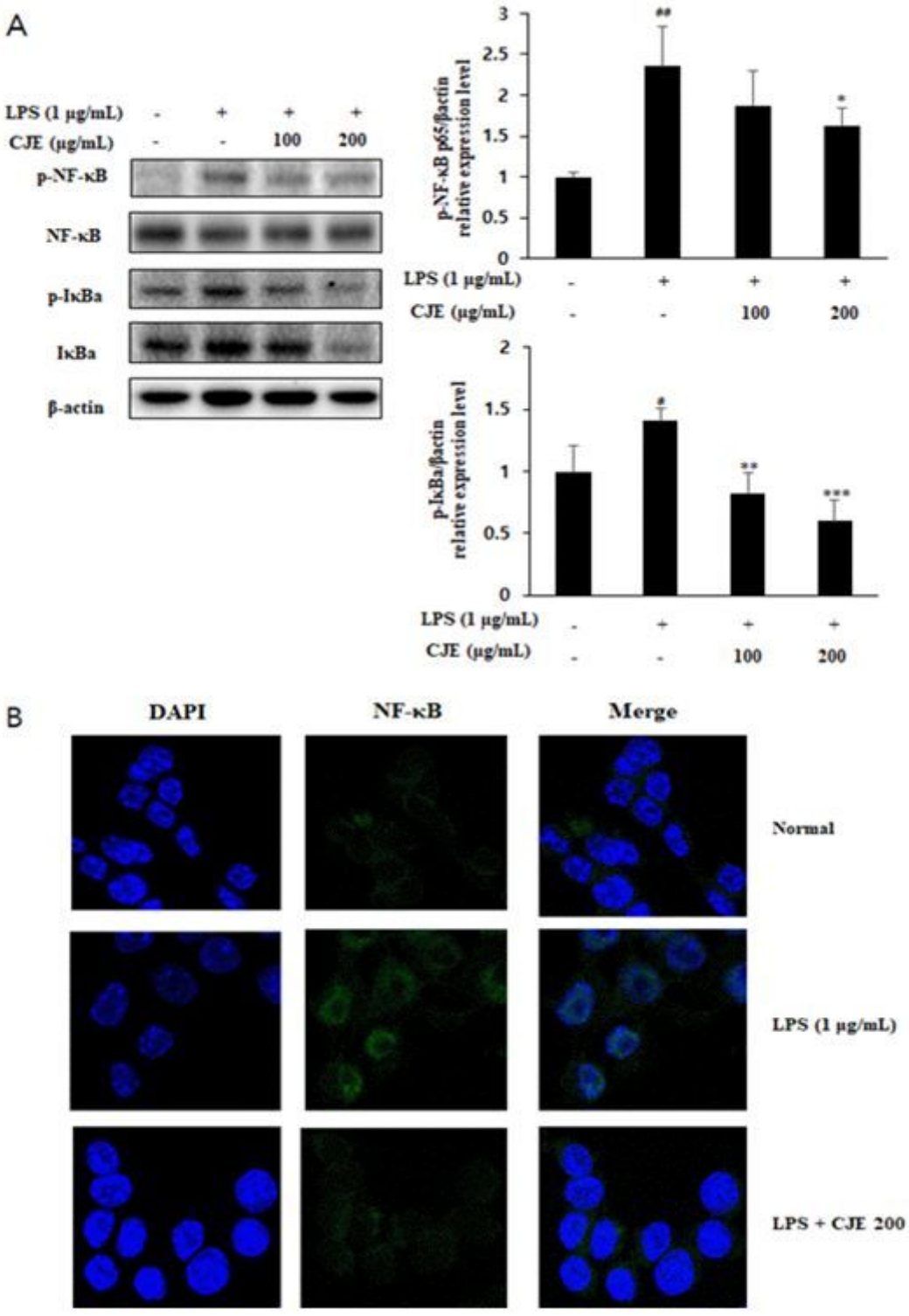


Figure 4

Effect of CJE on phosphorylation level of NF-κB, IκBα (A) and NF-κB nuclear translocation (B) in LPS-induced RAW264.7 cell was measured by western blotting and immunofluorescence assay. ## P<0.01, #P<0.05 vs. normal cells; ***P<0.001, **P<0.01, *P<0.05 vs. LPS-induced cells. Results are the mean ± SD from three independent experiments.

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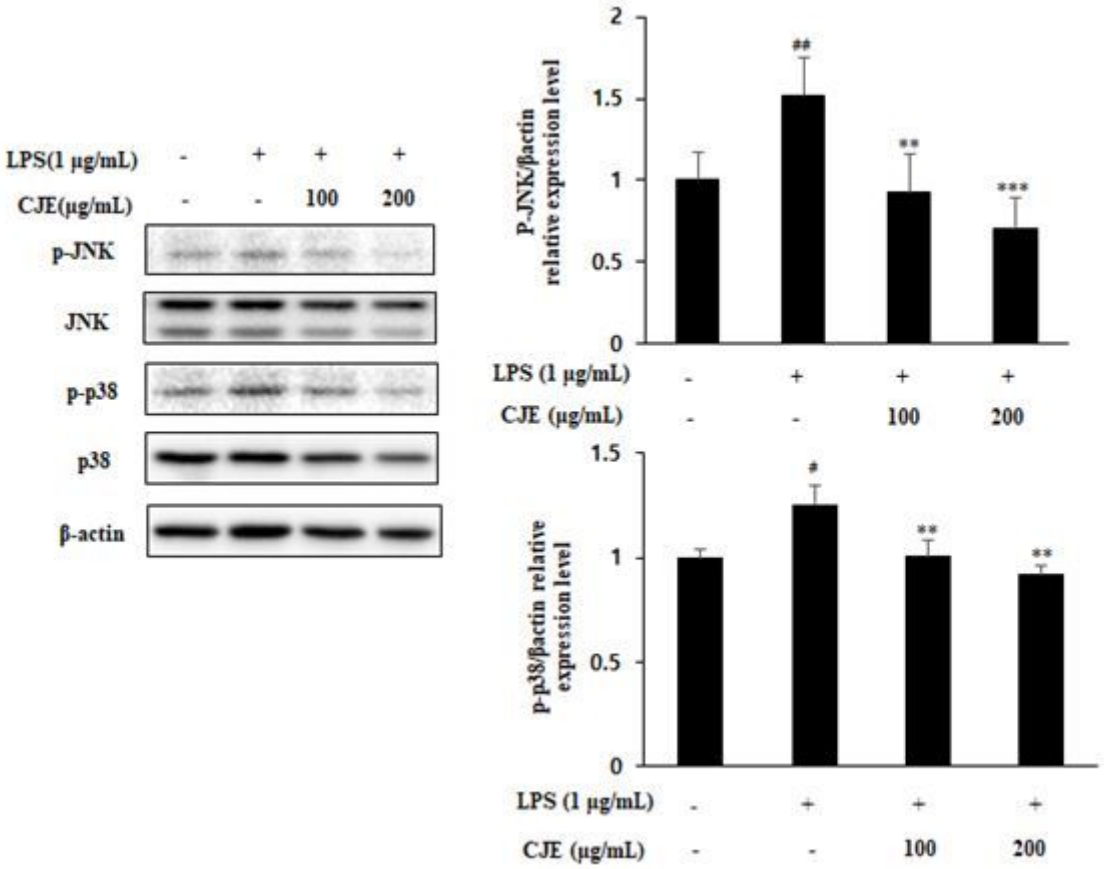


Figure 5

Effect of CJE on phosphorylation level of JNK and p-38 in LPS-induced RAW264.7 cell was measured by western blotting. ##P<0.01, #P<0.05 vs. normal cells; ***P<0.001, **P<0.01 vs. LPS-induced cells. Results are the mean ± SD from three independent experiments.

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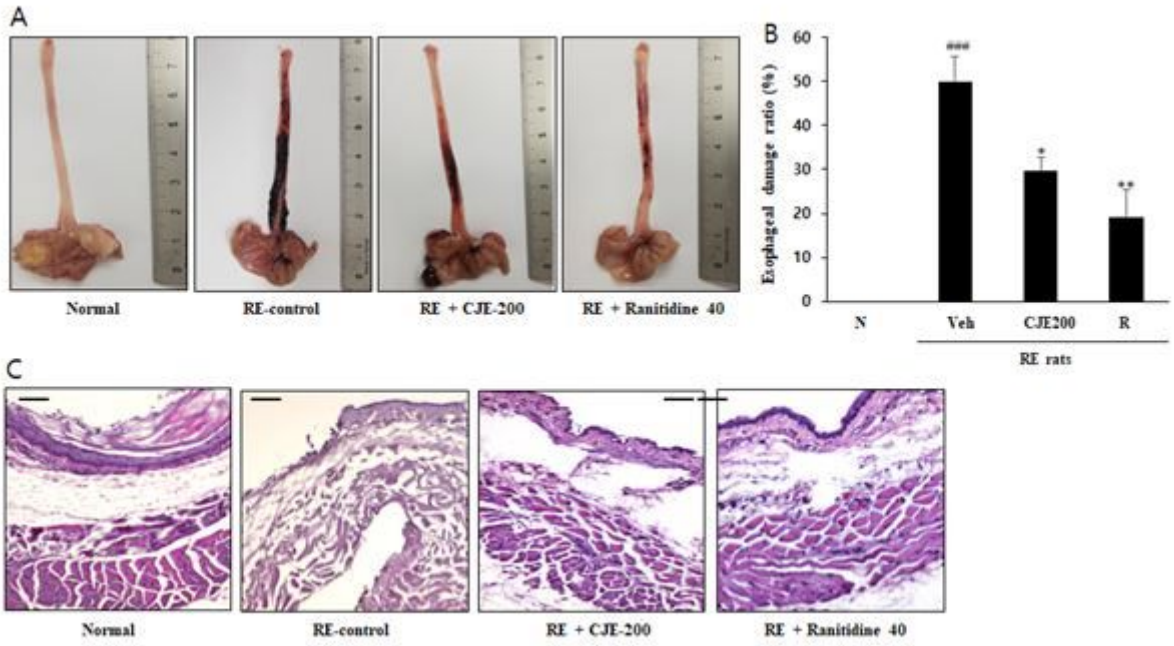


Figure 6

Effect of CJE on esophageal tissue damage (A), esophageal damage ratio (B) and histological changes of the esophagus in each group (C). N - Normal rat, Veh - RE control rat, CJE 200 - RE rats pretreated with CJE 200 mg/kg and R - RE rats treated with ranitidine 40 mg/kg. Values are presented as the mean ± standard deviation. ###P<0.001 vs. normal rat, **P<0.01, *P<0.05 vs. RE control rat.

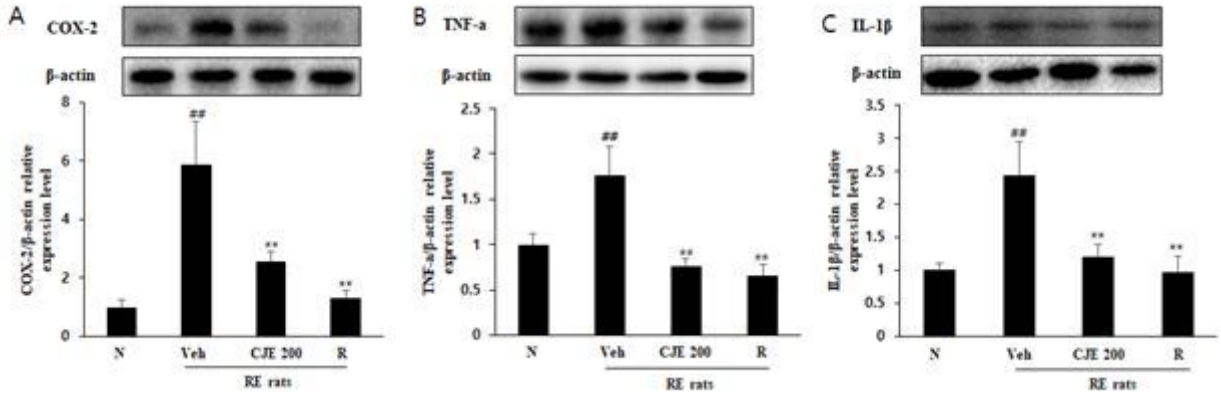


Figure 7

Effect of CJE on protein expression levels of COX-2 (A), TNF-α (B) and IL-1β (C) in rats esophageal tissue. The protein levels of COX-2, TNF-a and IL-1β were determined by western blotting. N - Normal rat, Veh - RE control rat, CJE 200 - RE rats pretreated with CJE 200 mg/kg and R - RE rats treated with ranitidine 40 mg/kg. The relative band intensity was measured as compared with β-actin. Values are presented as the mean ± standard deviation. ##P<0.01 vs. normal rat, **P<0.01 vs. RE-control rat.

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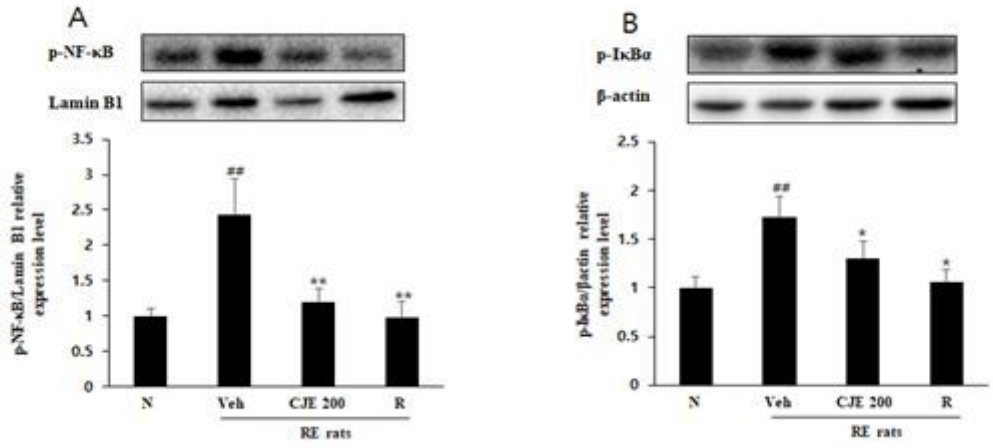


Figure 8

Effect of CJE on protein expression levels of phosphorylated NF-κB (A) and IκBa (B) in rats esophageal tissue. The protein levels of p-NF-κB and p-IκBa were determined by Western blotting. The relative band intensity of p-NF-κB and p-IκBa were measured as compared with Lamin B1 and β-actin. N - Normal rat, Veh - RE control rat, CJE 200 - RE rats pretreated with CJE 200 mg/kg and R - RE rats treated with ranitidine 40 mg/kg. Values are presented as the mean ± standard deviation. ##P<0.01 vs. normal rat, **P<0.01, *P<0.05 vs. RE-control rat.

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