

Research Article

Anti-Inflammatory Activities of *Cinnamomum cassia* Constituents *In Vitro* and *In Vivo*

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We have investigated the anti-inflammatory effects of *Cinnamomum cassia* constituents (cinnamic aldehyde, cinnamic alcohol, cinnamic acid, and coumarin) using lipopolysaccharide (LPS)-stimulated mouse macrophage (RAW264.7) and carrageenan (Carr)-induced mouse paw edema model. When RAW264.7 macrophages were treated with cinnamic aldehyde together with LPS, a significant concentration-dependent inhibition of nitric oxide (NO), tumor necrosis factor (TNF- α), and prostaglandin E₂ (PGE₂) levels productions were detected. Western blotting revealed that cinnamic aldehyde blocked protein expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nuclear transcription factor kappa B (NF- κ B), and I κ B α , significantly. In the anti-inflammatory test, cinnamic aldehyde decreased the paw edema after Carr administration, and increased the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in the paw tissue. We also demonstrated cinnamic aldehyde attenuated the malondialdehyde (MDA) level and myeloperoxidase (MPO) activity in the edema paw after Carr injection. Cinnamic aldehyde decreased the NO, TNF- α , and PGE₂ levels on the serum level after Carr injection. Western blotting revealed that cinnamic aldehyde decreased Carr-induced iNOS, COX-2, and NF- κ B expressions in the edema paw. These findings demonstrated that cinnamic aldehyde has excellent anti-inflammatory activities and thus has great potential to be used as a source for natural health products.

1. Introduction

Inflammation is recognized as a biological process in response to tissue injury. At the injury site, an increase in blood vessel wall permeability followed by migration of immune cells can lead edema formation during inflammation [1]. Inflammation leads to the upregulation of a series of enzymes and signaling proteins in affected cells and tissues. Inducible nitric oxide synthase (iNOS), a member of the NOS protein family, catalyzes the formation of nitric oxide (NO) from L-arginine [2]. Low concentration of NO produced by iNOS is likely to contribute to the antimicrobial

activity of macrophages against certain bacterial pathogens. Lipopolysaccharide (LPS) is an endotoxin and a constituent of the outer membrane of gram-negative bacteria. LPS stimulates innate immunity, by regulating the productions of inflammatory mediators, like, NO, TNF- α , and Interleukin-6 [3]. And in the animal the inflammation model of a carrageenan (Carr) induced edema is usually used to assess the contribution of natural products in resisting the biochemical changes associated with acute inflammation. Carr can induce acute inflammation beginning with infiltration of phagocytes, the production of free radicals as well as the release of inflammatory mediators [4]. The resulting inflammation has

been shown to be associated with a number of chronic diseases, including asthma, rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, and Alzheimer's disease, and also has a role in various human cancers [5].

Intracellular antioxidant mechanisms against these inflammatory stresses involve antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in tissues. Recently, it has been shown that faulty cellular antioxidant systems cause organisms to develop a series of inflammatory and cancer diseases [6]. However, it appears that the various roles of enzymatic antioxidants help to protect organisms from excessive generation of oxidative stress in the inflammatory process, which has triggered studies focusing on the role of natural products in suppressing the production of oxidation by increasing enzymatic antioxidants in tissues [7].

Cinnamomum cassia (*C. cassia*), bark, is the outer skin of an evergreen tall tree belonging to the family Lauraceae. It is commonly used as traditional Chinese medicine for treating dyspepsia, gastritis, blood circulation disturbances, and inflammatory diseases. Its extracts contain several active components such as essential oils (cinnamic aldehyde, cinnamic alcohol, cinnamic acid, and coumarin), tannin, mucus, and carbohydrates [8]. *C. cassia* has been shown to have many pharmacological properties, such as antiulcerogenic, anti-inflammatory, antipyretic, antimicrobial, antidiabetic and antitumor activity [9, 10]. However, in this paper we examined that cinnamic aldehyde was the most potent anti-inflammatory constituent of *C. cassia* on LPS-induced in RAW264.7 cells and Carr-induced on paw edema in mice. And we detected the levels of iNOS, COX-2, and NF- κ B in either RAW264.7 cell or paw edema. Also, the activities of CAT, SOD, and GPx in the paw tissue at the 5th h after Carr injection were measured to understand the relationship between the anti-inflammatory mechanism of cinnamic aldehyde and antioxidant enzymes.

2. Materials and Methods

2.1. Chemicals. LPS (endotoxin from *Escherichia coli*, serotype 0127:B8), Carr, indomethacin, cinnamic aldehyde ($\geq 98\%$), cinnamic alcohol ($\geq 98\%$), cinnamic acid ($\geq 99\%$), coumarin ($\geq 99\%$) (Figure 1(a)) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, USA). TNF- α and PGE₂ were purchased from Biosource International Inc. (Camarillo, CA, USA). Anti-iNOS, anti-COX-2, anti-NF- κ B, anti-I κ B α , and anti- β -actin antibody (Santa Cruz, USA) and a protein assay kit (Bio-Rad Laboratories Ltd., Watford, Herts, UK) were obtained as indicated. Poly-(vinylidene fluoride) membrane (Immobilon-P) was obtained from Millipore Corp. (Bedford, MA, USA).

2.2. Animals. 6–8 weeks male imprinting control region (ICR) mice were obtained from the BioLASCO Taiwan Co., Ltd. The animals were kept in plexiglass cages at a constant temperature of $22 \pm 1^\circ\text{C}$, and relative humidity of $55 \pm 5\%$ with 12 h dark-light cycle for at least 2 week before the

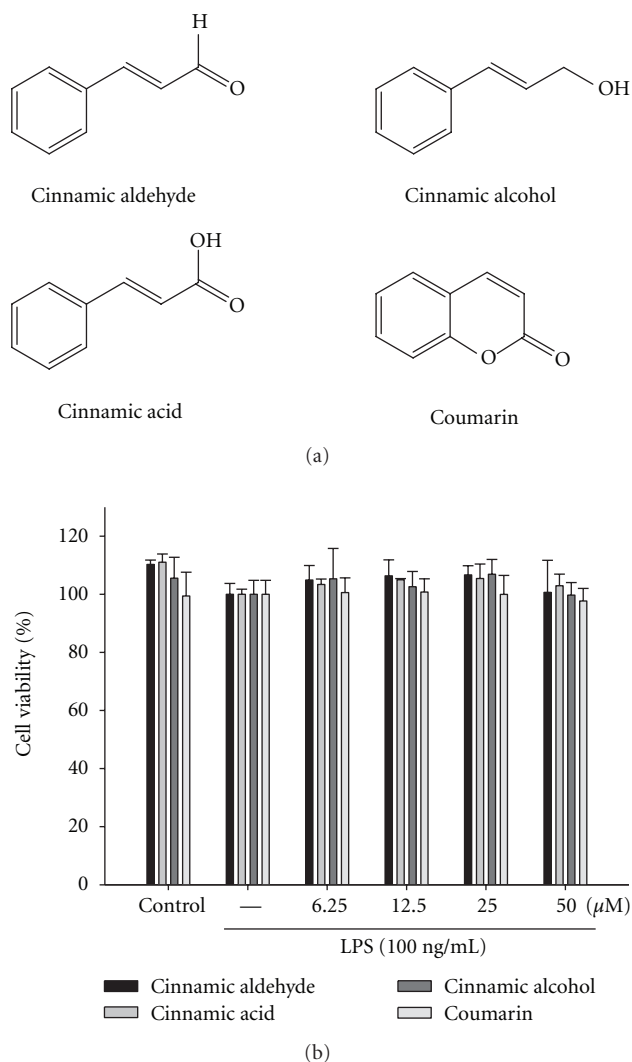


FIGURE 1: Chemical structure of *Cinnamomum cassia* constituents (cinnamic aldehyde, cinnamic alcohol, cinnamic acid, and coumarin) (a) and cytotoxic effects of *Cinnamomum cassia* constituents in RAW264.7 cells (b). Cells were incubated for 24 h with 100 ng/mL of LPS in the absence or presence of samples (0, 6.25, 12.5, 25, and 50 μM). Samples were added 1 h before incubation with LPS (lipopolysaccharide). Cell viability assay was performed using MTT assay. The data were presented as mean \pm S.D. for three different experiments performed in triplicate.

experiment. They were given food and water *ad libitum*. All experimental procedures were performed according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. In addition, all tests were conducted under the guidelines of the International Association for the Study of Pain.

After a 2-week adaptation period, male ICR mice (18–25 g) were randomly assigned to four groups ($n = 6$) of the animals in the study. The control group receives normal saline (i.p.). The other three groups include a Carr-treated, a positive control (Carr + Indo), and cinnamic aldehyde administered groups (Carr + cinnamic aldehyde).

2.3. Cell Culture. A murine macrophage cell line RAW264.7 (BCRC no. 60001) was purchased from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in plastic dishes containing Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA) in a CO₂ incubator (5% CO₂ in air) at 37°C and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin—0.02% EDTA in Ca²⁺-, Mg²⁺-free phosphate-buffered saline (DPBS).

2.4. Cell Viability. Cells (2×10^5) were cultured in 96-well plate containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with cinnamic aldehyde, cinnamic alcohol, cinnamic acid, and coumarin in the presence of 100 ng/mL LPS (lipopolysaccharide) for 24 h. After that, the cells were washed twice with DPBS and incubated with 100 μ L of 0.5 mg/mL MTT for 2 h at 37°C testing for cell viability {MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)}. The medium was then discarded and 100 μ L dimethyl sulfoxide (DMSO) was added. After 30-min incubation, absorbance at 570 nm was read using a microplate reader.

2.5. Measurement of Nitric Oxide/Nitrite. NO production was indirectly assessed by measuring the nitrite levels in the cultured media and serum determined by a colorimetric method based on the Griess reaction [4]. The cells were incubated with cinnamic aldehyde, cinnamic alcohol, cinnamic acid, coumarin (0, 6.25, 12.5, 25, and 50 μ M) in the presence of LPS (100 ng/mL) at 37°C for 24 h. Then, cells were dispensed into 96-well plates, and 100 μ L of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride and 5% phosphoric acid) and incubated at room temperature for 10 min, the absorbance was measured at 540 nm with a Micro-Reader (Molecular Devices, Orleans Drive, Sunnyvale, CA). Serum samples were diluted four times with distilled water and deproteinized by adding 1/20 volume of zinc sulfate (300 g/L) to a final concentration of 15 g/L. After centrifugation at 10,000 \times g for 5 min at room temperature, 100 μ L supernatant was applied to a microtiter plate well, followed by 100 μ L of Griess reagent. After 10 min of color development at room temperature, the absorbance was measured at 540 nm with a Micro-Reader. By using sodium nitrite to generate a standard curve, the concentration of nitrite was measured by absorbance at 540 nm.

2.6. Carr-Induced Edema. The Carr-induced hind paw edema model was used for determination of anti-inflammatory activity [1]. Animals were i.p. treated with cinnamic aldehyde (1.25, 2.5 and 5 mg/kg), Indo or normal saline, 30 min prior to injection of 1% Carr (50 μ L) in the plantar side of right hind paws of the mice. The paw volume was measured after Carr injection and at 1, 2, 3, 4, and 5 h intervals

after the administration of the edematogenic agent using a plethysmometer (model 7159, Ugo Basile, Varese, Italy). The degree of swelling induced was evaluated by the ratio a/b , where a is the volume of the right hind paw after Carr treatment, and b is the volume of the right hind paw before Carr treatment. Indo was used as a positive control. After 5 h, the animals were sacrificed and the Carr-induced edema feet were dissected and stored at -80°C. Also, blood was withdrawn and kept at -80°C. The protein concentration of the sample was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA).

2.7. MDA Assay. MDA from Carr-induced edema foot was evaluated by the thiobarbituric acid reacting substance (TRARS) method [1]. Briefly, MDA reacted with thiobarbituric acid in the acidic high temperature and formed a red-complex TBARS. The absorbance of TBARS was determined at 532 nm.

2.8. Myeloperoxidase Activity Assay. The activity of tissue MPO was assessed at the 5th h after injection of Carr into the mouse right hind paw according to the method of Bani et al. [11] with some modifications. Samples were placed in 0.75 mL of 80 mM phosphate-buffered saline (PBS), pH 5.4, and then homogenized in a motor-driven homogenizer. The homogenate was centrifuged at 12,000 \times g at 4°C for 15 min. Triplicate 0.1 mL of supernatant with 2.9 mL of potassium phosphate buffer (50 mM, pH 6) containing 0.19 mg/mL of o-dianisidine chloride and 0.0005% H₂O₂ was a substrate for myeloperoxidase. Oxidized o-dianisidine formed a soluble chromophore and absorbance (OD₄₆₀) was determined by spectrophotometry (Molecular Devices, Orleans Drive, Sunnyvale, CA) over 2 min. Myeloperoxidase activity (Δ OD₄₆₀) was calculated by subtracting the value of OD₄₆₀ at time 0 min from that at 2 min for each sample.

2.9. Measurement of TNF- α and PGE₂ by an Enzyme-Linked Immunosorbent Assay (ELISA). The levels of TNF- α and PGE₂ were determined using a commercially available ELISA kit (Biosource International Inc., Camarillo, CA) according to the manufacturer's instruction. TNF- α and PGE₂ were determined from a standard curve.

2.10. Antioxidant Enzyme Activity Measurements. The following biochemical parameters were analyzed to check the paw tissues activity of cinnamic aldehyde by the methods given below.

Total SOD activity was determined by the inhibition of cytochrome *c* reduction [12]. The reduction of cytochrome *c* was mediated by superoxide anions generated by the xanthine/xanthine oxidase system and monitored at 550 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the rate of cytochrome *c* reduction by 50%. Total CAT activity was based on that of Aebi [13]. In brief, the reduction of 10 mM H₂O₂ in 20 mM of phosphate buffer (pH 7.0) was monitored by measuring the absorbance at 240 nm. The activity was calculated using a molar absorption coefficient, and the enzyme activities were

defined as nanomoles of dissipating hydrogen peroxide per milligram protein per minute. Total GPx activity in cytosol was determined according to Paglia and Valentine's method [14]. The enzyme solution was added to a mixture containing hydrogen peroxide and glutathione in 0.1 mM Tris buffer (pH 7.2) and the absorbance at 340 nm was measured. Activity was evaluated from a calibration curve, and the enzyme activities were defined as nanomoles of NADPH oxidized per milligram protein per minute.

2.11. Protein Lysate Preparation and Western Blot Analysis of iNOS, COX-2, I κ B α , and NF- κ B. The stimulated murine macrophage cell line RAW264.7 cells were washed with PBS and lysed in an ice-cold lysis buffer (10% glycerol, 1% Triton X-100, 1 mM Na₃VO₄, 1 mM EGTA, 10 mM NaF, 1 mM Na₄P₂O₇, 20 mM Tris buffer (pH 7.9), 100 mM β -glycerophosphate, 137 mM NaCl, 5 mM EDTA, and one protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA)) on ice for 1 h, followed by centrifugation at 12,000 rpm for 30 min at 4°C. Soft tissues were removed from individual mice paws and homogenized in a solution containing 10 mM CHAPS, 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 μ g/mL, aprotinin, 1 μ M pepstatin and 10 μ M leupeptin. The homogenates were centrifuged at 12,000 g for 20 min, and 30 μ g of protein from the supernatants was then separated on 10% sodium dodecylsulphate-polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. After transfer, the membrane was blocked for 2 h at room temperature with 5% skim milk in Tris-buffered saline-Tween (TBST; 20 mM Tris, 500 mM NaCl, pH 7.5, 0.1% Tween 20). The membranes were then incubated with mouse monoclonal anti-iNOS, anti-COX-2, anti-I κ B α , or anti-NF- κ B antibody in 5% skim milk in TBST for 2 h at room temperature. The membranes were washed three times with TBST at room temperature and then incubated with a 1:2000 dilution of anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (Sigma, St. Louis, MO, U.S.A.) in 2.5% skim milk in TBST for 1 h at room temperature. The membranes were washed three times and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL reagent (Amersham International plc., Buckinghamshire, UK). The results of Western blot analysis were quantified by measuring the relative intensity compared to the control using Kodak Molecular Imaging Software (Version 4.0.5, Eastman Kodak Company, Rochester, NY) and represented in the relative intensities.

2.12. Histological Examination. For histological examination, biopsies of paws were taken 5 h following the interplanetary injection of Carr. The tissue slices were fixed in a solution (1.85% formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded ethanol and embedded in Paraffin (Sherwood Medical). Sections (thickness 5 μ m) were deparaffinized with xylene and stained with hematoxylin and eosin (H&E) stain. All samples were observed and photographed with BH-2 Olympus microscopy. Every 3~5 tissue slices were randomly chosen

from Carr, Indo and cinnamic aldehyde-treated (5 mg/kg) groups. Histological examination of these tissue slices revealed an excessive inflammatory response with massive infiltration of neutrophils (polymorphonuclear leukocytes (PMNs)) by microscopy. The numbers of neutrophils were counted in each scope (400 x) and thereafter obtain their average count from 5 scopes of every tissue slice [15].

2.13. Statistical Analysis. Data are expressed as mean \pm standard error of the mean (SEM). Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Scheffe's multiple range test). Statistical significance is expressed as * P < 0.05, ** P < 0.01, *** P < 0.001.

3. Results

3.1. Cell Viability. The effect of *C. cassia* constituents (cinnamic aldehyde, cinnamic alcohol, cinnamic acid, and coumarin) on RAW264.7 cell viability was determined by a MTT assay. Cells cultured with samples at the concentrations (0, 6.25, 12.5, 25, and 50 μ M) used in the presence of 100 ng/mL LPS for 24 h did not change cell viability (Figure 1(b)).

3.2. Effect of Cinnamic Aldehyde, Cinnamic Alcohol, Cinnamic Acid, and Coumarin on LPS-Induced NO Production in Macrophages. In the present study, effects of cinnamic aldehyde, cinnamic alcohol, cinnamic acid, and coumarin on LPS-induced NO production in RAW264.7 macrophages were investigated. Nitrite accumulated in the culture medium was estimated by the Griess reaction as an index for NO release from the cells. After treatment with LPS (100 ng/mL) for 24 h, the nitrite concentration increased in the medium. When RAW264.7 macrophages were treated with different concentrations of cinnamic aldehyde together with LPS for 24 h, the cinnamic aldehyde inhibited nitrite production significantly (Figure 2). Cinnamic aldehyde did not interfere with the reaction between nitrite and Griess reagents at 50 μ M (data not shown). Unstimulated macrophages, after 24 h of incubation in culture medium produced background levels of nitrite. When RAW264.7 macrophages were treated with different concentrations of cinnamic aldehyde (0, 6.25, 12.5, 25, and 50 μ M) together with LPS (100 ng/mL) for 24 h, a significant concentration-dependent inhibition of nitrite production was detected. There was either a significant decrease in the nitrite production of group treated with 12.5 μ M cinnamic aldehyde (P < 0.05), or very or highly significant decrease of groups treated, respectively, with 25 or 50 μ M of cinnamic aldehyde when compared with the LPS-alone group (P < 0.01 or P < 0.001). The IC₅₀ value for inhibition of nitrite production of cinnamic aldehyde was about 45.56 ± 1.36 μ M.

3.3. Inhibition of LPS-Induced iNOS, COX-2, I κ B α , and NF- κ B Protein by Cinnamic Aldehyde, Cinnamic Alcohol, Cinnamic Acid, and Coumarin. In order to investigate whether the inhibition of NO production was due to a decreased iNOS, COX-2, I κ B α , and NF- κ B protein level, the effect of

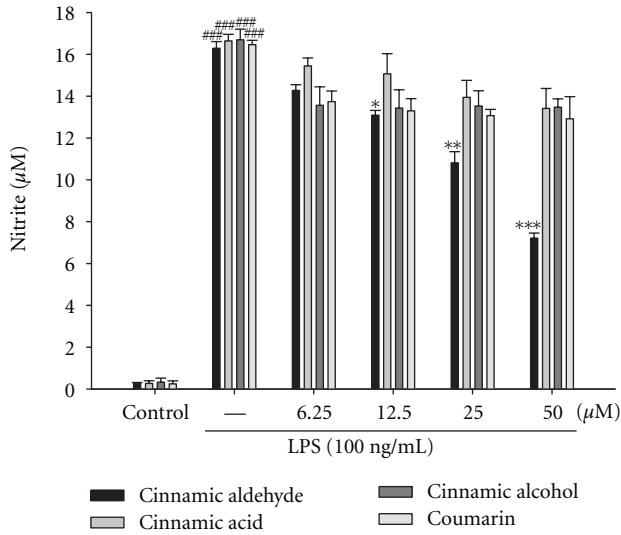


FIGURE 2: Effects of *Cinnamomum cassia* constituents (cinnamic aldehyde, cinnamic alcohol, cinnamic acid, and coumarin) on LPS-induced NO production of RAW264.7 macrophages. Cells were incubated for 24 h with 100 ng/mL of LPS in the absence or presence of samples (0, 6.25, 12.5, 25, and 50 µM). Samples were added 1 h before incubation with LPS. Nitrite concentration in the medium was determined using Griess reagent. The data were presented as mean ± S.D. for three different experiments performed in triplicate. ### compared with sample of control group. *P < 0.05, **P < 0.01, and ***P < 0.001 were compared with LPS-alone group.

cinnamic aldehyde, cinnamic alcohol, cinnamic acid, and coumarin was studied by immunoblot. The results showed the incubation with cinnamic aldehyde (50 µM) in the presence of LPS (100 ng/mL) for 24 h or 1 h inhibited iNOS, COX-2, IκBα, and NF-κB proteins expression in mouse macrophage RAW264.7 cells in the cytosol (Figures 3(a) and 4(a)). The detection of β-actin was also performed in the same blot as an internal control. The intensity of protein bands was analyzed by using Kodak Quantity software in three independent experiments and it showed an average of 77.4% and 84.8% downregulation of iNOS and COX-2 proteins, respectively, after treatment with cinnamic aldehyde at 50 µM compared with the LPS-alone (Figure 3(b)). And the intensity of protein bands showed an average of 82.6% and 86.2% upregulation of NF-κB and IκBα protein (P < 0.001) (Figure 4(b)).

3.4. Inhibition of LPS Induced the Level of TNF-α and PGE₂ by Cinnamic Aldehyde. TNF-α mediates the production of many other cytokines during inflammation, in particular, the production of interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) [16]. We examined the effect of cinnamic aldehyde on LPS induced upregulation of TNF-α. A very low amount of TNF-α protein was detected by a specific ELISA for TNF-α in controls (Figure 5(a)). When RAW264.7 macrophages were treated with different concentrations of cinnamic aldehyde (12.5, 25, and 50 µM) together with LPS (100 ng/mL) for

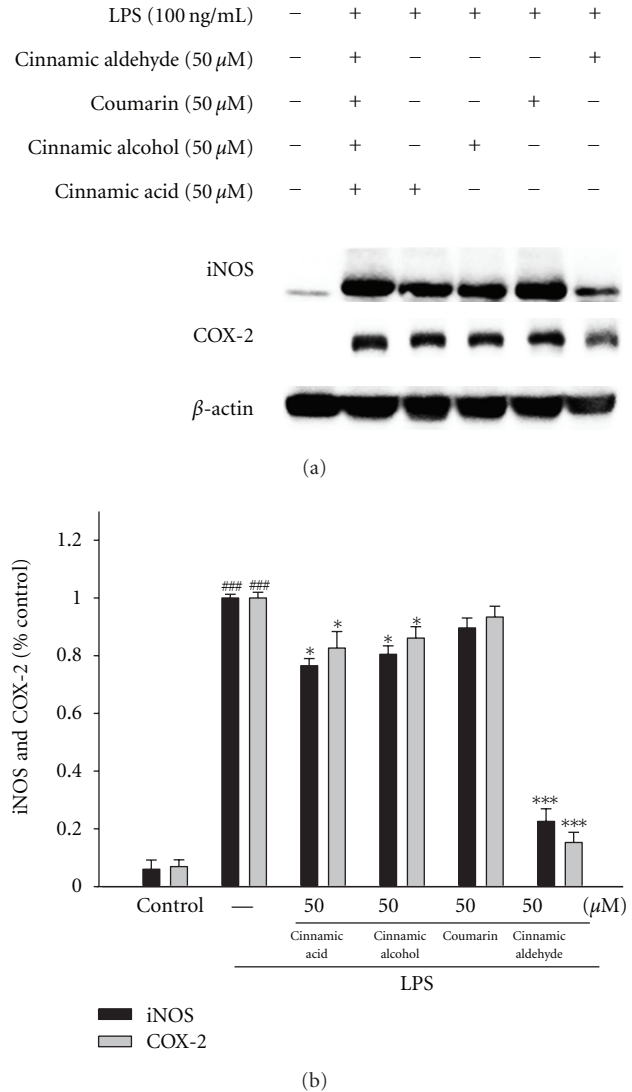


FIGURE 3: Inhibition of iNOS and COX-2 protein expression by *Cinnamomum cassia* constituents (cinnamic aldehyde, cinnamic alcohol, cinnamic acid, and coumarin) in LPS-stimulated RAW264.7 cells. Cells were incubated for 24 h with 100 ng/mL of LPS in the absence or the presence of samples (50 µM). Samples were added 1 h before incubation with LPS. Lysed cells were then prepared and subjected to western blotting using an antibody specific for iNOS and COX-2. β-actin was used as an internal control. (a) A representative western blot from two separate experiments is shown. (b) Relative iNOS and COX-2 protein levels were calculated with reference to an LPS-stimulated culture. ### compared with sample of control group. The data were presented as mean ± S.D. for three different experiments performed in triplicate. *P < 0.05 and ***P < 0.001 were compared with LPS-alone group.

24 h, a significant concentration-dependent inhibition of TNF-α production was detected. There was either a significant decrease in the TNF-α production of group treated with 12.5 µM cinnamic aldehyde (P < 0.05), or highly significant decrease of groups treated, respectively, with 25 and 50 µM of cinnamic aldehyde when compared with the LPS-alone group (P < 0.01 or P < 0.001). The IC₅₀ value for

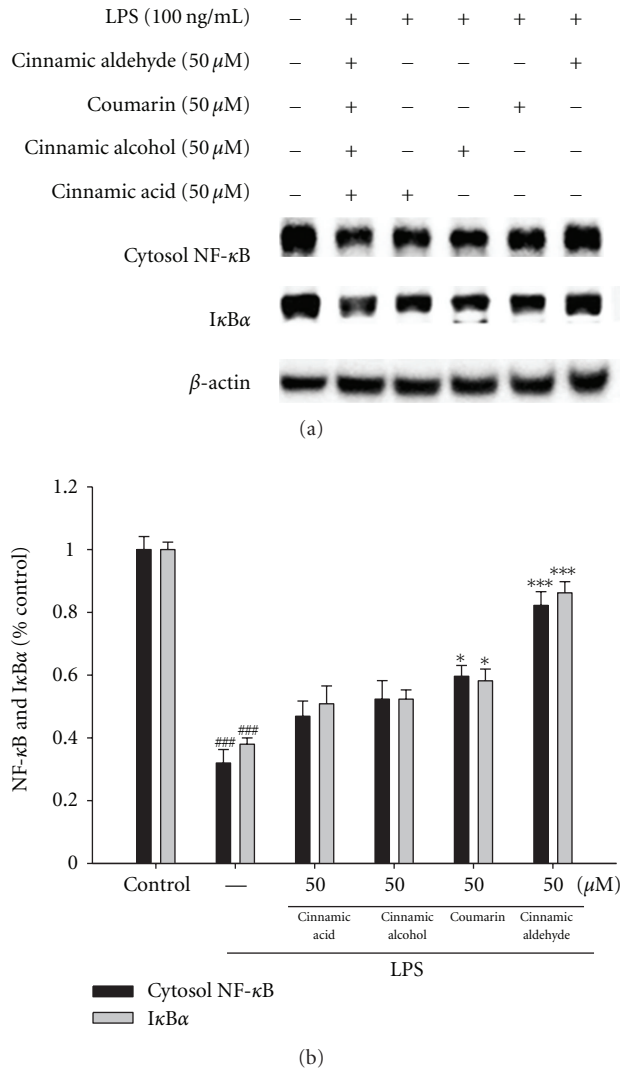


FIGURE 4: Inhibition of NF- κ B and I κ B α (a) protein expressions by *Cinnamomum cassia* constituents (cinnamic aldehyde, cinnamic alcohol, cinnamic acid, and coumarin) in LPS-stimulated RAW264.7 cells. Samples (50 μ M) were added into cells 1 h before LPS (100 ng/mL) stimulation and protein samples were prepared for 1 h after LPS stimulation. Activations of signaling molecules were then evaluated by Western blot analysis. Lysed cells were then prepared and subjected to western blotting using an antibody specific for NF- κ B (P65) and I κ B α in the cytosol. β -actin was used as an internal control. A representative western blot from two separate experiments is shown. Relative NF- κ B and I κ B α protein levels were calculated with reference to an LPS-stimulated culture (b). ### compared with sample of control group. The data were presented as mean \pm S.D. for three different experiments performed in triplicate. * P < 0.05 and *** P < 0.001 were compared with LPS-alone group.

inhibition of TNF- α production of cinnamic aldehyde was about $29.58 \pm 0.34 \mu$ M.

PGE₂ represents the most important inflammatory product of COX-2 activity and it was quantified in cell-free culture supernatant [16]. As shown in Figure 5(b), cells were stimulated with LPS alone raised significant amount

of PGE₂ in RAW264.7 macrophages. When RAW264.7 macrophages were treated with different concentrations of cinnamic aldehyde (12.5, 25, and 50 μ M) together with LPS (100 ng/mL) for 24 h, a significant concentration-dependent inhibition of PGE₂ production was detected. The IC₅₀ value for inhibition of PGE₂ production of cinnamic aldehyde was about $37.67 \pm 0.58 \mu$ M.

3.5. Effects of Cinnamic Aldehyde on Carr-Induced Mice Paw Edema. In this study, we used Carr-induced edema because this model is widely employed for screening the effects of anti-inflammatory drugs. Carr-induced paw edema is shown in Figure 6(a). Cinnamic aldehyde (5 mg/kg) inhibited (P < 0.001) the development of paw edema induced by Carr after the 4th and the 5th h of treatment, significantly. Indo (10 mg/kg) significantly decreased the Carr-induced paw edema after the 4th and the 5th h of treatment (P < 0.001).

3.6. Effects of Cinnamic Aldehyde on the MDA Level. The MDA level increased significantly in the edema paw at the 5th h after Carr injection (P < 0.001). However, the MDA level was decreased significantly by treatment with cinnamic aldehyde (5 mg/kg) (P < 0.001), as well as 10 mg/kg Indo (Figure 6(b)).

3.7. Effects of Cinnamic Aldehyde on the MPO Activity. The MPO activity increased significantly in the edema paw at the 5th h after Carr injection (P < 0.001). However, the MPO activity was decreased significantly by the treatment with cinnamic aldehyde (5 mg/kg) (P < 0.001), as well as 10 mg/kg Indo (Figure 6(c)).

3.8. Effects of Cinnamic Aldehyde on the NO Level. In Figure 6(d), the NO level increased significantly in the edema serum at the 5th h after Carr injection (P < 0.001). Cinnamic aldehyde (5 mg/kg) significantly decreased the serum NO level (P < 0.001). The inhibitory potency was similar to that of Indo (10 mg/kg) at the 5th h after induction.

3.9. Effects of Cinnamic Aldehyde on the TNF- α and PGE₂ Level. The TNF- α and PGE₂ level increased significantly in serum at the 5th h after Carr injection (P < 0.001). However, cinnamic aldehyde (1.25 or 2.5 mg/kg) decreased the TNF- α and PGE₂ level in serum at the 5th h after Carr injection (P < 0.05 or P < 0.01), as well as 10 mg/kg Indo (Figures 6(e) and 6(f)).

3.10. Effects of Cinnamic Aldehyde on Activities of Antioxidant Enzymes. At the 5th h after the intrapaw injection of Carr, paw tissues were also analyzed for the biochemical parameters such as CAT, SOD, and GPx activities. CAT, SOD, and GPx activities in paw tissue were decreased significantly by Carr administration. CAT, SOD, and GPx activity were increased significantly after being treated with 5 mg/kg cinnamic aldehyde and 10 mg/kg Indo (P < 0.01 or P < 0.001) (Table 1).

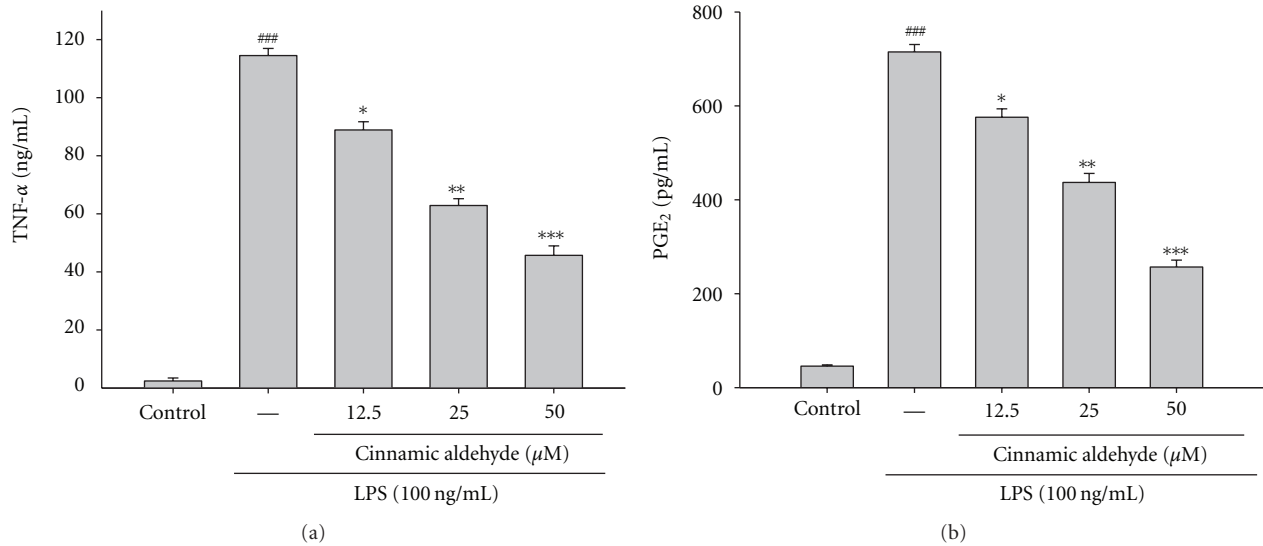


FIGURE 5: The effects of cinnamic aldehyde on lipopolysaccharide (LPS)-induced TNF- α (a) and PGE₂ (b) in LPS-stimulated RAW264.7 cells. Cells were incubated for 24 h with 100 ng/mL of LPS in the absence or in the presence of cinnamic aldehyde (0, 12.5, 25, and 50 μ M). Cinnamic aldehyde was added 1 h before the incubation with LPS. TNF- α and PGE₂ concentrations in the medium were determined using ELISA kit. The data were presented as mean \pm S.D. for three different experiments performed in triplicate. ^{###} $P < 0.001$ compared with sample of control group. ^{*} $P < 0.05$, ^{**} $P < 0.01$, and ^{***} $P < 0.001$ were compared with LPS-alone group.

TABLE 1: Effects of cinnamic aldehyde and indomethacin (Indo) on changes in CAT, SOD, and GPx activities were studied in Carr-induced paw edema (5th h) in mice.

Groups	Catalase	SOD	GPx
	(U/mg protein)	(U/mg protein)	(U/mg protein)
Control	5.89 \pm 0.12	21.58 \pm 0.08	28.26 \pm 0.21
Carr	3.68 \pm 0.04 ^{###}	12.46 \pm 0.13 ^{###}	16.68 \pm 0.19 ^{###}
Carr + Indo	5.27 \pm 0.16 ^{**}	20.06 \pm 0.19 ^{**}	26.32 \pm 0.19 ^{***}
Carr + cinnamic aldehyde (1.25 mg/Kg)	3.89 \pm 0.13	14.59 \pm 0.15	17.59 \pm 0.16
Carr + cinnamic aldehyde (2.5 mg/Kg)	4.59 \pm 0.19 [*]	16.61 \pm 0.21 [*]	21.27 \pm 0.25 [*]
Carr + cinnamic aldehyde (5 mg/Kg)	5.21 \pm 0.19 ^{**}	20.83 \pm 0.17 ^{**}	25.26 \pm 0.24 ^{**}

Each value represents as mean \pm S.E.M. ^{###} $P < 0.001$ as compared with control group. ^{*} $P < 0.05$, ^{**} $P < 0.01$, and ^{***} $P < 0.001$ as compared with the Carr (λ -carrageenan) group (one-way ANOVA followed by Scheffe's multiple range test).

3.11. Effects of Cinnamic Aldehyde on Carr-Induced iNOS, COX-2, and NF- κ B Protein Expressions in Mice Paw Edema.

To investigate whether the inhibition of NO production was due to a decreased iNOS, COX-2, and NF- κ B protein level, the effect of cinnamic aldehyde on iNOS, COX-2, and NF- κ B proteins expression was studied by western blot. The results showed that injection of cinnamic aldehyde (5 mg/kg) on Carr-induced for 5 h inhibited iNOS, COX-2, and NF- κ B proteins expression in mouse paw edema (Figure 7(a)). The detection of β -actin was also performed in the same blot as an internal control. The intensity of protein bands was analyzed by using Kodak Quantity software in three independent experiments and showed an average of 76.1% and 63.3% downregulation of iNOS and COX-2 protein, respectively, after treatment with cinnamic aldehyde at 5 mg/kg compared with the Carr-induced alone (Figure 7(b)). In addition, the protein expression showed

an average of 57.1% and 45.1% down-regulation of iNOS, and COX-2 protein after treatment with Indo at 10 mg/kg compared with the Carr-induced alone (Figure 7(b)). And the intensity of protein bands showed an average of 87.6% upregulation of NF- κ B protein ($P < 0.001$) (Figure 7(b)). The down-regulation of iNOS, COX-2, and NF- κ B activity of the cinnamic aldehyde (5 mg/kg) was better than Indo (10 mg/kg).

3.12. Histological Examination.

Paw biopsies of Carr model animals showed marked cellular infiltration in the connective tissue. The infiltrates accumulated between collagen fibers and into intercellular spaces. Paw biopsies of animals treated with cinnamic aldehyde (5 mg/kg) showed a reduction in Carr-induced inflammatory response. Actually inflammatory cells were reduced in number and confined to near

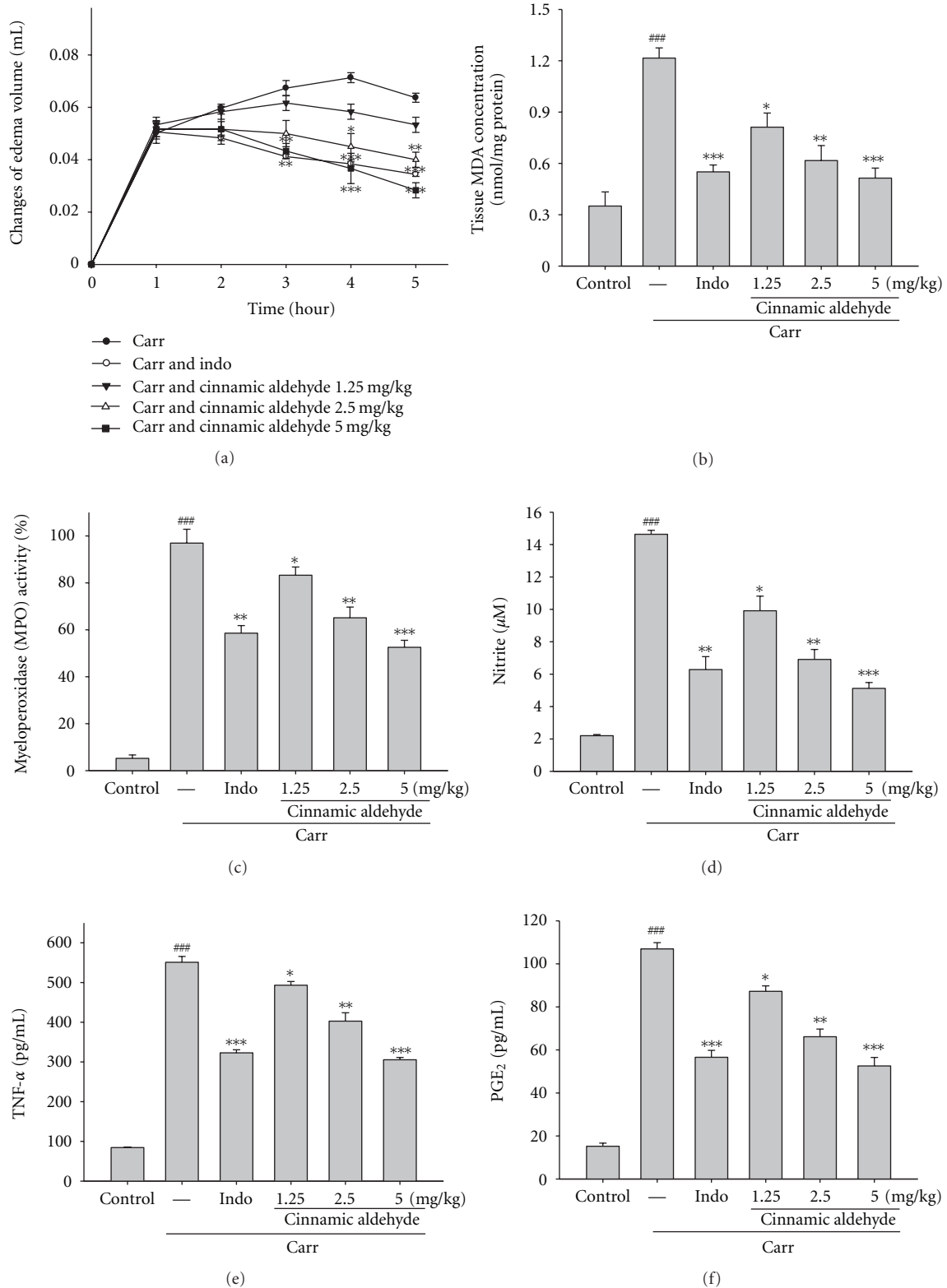


FIGURE 6: Effects of cinnamic aldehyde and Indo on hind paw edema induced by Carr in mice (a), the tissue MDA (b) and MPO (c) concentrations of foot in mice, Carr-induced NO (d), TNF- α , (e) and PGE₂ (f) concentrations of serum at the 5th h in mice. The values are averaged, obtained in individual animals ($n = 6$). Each value represents as mean \pm S.E.M. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ as compared with the Carr group.

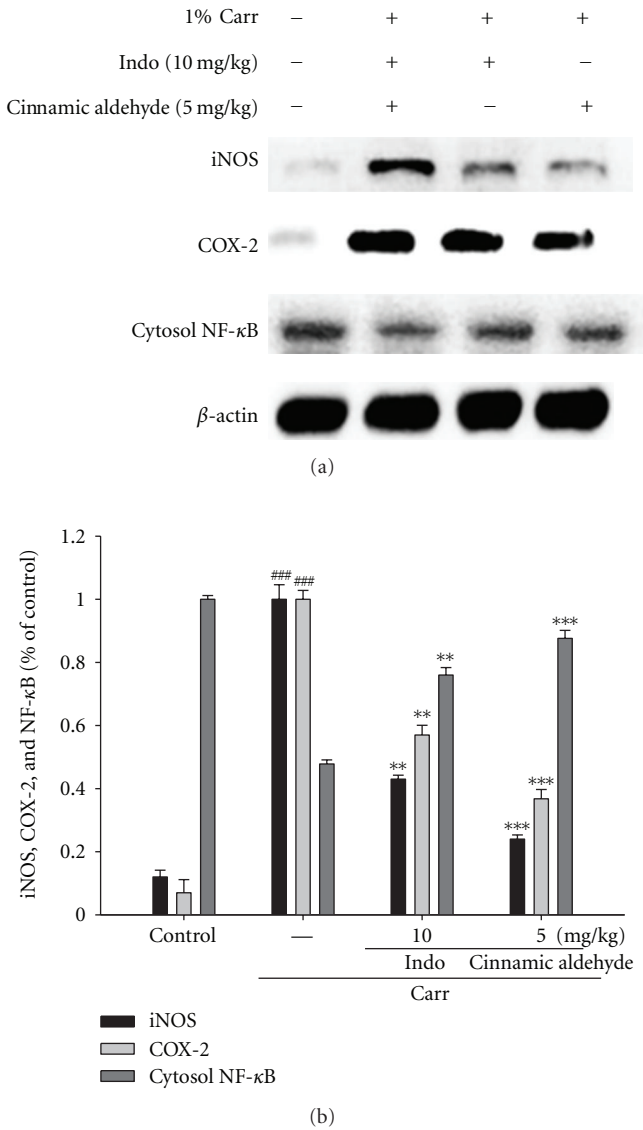


FIGURE 7: Inhibition of iNOS, COX-2, and NF-κB protein expressions by cinnamic aldehyde induced by Carr of foot at the 5th h in mice. Tissues suspended were then prepared and subjected to western blotting using an antibody specific for iNOS, COX-2, and NF-κB. β-actin was used as an internal control. (a) A representative western blot from two separate experiments is shown. (b) Relative iNOS, COX-2, and NF-κB protein levels were calculated with reference to a Carr-injected mouse. ### compared with sample of control group. The data were presented as mean ± S.D. for three different experiments performed in triplicate. ** $P < 0.01$ and *** $P < 0.001$ were compared with Carr-alone group.

the vascular areas. Intercellular spaces did not show any cellular infiltrations. Collagen fibers were regular in shape and showed a reduction of intercellular spaces. Moreover, the hypoderm connective tissue was not damaged (Figure 8(a)). Neutrophils increased with Carr treatment ($P < 0.01$), as Indo and cinnamic aldehyde (5 mg/kg) could significantly decrease the neutrophils numbers as compared to the Carr-treated group ($P < 0.001$) (Figure 8(b)).

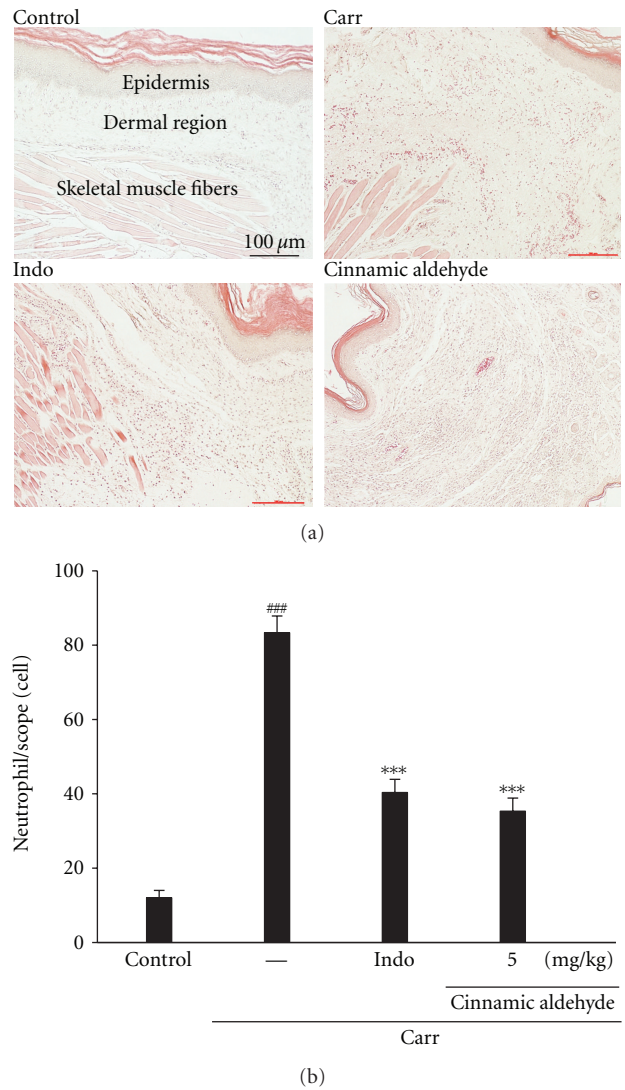


FIGURE 8: Representative light micrographs of mouse hind footpad H&E stained to reveal hemorrhage, edema, and inflammatory cell infiltration in control mice (a), Carr-treated mice demonstrates hemorrhage with moderately extravascular red blood cell and large amount of inflammatory leukocyte mainly neutrophils infiltration in the subdermis interstitial tissue of mice (b), and mice given indomethacin (Indo) (10 mg/kg) before Carr. Cinnamic aldehyde (5 mg/kg) significantly shows morphological alterations (100x) and the numbers of neutrophils in each scope (400x) compared to subcutaneous injection of Carr only. ### $P < 0.001$ as compared with the control group. *** $P < 0.001$ compared with Carr group. Scale bar = 100 μm.

4. Discussion

In the present study, we demonstrated anti-inflammatory activities of *C. cassia* constituents (cinnamic aldehyde, cinnamic alcohol, cinnamic acid, and coumarin) in both in *vitro* and in *vivo* experimental systems, using LPS-stimulated RAW264.7 macrophages and a mouse model of topical inflammation, respectively. Dual inhibitory activities against iNOS, COX-2, and NF-κB as shown in *in vitro* assays appear

to confer on cinnamic aldehyde a potent *in vivo* efficacy in mouse, Carr-induced, paw edema, comparable with a potent COX inhibitor, indomethacin, suggesting its potential therapeutic usage as a novel topical anti-inflammatory source of health food.

The pathology of inflammation is initiated by complex processes triggered by microbial pathogens such as LPS, which is a prototypical endotoxin. LPS can directly activate macrophages, which trigger the production of inflammatory mediators, such as NO and TNF- α [17]. The pharmacological reduction of LPS-inducible inflammatory mediators is regarded as one of the essential conditions to alleviate a variety of disorders caused by activation of macrophages. Thus, RAW264.7 macrophages provide us with a good model for anti-inflammatory drug screening and for subsequently evaluating the inhibitors of the signal pathways that lead to the induction of proinflammatory enzymes and to the production of pro-inflammatory cytokines.

Cinnamic aldehyde, the major constituent of leaf essential oil from *C. cassia*. Cinnamic aldehyde has been demonstrated to exhibit antitumor activities, antibacteria activities, anti-LPS-induced NF- κ B transcriptional activities [18, 19]. Cinnamic aldehyde which has α , β unsaturated carbonyl moiety exerted suppressive effect on toll-like-receptor-4-(TLR4-) mediated signaling [20]. And in this paper, we first evaluated that cinnamic alcohol, cinnamic acid, and coumarin have only little or less anti-inflammatory activities in LPS-inducible inflammatory model *in vitro*. Our current results provided a potential medical application in modulating inflammatory diseases.

As many of these conditions exhibit rapid onset and development, often resulting in the failure of conventional anti-inflammatory therapies and extremely high mortality rates, a simultaneous suppression of NO production pathways, as shown by cinnamic aldehyde, may satisfy the so far unmet need for control of the rapid progression of the inflammatory process. *In vitro* models such as macrophage cells or other cell lines are useful materials with a steady high-level production of NO. The mechanisms by which cinnamic aldehyde inhibits macrophage functions have not been elucidated. Results *in vitro* showed that cinnamic aldehyde suppressed LPS-induced production of NO, the expression of inflammatory protein products such as iNOS, COX-2, I κ B α , and NF- κ B. Examination of the cytotoxicity of cinnamic aldehyde in RAW264.7 macrophages using MTT assay has indicated that cinnamic aldehyde even at 50 μ M did not affect the viability of RAW264.7 cells. Therefore, inhibition of LPS-induced nitrite production by cinnamic aldehyde was not the result of a possible cytotoxic effect on these cells.

Excess amounts of NO and PGE₂ play a critical role in the aggravation of chronic inflammatory diseases, such as hepatic dysfunction and pulmonary disease. Recently, *in vitro* and *in vivo* have indicated an existing cross talk between the release of NO and prostaglandins (PGs) in the modulation of molecular mechanisms that regulate PGs generating pathway [21]. Scientific papers observed that while the production of both NO and PGE₂ was blocked by the NOS inhibitors in mouse macrophages RAW264.7 cells,

these inhibitory effects were reversed by coincubation with the precursor of NO synthesis, L-Arginine. Furthermore, inhibition of iNOS activity by nonselective NOS inhibitors attenuated the release of NO and PGs simultaneously in LPS-activated macrophages, which suggested that endogenously released NO from macrophages exerted a stimulatory action on enhancing the PGs production. Conversely, it has been shown that COX activation in turn modulates L-arginine-NO pathway, whereas COX inhibition decreases NOS activity in human platelets [22]. These results are indicative of the cross-talk between NO and PGs pathways.

The Carr-induced mice paw edema is a suitable test for evaluating anti-inflammatory drugs and has frequently been used to assess the antiedematous effect of natural products [23]. The degree of swelling of the Carr-injected paws was maximal 3th h after injection. Cinnamic aldehyde and Indo significantly inhibited the development of edema the 4th and the 5th h after treatment ($P < 0.001$). They both showed anti-inflammatory effects in Carr-induced mice edema paw. It is well known that the third phase of the edema induced by Carr, in which the edema reaches its highest volume, is characterized by the presence of prostaglandins and other compounds of slow reaction found that the injection of Carr into the rat paw induces the liberation of bradykinin, which later induces the biosynthesis of prostaglandin and other autacoids, which are responsible for the formation of the inflammatory exudates [24].

In the studies of the mechanism on the inflammation, NO plays an important role in the Carr-induced inflammatory response [25]. Our present results confirm that Carr-induced paw edema model results in the production of NO. The expression of the inducible isoform of NO synthase has been proposed as an important mediator of inflammation. In our study, the level of NO was decreased significantly by treatment with 1.25, 2.5, and 5 mg/kg cinnamic aldehyde. We suggest the anti-inflammatory mechanism of cinnamic aldehyde may be through the L-arginine-NO pathway because cinnamic aldehyde significantly inhibits the NO production.

The proinflammatory cytokines such as TNF- α and IL-1 are small secreted proteins, which mediate and regulate immunity and inflammation. The production of TNF- α is crucial for the synergistic induction of NO synthesis in LPS-stimulated macrophages. TNF- α induces a number of physiological effects including septic shock, inflammation, and cytotoxicity [26]. Also, TNF- α is a mediator of Carr-induced inflammatory incapacitation and is able to induce the further release of kinins, and leukotrienes, which is suggested to have an important role in the maintenance of long-lasting nociceptive response [27]. In this study, we found that cinnamic aldehyde decreased the TNF- α level in serum after Carr injection by treatment with 1.25, 2.5, and 5 mg/kg, significantly.

Neutrophils and macrophages are critical to the pathogenesis of acute injury, rheumatoid arthritis and other inflammatory diseases [15]. The Carr-induced inflammatory response has been linked to neutrophils infiltration and the production of neutrophils-derived free radicals, such as hydrogen peroxide as well as the release of other neutrophils-derived mediators [1]. Some researches demonstrate that

inflammatory effect induced by Carr is associated with free radical. Free radical, prostaglandin, and NO will be released when administrating with Carr for 1 ~ 6 h. ROS play an important role in modulating the extent of inflammatory response and consequent tissue and cell injury. MDA is a metabolic product of lipid peroxidation, the level of which is raised in oxidative stress. MDA production is due to free radical attack plasma membrane. Increasing evidence regarding free radical-generating agents and inflammatory processes suggests that accumulation of reactive oxygen species can cause tissue injury [28]. Thus, inflammatory effect would result in the accumulation of MDA. In this study, there is significant increase in CAT, SOD, and GPx activities with cinnamic aldehyde treatment. Furthermore, there are significant decreases in MDA level with cinnamic aldehyde treatment. We assume the suppression of MDA production is probably due to the increases of CAT, SOD, and GPx activities.

Activation of polymorphonuclear neutrophils (PMNs) reflects a primary immunological response to invading pathogens [29]. In Carr-induced inflammation, cinnamic aldehyde significantly inhibited cellular infiltration (neutrophils and granulocytes) into the air-pouch fluid. Also, MPO from the neutrophil's azurophilic granules is responsible for invoking tissue injury [30]. Results indicate that cinnamic aldehyde has considerable potential as a therapeutic inhibitor of MPO-mediated tissue damage.

NF- κ B is known to be a major transcription factor to regulate the expressions of pro-inflammatory enzymes and cytokines, such as iNOS, COX-2, and TNF- α [31]. NF- κ B subunits (p65 and/or p50) are normally sequestered in the cytosol as an inactive complex by binding to inhibitory factor I κ B- α in unstimulated cells. Upon stimulation of pro-inflammatory signals including LPS, I κ B- α is phosphorylated by I κ B kinase (IKK) and inactivated through ubiquitin-mediated degradation [32]. The resulting free NF- κ B is translocated into the nucleus and it acts as a transcription factor. As shown in Figure 4(a), the treatment with cinnamic aldehyde blocks the degradation of NF- κ B in LPS-induced macrophage and Carr-induced paw edema. Therefore, these results suggest that cinnamic aldehyde inhibits the expression of iNOS and COX-2, and thus NO production through inactivation of NF- κ B activation.

The anti-inflammatory properties of cinnamic aldehyde would appear to be similar to the anti-inflammatory properties of certain other essential oils deriving from certain other plants. *Hyptis pectinata* essential oil exhibits antinociceptive and anti-inflammatory activity through the inhibition of NO and PGE₂ production after Carr injection [33]. And, the essential oil of *Cordia verbenacea* significantly decreased TNF- α production in Carr-injected rat paws [34].

In conclusion, these results suggested that cinnamic aldehyde possessed anti-inflammatory effects. The anti-inflammatory mechanism of cinnamic aldehyde may be related to iNOS and it is associated with the increase in the activities of antioxidant enzymes (CAT, SOD, and GPx). Cinnamic aldehyde may be used as a pharmacological agent in the prevention or treatment of disease in which free radical formation is a pathogenic factor.

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