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Anti-inflammatory Activities of Cinnamomum cassia Constituents in vitro and in vivo

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Abstract

In this study, 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) *in vitro* and carrageenan (Carr)-induced mouse paw edema model *in vivo*. 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 (PGE_2) 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-κB), and IκBα, significantly.

In the anti-inflammatory test, cinnamic aldehyde decreased the paw edema at the 4^{th} and the 5^{th} h after λ-carrageenin (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 significantly attenuated the malondialdehyde (MDA) level and myeloperoxidase (MPO) activity in the edema paw at the 5^{th} h after Carr injection. Cinnamic aldehyde decreased the NO, TNF-α, and PGE_2 levels on the serum level at the 5^{th} h after Carr injection. Western blotting revealed that cinnamic aldehyde decreased Carr-induced iNOS, COX-2, and NF-κB expressions at the 5^{th} h in the edema paw. An intraperitoneal (*i.p.*) injection treatment with cinnamic aldehyde also diminished neutrophil infiltration into sites of inflammation as did indomethacin (Indo). The anti-inflammatory mechanisms of cinnamic aldehyde might be related to the decrease in the level of MDA, MPO, iNOS, and COX-2 *via* increasing the

activities of CAT, SOD, and GPx in the edema paw through the suppression of NO, TNF-α, and PGE_2. These findings demonstrated that cinnamic aldehyde has excellent anti-inflammatory activities *in vitro* and *in vivo* and thus have great potential to be used as a source for natural health products.

KEY WORDS: Chinese medicine; Cinnamic aldehyde; Anti-inflammation; NO; TNF-*α*.

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 up-regulation 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 anti-tumor 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-κB in either RAW264.7 cell or paw edema. Also, the activities of CAT, SOD, and GPx in the paw tissue at the 5^{th} h after Carr injection were measured to understand the relationship between the anti-inflammatory mechanism of cinnamic aldehyde and antioxidant enzymes.

Materials and methods

Chemicals

LPS (endotoxin from *Escherichia coli*, serotype 0127:B8), Carr, indomethacin, cinnamic aldehyde (≥ 98%), cinnamic alcohol (≥ 98%), cinnamic acid (≥ 99%), coumarin (≥ 99%) (Fig. 1A) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, USA). TNF-α and PGE_2 were purchased from Biosource International Inc. (Camarillo, CA, USA). Anti-iNOS, anti-COX-2, anti-NF-κB, anti-IκBα, and anti-β-actin antibody (Santa Cruz, USA) and a protein assay kit (Bio-Rad Laboratories Ltd., Watford, Herts, U.K.) were obtained as indicated. Poly-(vinylidene fluoride) membrane (Immobilon-P) was obtained from Millipore Corp. (Bedford, MA, USA).

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 ±1℃, and relative humidity of 55 ± 5 % with 12 h dark-light cycle for at least 2 week before the 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).

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 CO_2 incubator (5% CO_2 in air) at 37℃ and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin–0.02% EDTA in Ca^{2+}-, Mg^{2+}- free phosphate-buffered saline (DPBS).

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 μL of 0.5 mg/mL MTT for 2 h at 37℃ testing for cell viability {MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)}. The medium was then discarded and 100 μL dimethyl sulfoxide (DMSO) was added. After 30-min incubation, absorbance at 570 nm was read using a microplate reader.

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 μM) in the presence of LPS (100 ng/mL) at 37^0C for 24 h. Then, cells were dispensed into 96-well plates, and 100 mL 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×*g* for 5 min at room temperature, 100 μL supernatant was applied to a microtiter plate well, followed by 100 μL 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.

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 μL) 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 ℃. Also, blood were withdrawn and kept at -80 ℃. The protein concentration of the sample was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA).

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.

Myeloperoxidase Activity Assay

The activity of tissue MPO was assessed at the 5^{th} 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 × *g* at 4 ℃ 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% H_2O_2 was a substrate for myeloperoxidase. Oxidized o-dianisidine formed a soluble chromophore and absorbance (OD_{460}) was determined by spectrophotometry (Molecular Devices, Orleans Drive, Sunnyvale, CA)

over 2 min. Myeloperoxidase activity (Δ OD_{460}) was calculated by subtracting the value of OD_{460} at time 0 min from that at 2 min for each sample.

Measurement of TNF-α and PGE_2 by an Enzyme-Linked Immunosorbent Assay (ELISA). The levels of TNF-α and PGE_2 were determined using a commercially available ELISA kit (Biosource International Inc., Camarillo, CA) according to the manufacturer's instruction. TNF-α and PGE_2 were determined from a standard curve.

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 10mM H_2O_2 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.

Protein Lysate Preparation and Western blot Analysis of iNOS, COX-2, IκBα, and NF-κB

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, 1mM Na_3VO_4, 1mM EGTA, 10mM NaF, 1mM $Na_4P_2O_7$, 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℃. Soft tissues were removed from individual mice paws and homogenized in a solution containing 10 mM CHAPS, 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 μg/mL, aprotinin, 1 μM pepstatin and 10 μM leupeptin. The homogenates were centrifuged at 12,000*g* for 20 min, and 30 μg 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-IκBα, or anti-NF-κB 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, U.K.). 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.

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 μm) 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 [ploymorphonuclear 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].

Statistical Analysis. Data are expressed as mean ± 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 *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Results

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 μM) used in the presence of 100 ng/mL LPS for 24 h did not change cell viability (Fig. 1B).

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 (Fig. 2). Cinnamic aldehyde did not interfere with the reaction between nitrite and Griess reagents at 50 μM (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 μM) together with LPS (100 ng/mL) for 24

297 h, a significant concentration-dependent inhibition of nitrite production was detected.
298 There was either a significant decrease in the nitrite production of group treated with 12.5
299 μM cinnamic aldehyde ($p < 0.05$), or very or highly significant decrease of groups treated
300 respectively with 25 or 50 μM of cinnamic aldehyde when compared with the LPS-alone
301 group ($p < 0.01$ or $p < 0.001$). The IC_{50} value for inhibition of nitrite production of
302 cinnamic aldehyde was about 45.56 ± 1.36 μM

303

304 **Inhibition of LPS-induced iNOS, COX-2, IκBα, and NF-κB Protein by Cinnamic**
305 **aldehyde, Cinnamic alcohol, Cinnamic acid, and Coumarin.** In order to investigate
306 whether the inhibition of NO production was due to a decreased iNOS, COX-2, IκBα**,**
307 and NF-κB protein level, the effect of cinnamic aldehyde, cinnamic alcohol, cinnamic
308 acid, and coumarin was studied by immunoblot. The results showed the incubation with
309 cinnamic aldehyde (50 μM) in the presence of LPS (100 ng/mL) for 24 h or 1h inhibited
310 iNOS, COX-2, IκBα**,** and NF-κB proteins expression in mouse macrophage RAW264.7
311 cells in the cytosol (Fig. 3A and Fig. 4A). The detection of β-actin was also performed in
312 the same blot as an internal control. The intensity of protein bands was analyzed by using
313 Kodak Quantity software in three independent experiments and it showed an average of
314 77.4% and 84.8% down-regulation of iNOS and COX-2 proteins, respectively, after
315 treatment with cinnamic aldehyde at 50 μM compared with the LPS-alone (Fig. 3B). And
316 the intensity of protein bands showed an average of 82.6% and 86.2% up-regulation of
317 NF-κB and IκBα protein ($p<0.001$) (Fig.4B).

Inhibition of LPS-induced the level of TNF-α and PGE_2 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 up-regulation of TNF-α. A very low amount of TNF-α protein was detected by a specific ELISA for TNF-α in controls (Fig. 5A). 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 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 IC_{50} value for inhibition of TNF-α production of cinnamic aldehyde was about 29.58 ± 0.34 μM.

PGE_2 represents the most important inflammatory product of COX-2 activity and it was quantified in cell-free culture supernatant [16]. As shown in Fig. 5B, cells were stimulated with LPS alone raised significant amount of PGE_2 in RAW264.7 macrophages. 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 24 h, a significant concentration-dependent inhibition of PGE_2 production was detected. The IC_{50} value for inhibition of PGE_2 production of cinnamic aldehyde was about 37.67 ± 0.58 μM.

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 Fig. 6A. Cinnamic aldehyde (5 mg/kg) inhibited ($p < 0.001$) the development of paw edema induced by Carr after the 4^{th} and the 5^{th} h of treatment, significantly. Indo (10 mg/kg) significantly decreased the Carr induced paw edema after the 4^{th} and the 5^{th} h of treatment ($p < 0.001$).

Effects of Cinnamic aldehyde on the MDA level. The MDA level increased significantly in the edema paw at the 5^{th} 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 (Fig. 6B).

Effects of Cinnamic aldehyde on the MPO activity. The MPO activity increased significantly in the edema paw at the 5^{th} 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 (Fig. 6C).

Effects of Cinnamic aldehyde on the NO Level. In Fig. 6D, the NO level increased significantly in the edema serum at the 5^{th} 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 5^{th} h after induction.

Effects of Cinnamic aldehyde on the TNF-α and PGE_2 Level. The TNF-*α* and PGE_2 level increased significantly in serum at the 5^{th} h after Carr injection ($p < 0.001$). However, cinnamic aldehyde (1.25 or 2.5 mg/kg) decreased the TNF-α and PGE_2 level in serum at the 5^{th} h after Carr injection ($p < 0.05$ or $p < 0.01$), as well as 10 mg/kg Indo (Fig. 6E and 6F).

Effects of Cinnamic aldehyde on activities of Antioxidant Enzymes. At the 5^{th} 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 treated with 5 mg/kg cinnamic aldehyde and 10 mg/kg Indo ($P<0.01$ or $P<0.001$) (Table 1).

Effects of Cinnamic aldehyde on Carr-induced iNOS, COX-2, and NF-κB protein expressions in Mice Paw Edema. To investigate whether the inhibition of NO production was due to a decreased iNOS, COX-2, and NF-κB protein level, the effect of cinnamic aldehyde on iNOS, COX-2, and NF-κB proteins expression were 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-κB proteins expression in mouse paw edema (Fig. 7A). 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%

384 down-regulation of iNOS and COX-2 protein respectively after treatment with cinnamic
385 aldehyde at 5 mg/kg compared with the Carr-induced alone (Fig. 7B). In addition, the
386 protein expression showed an average of 57.1% and 45.1% down-regulation of iNOS,
387 and COX-2 protein after treatment with Indo at 10 mg/kg compared with the
388 Carr-induced alone (Fig. 7B). And the intensity of protein bands showed an average of
389 87.6% up-regulation of NF-κB protein (p<0.001) (Fig.7B). The down-regulation of
390 iNOS, COX-2, and NF-κB activity of the cinnamic aldehyde (5 mg/kg) was better than
391 Indo (10 mg/kg).
392
393 **Histological Examination.** Paw biopsies of Carr model animals showed marked cellular
394 infiltration in the connective tissue. The infiltrates accumulated between collagen fibers
395 and into intercellular spaces. Paw biopsies of animals treated with cinnamic aldehyde (5
396 mg/kg) showed a reduction in Carr-induced inflammatory response. Actually
397 inflammatory cells were reduced in number and confined to near the vascular areas.
398 Intercellular spaces did not show any cellular infiltrations. Collagen fibers were regular in
399 shape and showed a reduction of intercellular spaces. Moreover, the hypoderm
400 connective tissue was not damaged (Fig. 8A). Neutrophils increased with Carr treatment
401 (P < 0.01). As Indo and cinnamic aldehyde (5 mg/kg) could significantly decrease the
402 neutrophils numbers as compared to the Carr-treated group (P < 0.001) (Fig. 8B).
403
404 **Discussion**
405 In the present study, we demonstrated anti-inflammatory activities of *C. cassia*
406 constituents (cinnamic aldehyde, cinnamic alcohol, cinnamic acid, and coumarin) in both

in *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 an good model for anti-inflammatory drug screening and for subsequently evaluating the inhibitors of the signal pathways that lead to the induction of pro-inflammatory 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 anti-tumor activities, anti-bacteria activities, anti LPS-induced NF-κB 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 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, IκBα, and NF-κB. Examination of the cytotoxicity of cinnamic aldehyde in RAW264.7 macrophages using MTT assay has indicated that cinnamic aldehyde even at 50 μM 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 PGE_2 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 were observed that while the production of both NO and PGE_2 was blocked by the NOS inhibitors in mouse macrophages RAW264.7 cells, these inhibitory effects were reversed by co-incubation 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 anti-edematous effect of natural products [23]. The degree of swelling of the Carr-injected paws was maximal 3^{th} h after injection. Cinnamic aldehyde and Indo significantly inhibited the development of edema the 4^{th} and the 5^{th} 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.

476 The proinflammatory cytokines such as TNF-α and IL-1 are small secreted proteins,
477 which mediate and regulate immunity and inflammation. The production of TNF-α is
478 crucial for the synergistic induction of NO synthesis in LPS-stimulated macrophages.
479 TNF-α induces a number of physiological effects including septic shock, inflammation,
480 and cytotoxicity [26]. Also, TNF-α is a mediator of Carr-induced inflammatory
481 incapacitation, and is able to induce the further release of kinins and leukotrienes, which
482 is suggested to have an important role in the maintenance of long-lasting nociceptive
483 response [27]. In this study, we found that cinnamic aldehyde decreased the TNF-α level
484 in serum after Carr injection by treatment with 1.25, 2.5, and 5 mg/kg, significantly.
485 Neutrophils and macrophages are critical to the pathogenesis of acute injury,
486 rheumatoid arthritis and other inflammatory diseases [15]. The Carr-induced
487 inflammatory response has been linked to neutrophils infiltration and the production of
488 neutrophils-derived free radicals, such as hydrogen peroxide as well as the release of
489 other neutrophils-derived mediators [1]. Some researches demonstrate that inflammatory
490 effect induced by Carr is associated with free radical. Free radical, prostaglandin and NO
491 will be released when administrating with Carr for 1 ~ 6 h. ROS play an important role in
492 modulating the extent of inflammatory response and consequent tissue and cell injury.
493 MDA is a metabolic product of lipid peroxidation, the level of which is raised in
494 oxidative stress. MDA production is due to free radical attack plasma membrane.
495 Increasing evidence regarding free radical-generating agents and inflammatory processes
496 suggests that accumulation of reactive oxygen species can cause tissue injury [28]. Thus,
497 inflammatory effect would result in the accumulation of MDA. In this study, there is
498 significantly increased in CAT, SOD, and GPx activities with cinnamic aldehyde

499 treatment. Furthermore, there are significantly decreases in MDA level with cinnamic
500 aldehyde treatment. We assume the suppression of MDA production is probably due to
501 the increases of CAT, SOD, and GPx activities.

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

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

520 The anti-inflammatory properties of cinnamic aldehyde would appear to be similar
521 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 PGE_2 production after Carr injection [33]. And, the essential oil of *Cordia verbenacea* significantly decreased TNF-a 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 in a pathogenic factor.

Acknowledgement

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Figure Legends

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 ± S.D. for three different experiments performed in triplicate.

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.

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 a 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.

Figure 4. Inhibition of NF-κB and IκBα (A) protein expressions by *cinnamomum cassia* constituents (cinnamic aldehyde, cinnamic alcohol, cinnamic acid, and coumarin) in LPS-stimulated RAW264.7 cells. Samples (50 μM) 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-κB (P65) and IκBα in the cytosol. β-actin was used as an internal control. A representative western blot from two separate experiments is shown. Relative NF-κB and IκBα protein levels were calculated with reference to a LPS-stimulated culture (B). ###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.

Figure 5. The effects of cinnamic aldehyde on lipopolysaccharide (LPS)-induced TNF-α (A) and PGE_2 (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 μM). Cinnamic aldehyde was added 1 h before the incubation with LPS. TNF-α and PGE_2 concentrations in the medium were determined using ELISA kit. The data were presented as mean ± 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.

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 PGE_2 (F) concentrations of serum at the 5^{th} hr in mice. The values are averaged, obtained in individual animals (n=6). Each value represents as mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ as compared with the Carr group.

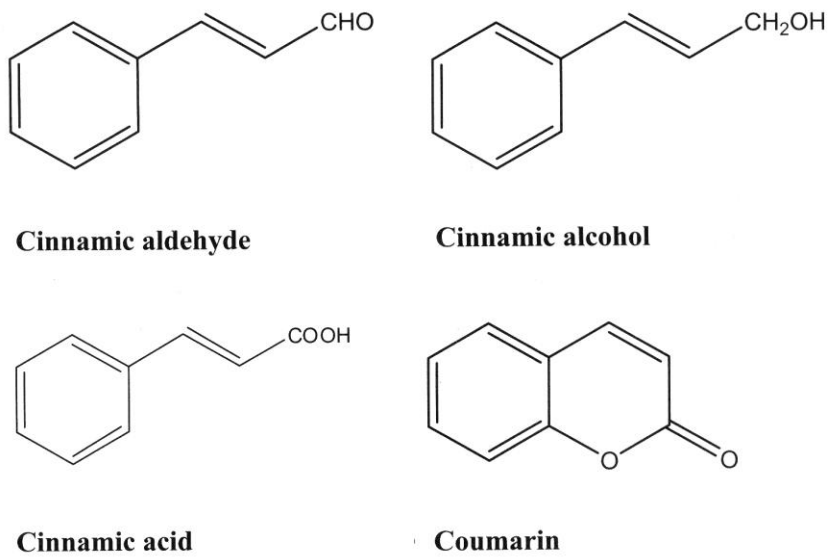
Figure 7. Inhibition of iNOS, COX-2, and NF-κB protein expressions by cinnamic aldehyde induced by Carr of foot at the 5^{th} h in mice. Tissue suspended were then prepared and subjected to western blotting using an antibody specific for iNOS, COX-2, and NF-κB. β-actin was used as an internal control. (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.

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. **(**C**).** Cinnamic aldehyde (5 mg/kg) significantly show morphological alterations (100×) (D) and the numbers of neutrophils in each scope (400x) (E) 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.

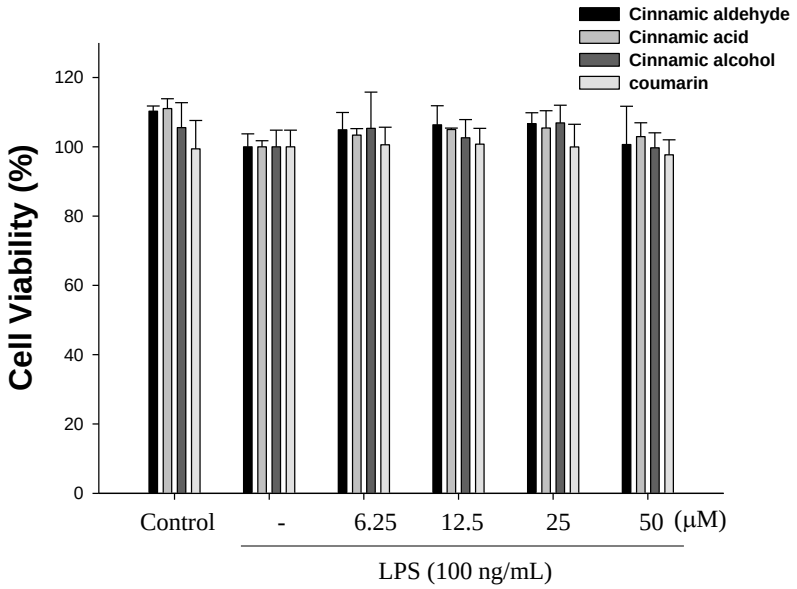
768 **Figure 1.**

769 **A.**



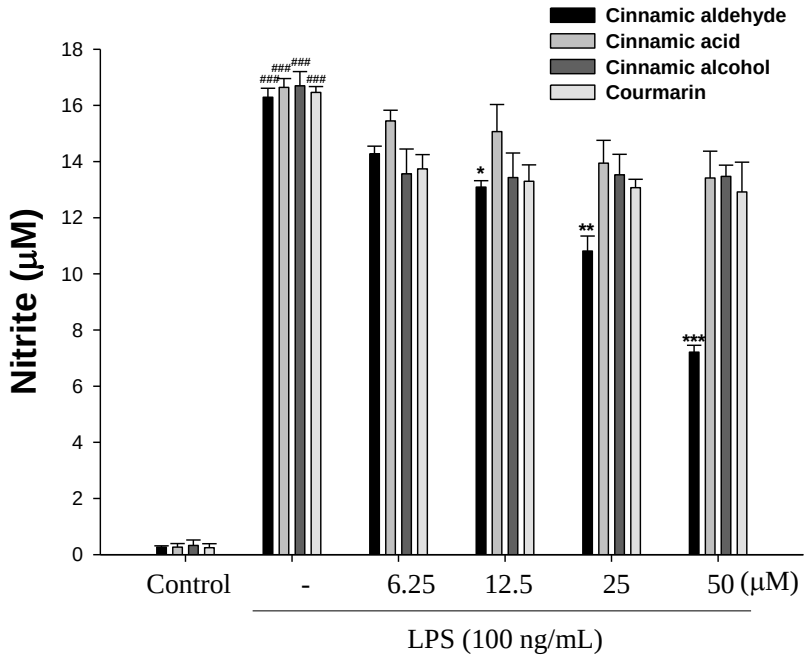
770

771 **B.**



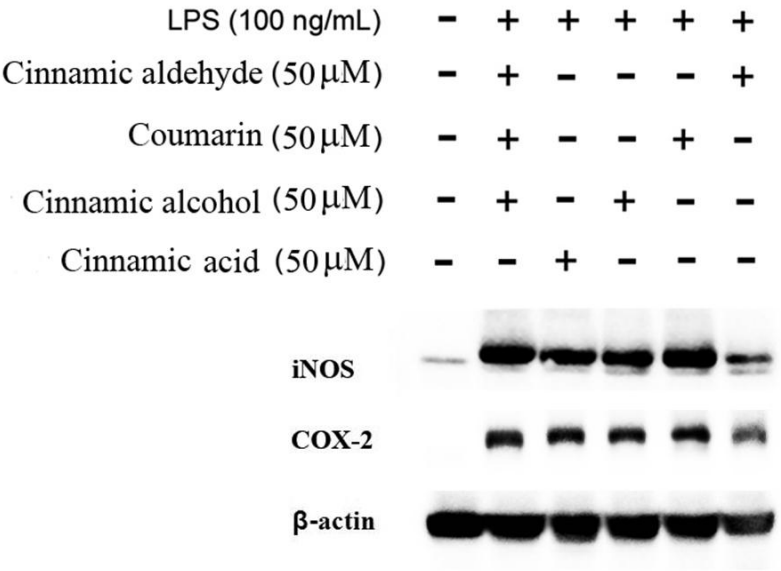
772

Figure 2.


Cinnamic aldehyde
Cinnamic acid
Cinnamic alcohol
Courmarin
Nitrite (μM)
18
16
14
12
10
8
6
4
2
0
###
###
###
###
*
**
***
Control
-
6.25
12.5
25
50 (μM)
LPS (100 ng/mL)

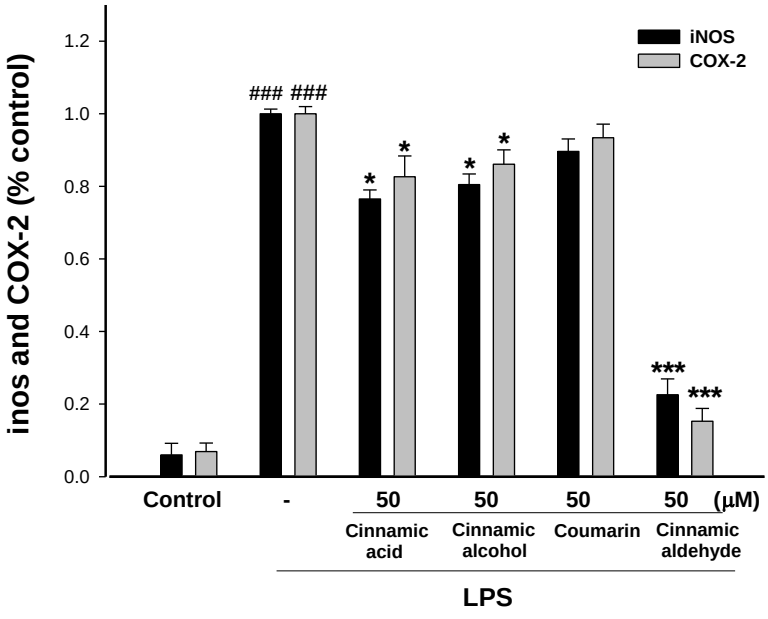
784 **Figure 3.**

785 **A.**



786

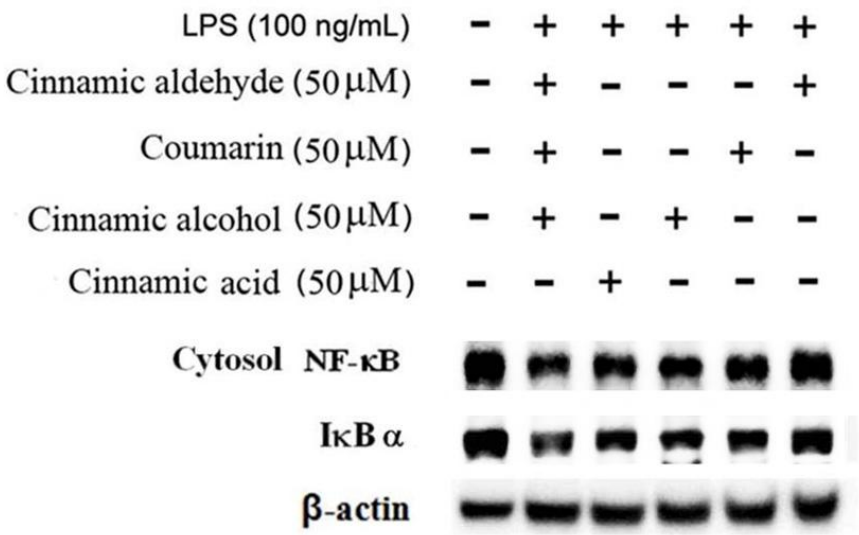
787 **B.**



788

Figure 4.

A.


LPS (100 ng/mL) - + + + + +
Cinnamic aldehyde (50 μM) - + - - - +
Coumarin (50 μM) - + - - + -
Cinnamic alcohol (50 μM) - + - + - -
Cinnamic acid (50 μM) - - + - - -
Cytosol NF-κB
IκB α
β-actin

B.

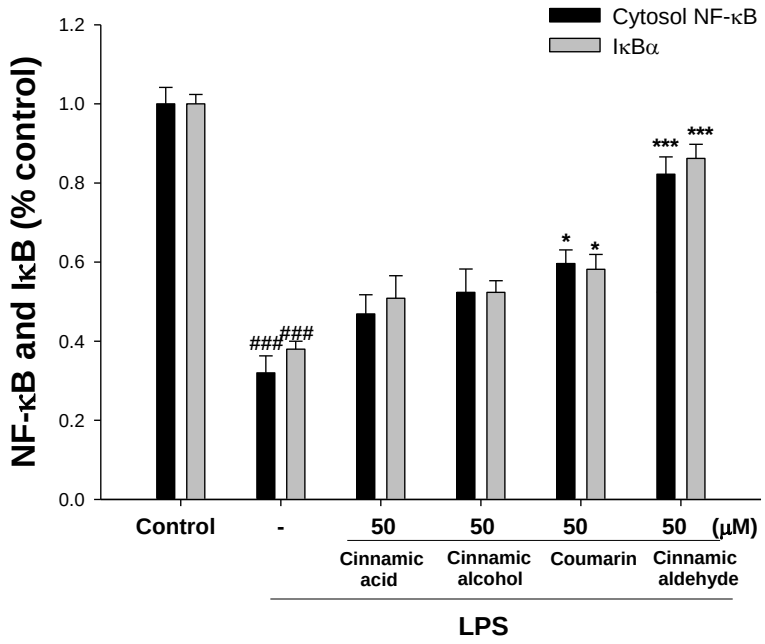
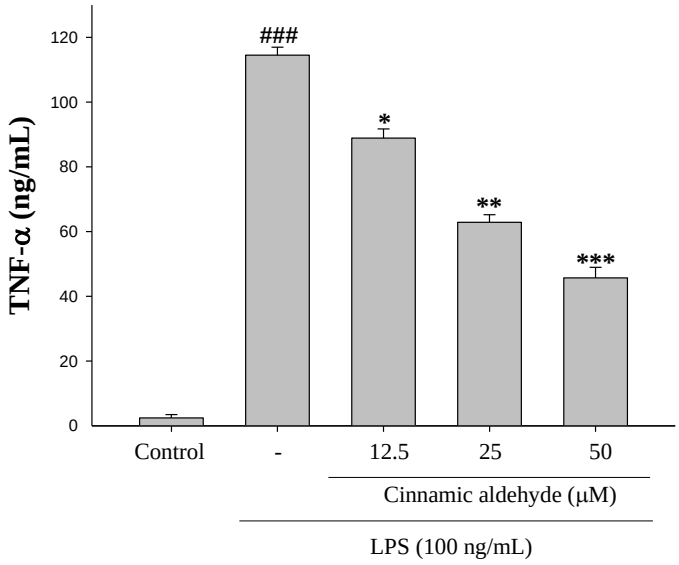

Cytosol NF-κB
IκBα
NF-κB and IκB (% control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
###
###
*
*
***
***
Control
-
50
50
50
50
(μM)
Cinnamic acid
Cinnamic alcohol
Coumarin
Cinnamic aldehyde
LPS

Figure 5.

A.


TNF-α (ng/mL)
120
100
80
60
40
20
0
###
*
**
***
Control
-
12.5
25
50
Cinnamic aldehyde (μM)
LPS (100 ng/mL)

B.

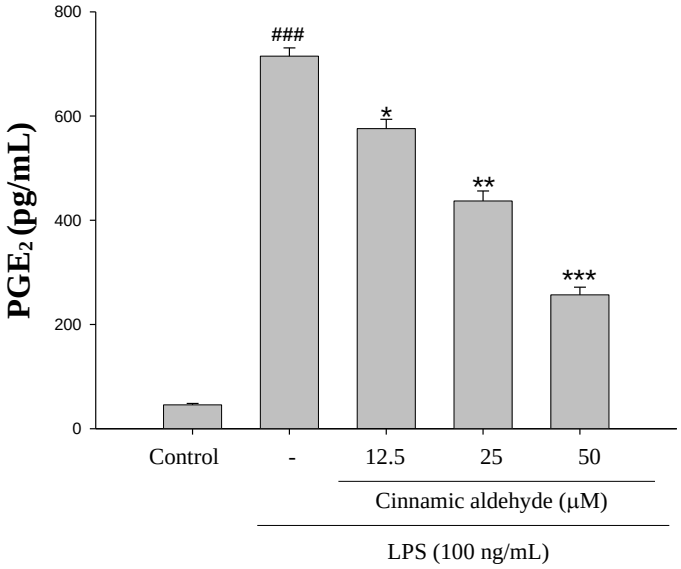
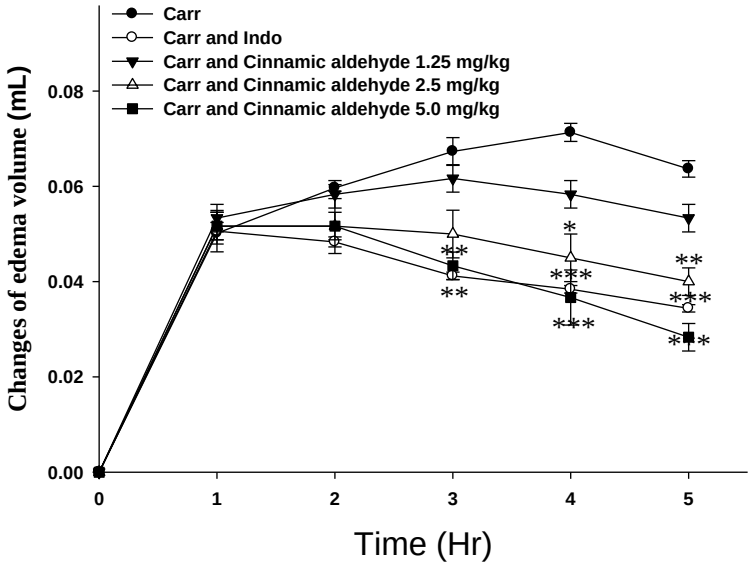
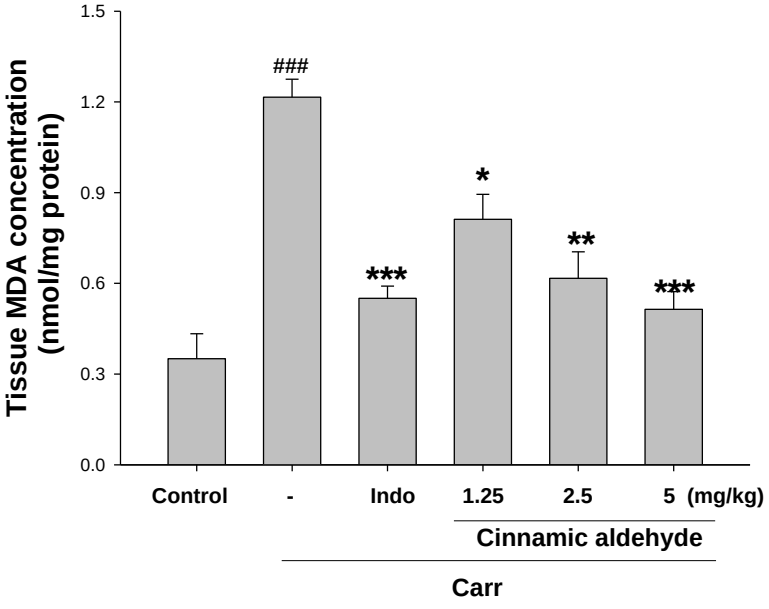

PGE2 (pg/mL)
800
600
400
200
0
###
*
**
***
Control
-
12.5
25
50
Cinnamic aldehyde (μM)
LPS (100 ng/mL)

Figure 6.

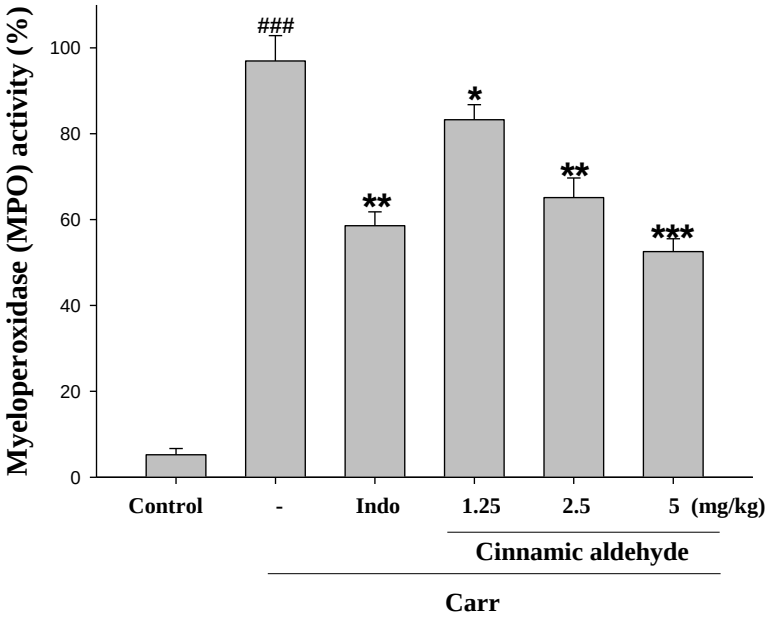
A.


Carr
Carr and Indo
Carr and Cinnamic aldehyde 1.25 mg/kg
Carr and Cinnamic aldehyde 2.5 mg/kg
Carr and Cinnamic aldehyde 5.0 mg/kg
Changes of edema volume (mL)
0.00
0.02
0.04
0.06
0.08
0
1
2
3
4
5
Time (Hr)

B.

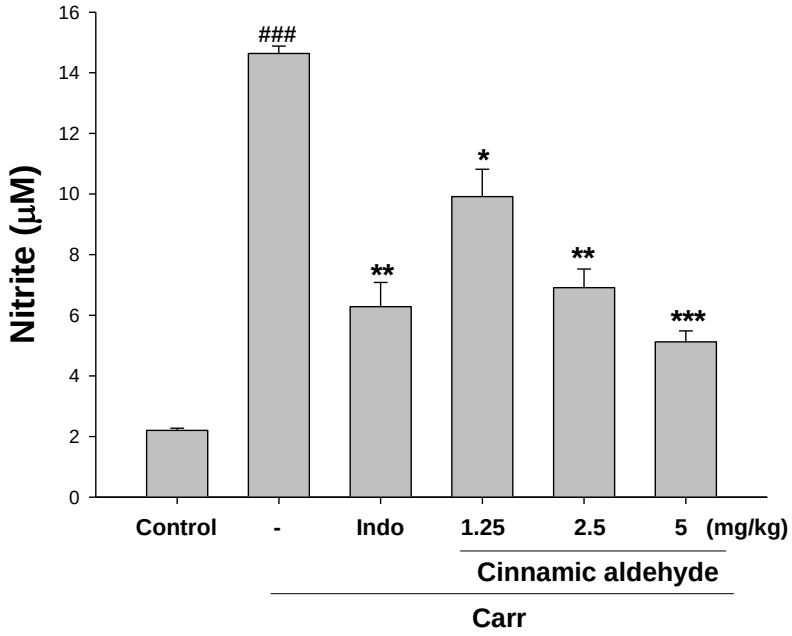

Tissue MDA concentration (nmol/mg protein)
0.0
0.3
0.6
0.9
1.2
1.5
###
***
*
**
***
Control
-
Indo
1.25
2.5
5 (mg/kg)
Cinnamic aldehyde
Carr

804 **C.**



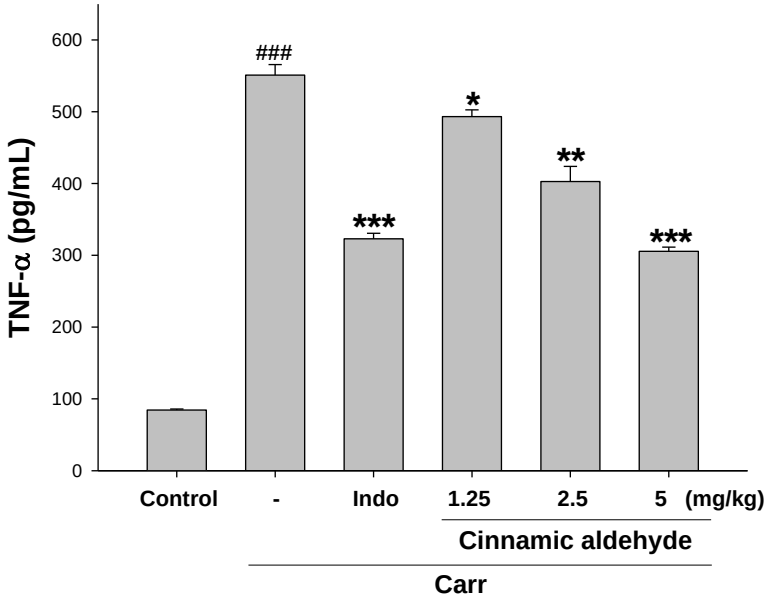
805

806 **D.**



807

E.


TNF-α (pg/mL)
600
500
400
300
200
100
0
###
***
*
**
***
Control
-
Indo
1.25
2.5
5 (mg/kg)
Cinnamic aldehyde
Carr

F.

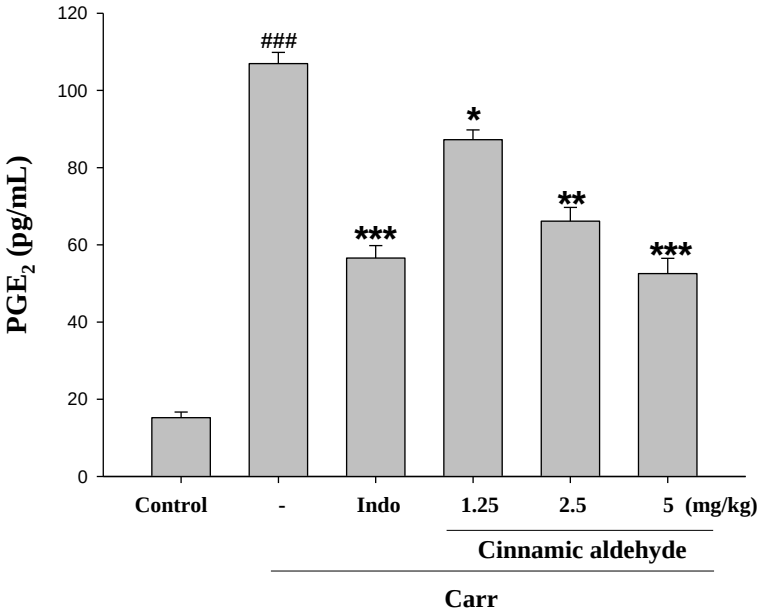
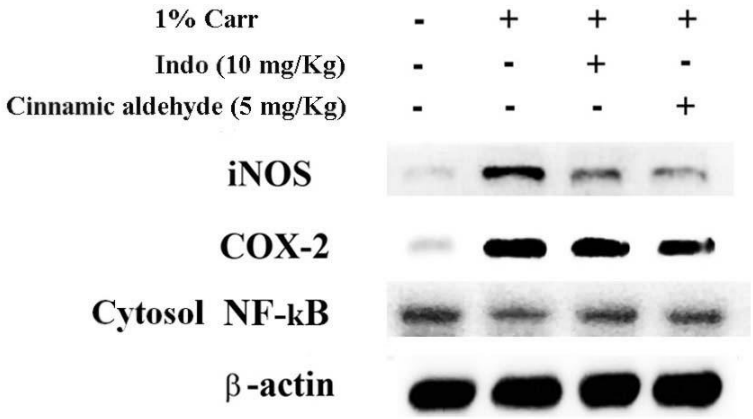

PGE2 (pg/mL)
120
100
80
60
40
20
0
###
***
*
**
***
Control
-
Indo
1.25
2.5
5 (mg/kg)
Cinnamic aldehyde
Carr

Figure 7.

A.


1% Carr - + + +
Indo (10 mg/Kg) - - + -
Cinnamic aldehyde (5 mg/Kg) - - - +
iNOS
COX-2
Cytosol NF-kB
β-actin

B.

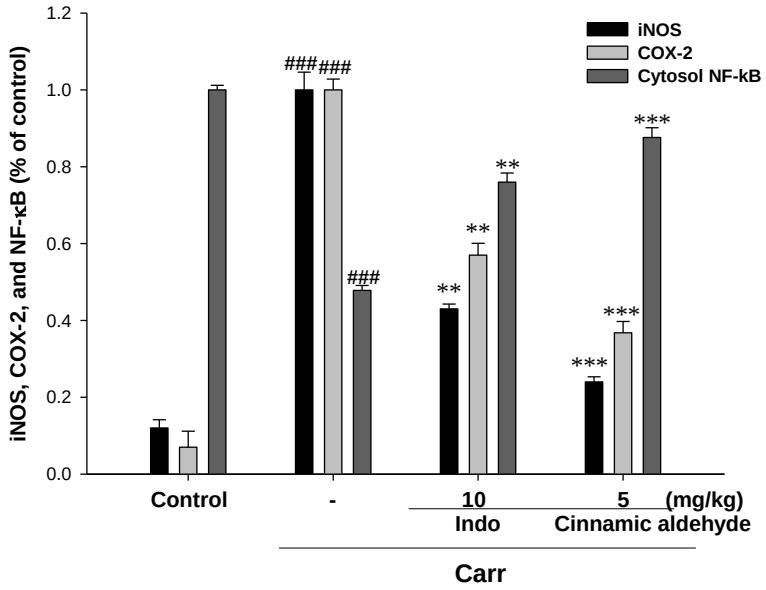
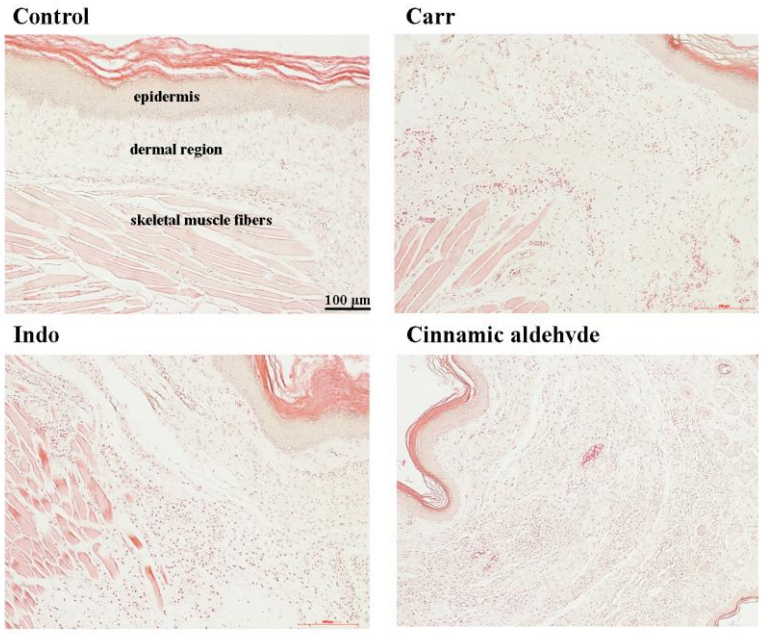
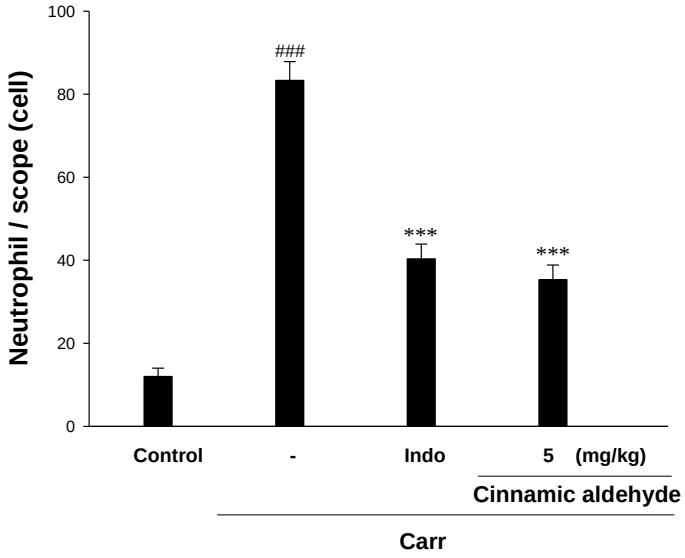

iNOS
COX-2
Cytosol NF-kB
iNOS, COX-2, and NF-κB (% of control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
###
**
***
Control
-
10
5
(mg/kg)
Indo
Cinnamic aldehyde
Carr

Figure 8.

A.


Control
epidermis
dermal region
skeletal muscle fibers
100 μm
Carr
Indo
Cinnamic aldehyde

B.


Neutrophil / scope (cell)
100
80
60
40
20
0
###
***
***
Control
-
Indo
5 (mg/kg)
Cinnamic aldehyde
Carr