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Analgesic and Anti-Inflammatory Activities of Aqueous Extracts of Fructus *Ligustri Lucidi*

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ABSTRACT

Fructus *Ligustri Lucidi* has been used for its anti-inflammatory effects in traditional Chinese medicine. This study investigated the analgesic and anti-inflammatory effects of aqueous extracts of Fructus *Ligustri Lucidi* (AFLL) in mice. AFLL significantly inhibited the production of radicals and lipid oxidation in various models. The reference compounds present in AFLL, including quercetin, myricetin, caffeic acid, gallic acid and ellagic acid, exhibited the pharmacological activities by scavenging radicals and decreasing LPS-induced nitric oxide (NO) production in macrophages. Administration of AFLL showed a concentration dependent inhibition on the number of acetic acid-induced writhing responses and formalin-induced pain in the late phase, and paw edema development after Carr treatment in mice. The anti-inflammatory activities of AFLL could be via NO and tumor necrosis factor α (TNF- α) suppression and associated with the increase in the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). Western blotting revealed that AFLL decreased Carr-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions. These results suggest that anti-inflammatory mechanisms of AFLL might be correlated to the decrease in the level of malondialdehyde (MDA), iNOS and COX-2 in the edema paw via increasing the activities of CAT, SOD and GPx in the liver. Overall, the results showed that AFLL could serve as a natural source of antioxidant, analgesic and anti-inflammation.

Key words: Fructus *Ligustri Lucidi*, antioxidant, analgesic, NO, anti-inflammation

INTRODUCTION

Intracellular antioxidant mechanisms against inflammatory stresses involve antioxidant enzymes of catalase (CAT), superoxide dismutase (SOD) 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⁽¹⁾. However, it appears that the various roles of enzymatic antioxidants help to protect organisms from excessive generation of oxidative and nitrative stress in the inflammatory process⁽²⁾. This has triggered studies focusing on the role of natural products in suppressing the production of oxidation by increasing enzymatic antioxidants in tissues⁽³⁾.

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 to edema formation during inflammation. Meanwhile, many other mechanisms such as the production of reactive nitrogen species (RNS) and proinflammatory cytokines are activated and exacerbate the inflammatory damage. 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 the infiltration of phagocytes, the production of free radicals as well as the release of inflammatory mediators⁽⁴⁾. Therefore, free radicals are recognized as the reactive species in inflammation induced biological damage. They are generated under oxidative and nitrative stress⁽⁵⁾. Oxidative and nitrative stress derived not only from disequilibrium cellular

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metabolism but also from inflammatory status⁽⁶⁾. Diverse free radicals are produced by inflammatory cells and can oxidize biomolecules while free radicals augment the progression of inflammation. For example, macrophages can generate nitrate stress in response to lipopolysaccharide (LPS) by expressing an inducible nitric oxide synthase (iNOS) to execute massive nitric oxide (NO) production.

Fructus *Ligustrum lucidum* (FLL), also known as “Nu zhen zi”, is well known as a tonic for liver in traditional Chinese medicine. It was reported to possess anti-mutagenic⁽⁷⁾, anti-inflammatory⁽⁸⁾, hepatoprotective⁽⁹⁾, hypolipidemic⁽¹⁰⁾, anti-aging⁽¹¹⁾ and anti-osteoporotic activity⁽¹²⁾. Although FLL has shown some physiological effects, there are few studies focusing on its antioxidant and analgesic effects as well as its anti-inflammatory effects on Carr-induced inflammatory damage. Consequently, the objective of the present study is to determine the antioxidant, analgesic and anti-inflammatory effects of the aqueous extract of Fructus *Ligustrum Lucidi* (AFLF).

MATERIALS AND METHODS

I. Materials

Lipopolysaccharide (LPS, *Escherichia coli* O127:B8), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), *N*-(1-naphthyl) ethylenediamine dihydrochloride, sulfanilamide, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), thiobarbituric acid (TBA), 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), λ -Carrageenan (Carr), indomethacin (Indo), ursolic acid, oleanolic acid, ellagic acid, caffeic acid and other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dried samples of FLL were obtained from Sun Ten Pharmaceutical Co., Ltd. in Taipei, Taiwan.

II. Sample Preparation

A 100 g sample of FLL was extracted with water (1 L) at 100°C for 60 min and then centrifuged at 10,000 \times g for 20 min. The extraction was repeated three times. The extracts were combined and filtered through a Whatman No. 1 filter paper. The filtrates were collected, concentrated with a vacuum evaporator until the volume was below 10 mL and then freeze-dried. The yield obtained was 4.5% (w/w).

III. Determination of Total Polyphenols

Total polyphenols were determined as gallic acid equivalents⁽¹³⁾. Two milliliters of sodium carbonate (20%, w/v) was added to different concentrations of samples in a 10-mL volumetric flask. After 5 min, 0.1 mL of Folin-Ciocalteu reagent (50%, v/v) was added and the volume was adjusted to 10 mL with H₂O. After incubation at 30°C for 1 h, the absorbance was measured at 750 nm and compared to a gallic acid calibration curve.

IV. Determination of ABTS Radical Inhibition

This assay determined the capacity of the samples to scavenge ABTS^{•+} as previously described⁽¹⁴⁾. The ABTS^{•+} was generated by reacting 1 mM of ABTS with 0.5 mM of hydrogen peroxide and 10 units/mL of horseradish peroxidase in the dark at 30°C for 2 h. Ten minutes after the addition of 1 mL of ABTS^{•+} to the samples, the absorbance at 734 nm was recorded. A lower level of absorbance indicated better radical scavenging activity.

V. Determination of DPPH Radical Inhibition

The effects of the samples on the DPPH radical were estimated according to the method described in a previous study⁽¹⁵⁾. The samples were added to a methanolic solution (1 mL) of DPPH radical (the final concentration of DPPH was 0.2 mM). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance of the resulting solution was then measured spectrophotometrically at 517 nm.

VI. Determination of Reducing Activity

The reducing power of the samples was determined as previously described⁽¹⁶⁾. Potassium ferricyanide (2.5 mL, 10 mg/mL) was added to the samples in phosphate buffer (2.5 mL, 200 mM, pH 6.6) and the mixture was incubated at 50°C for 20 min. Trichloroacetic acid (2.5 mL, 100 mg/mL) was added to the mixture, which was then centrifuged at 1,000 \times g for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 1.0 mg/mL), and the absorbance of the mixture was read at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing activity.

VII. Determination of Chelating Activity

The chelating activity of the samples on Fe²⁺ was measured as previously described⁽¹⁷⁾. Samples (0.6 mL) were reacted with FeCl₂ (2 mM, 0.2 mL) and ferrozine (5 mM, 0.2 mL) for 10 min, and the absorbance was determined at 562 nm. A lower level of absorbance indicated stronger chelating activity.

VIII. Determination of Liposome Oxidation

Lecithin (100 mg) was sonicated in an ultrasonic cleaner (Branson 8210, Branson Ultrasonic Corporation, Danbury, CT, USA) in phosphate buffer (10 mL, 10 mM, pH 7.4) for 2 h in an ice-cold water bath. The sonicated solution, FeCl₃, ascorbic acid and samples (0.2 mL) were mixed to produce a final concentration of 2 mg/mL of lecithin, 100 μ M of FeCl₃ and 200 μ M of ascorbic acid. The mixture was incubated for 1 h at 37°C. The oxidation of liposome was determined in boiling water bath by the thiobarbituric acid (TBA) method⁽¹⁸⁾. The absorbance of the sample was read at 532 nm against a

blank, which contained all reagents except lecithin. A lower level of absorbance indicated stronger protective activity.

IX. Cell Culture

A murine macrophage cell line RAW 264.7 (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.

X. Cell Viability

Cells seeded at 2×10^5 per well were cultured in 96-well plates containing DMEM supplemented with 10% FBS for 24 h. Then the cells were cultured with samples in the presence of 100 ng/mL of LPS (lipopolysaccharide) for 24 h. After that, the cells were washed twice with the medium and incubated with 100 μ L of 0.5 mg/mL of MTT at 37°C for 2 h. The medium was then discarded and 100 μ L of dimethyl sulfoxide (DMSO) was added. After 30 min of incubation, the absorbance at 570 nm was read by using a microplate reader.

XI. Measurement of Nitric Oxide/Nitrite

Nitrite levels in the cultured media and serum, which reflect intracellular nitric oxide synthase activity, were determined by Griess reaction⁽¹⁹⁾. The cells were incubated with samples in the presence of LPS (100 ng/mL) at 37°C for 24 h. Then, 100 μ L of the cultured media 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. Using sodium nitrite to generate a standard curve, the concentration of nitrite was measured from absorbance at 540 nm.

XII. Animals

This study was conducted in conformity with the policies and procedure details in the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23 1985) and was approved by the Ethics Committee of the Institutional Animal Care and Use Committee (IACUC) of China Medical University, Taichung, Taiwan. ICR strain male mice (6-8 weeks old) were obtained from BioLASCO Taiwan Co. Ltd., Taipei, Taiwan. The animals were housed in an environmentally controlled room (temperature $22 \pm 1^\circ\text{C}$; relative humidity $55 \pm 5\%$; 12 h dark-light cycle). They were given food and water *ad libitum*.

XIII. Acetic Acid-Induced Writhing Response

After a 2-week adaptation period, mice (18 to 25 g) were randomly assigned to five groups including a normal control, an Indo positive control and three AFLL-treated groups. The control group received 1% acetic acid (10 mL/kg body weight) and the positive control group received Indo (10 mg/kg, i.p.) 25 min before intraperitoneal injection of 1% acetic acid (10 mL/kg body weight). AFLL-treated groups received AFLL (125, 250 and 500 mg/kg, p.o.) 55 min before intraperitoneal injection of 1% acetic acid (10 mL/kg body weight). Five minutes after the i.p. injection of acetic acid, the number of writhing during the following 10 min was recorded⁽¹⁹⁾.

XIV. Formalin Test

The antinociceptive activity of the drugs was determined using the formalin test⁽¹⁹⁾. The control group received 5% formalin. Twenty microliters of 5% formalin was injected into the dorsal surface of the right hind-paw 60 min after administration of AFLL (125, 250 and 500 mg/kg, p.o.) and 30 min after administration of Indo (10 mg/kg, i.p.). The mice were observed for 30 min after the injection of formalin, and the amount of time spent licking the injected hind paw was recorded. The first 5 min post formalin injection was referred to as the early phase and the period between 15 min and 40 min as the late phase. The total time licking or biting the injured paw (pain behavior) was measured with a stop watch. The activity was recorded at 5 min intervals.

XV. Determination of Carrageenan (Carr) Induced Edema

Carr-induced hind paw edema model was used for the determination of anti-inflammatory activity⁽¹⁹⁾. After a 2-week adaptation period, mice (18 to 25 g) were randomly assigned to five groups including Carr, positive Indo control and three AFLL-treated groups. The Carr group received 1% Carr (50 μ L). AFLL at doses of 125, 250 and 500 mg/kg were orally administered 2 h before the injection with 1% Carr (50 μ L) in the plantar side of the right hind paws of the mice. Indo (10 mg/kg) was intraperitoneally administered 90 min before the injection with 1% Carr (50 μ L) in the plantar side of right hind paws of the mice. Paw volume was measured immediately after Carr injection at 1, 2, 3, 4 and 5 h intervals using a plethysmometer (Model 7159, Ugo Basile, Varese, Italy). The degree of swelling induced was evaluated by $a - b$, where a was the volume of the right hind paw after Carr treatment and b was 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 right hind paw tissue and liver tissue were dissected. The tissues were rinsed in ice-cold normal saline, and immediately placed in cold normal saline four times their volume and homogenized at 4°C. Then the homogenate was centrifuged at 12,000 $\times g$ for 5 min. The supernatant of hind paw tissue was stored at -20°C for malondialdehyde (MDA) assays and immunoblot (iNOS and COX-2). The supernatant of whole liver tissue was obtained for the antioxidant enzymes (CAT, SOD and

GPx) activity assays. In addition, blood was withdrawn for NO and TNF- α assay.

XVI. Determination of Tissue Lipid Peroxidation in Paw

Malondialdehyde (MDA) was evaluated by the thiobarbituric acid (TBA) method⁽²⁰⁾. Briefly, MDA was reacted with thiobarbituric acid in an acidic condition at high temperature and a red-complex TBARS was formed. The absorbance of TBARS was determined at 532 nm.

XVII. Measurement of Tumor Necrosis Factor (TNF- α) in Serum

Serum levels of TNF- α were determined using a commercially available ELISA kit (Biosource International, Inc., Camarillo, CA, USA) according to the instructions of the manufacturer. TNF- α was determined from a standard curve.

XVIII. Determination of Antioxidant Enzyme Activity in Liver

Total SOD activity was determined by the inhibition of cytochrome *c* reduction⁽²¹⁾. 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 estimation was based on a previously report⁽²²⁾. In brief, the reduction of 10 mM of H₂O₂ in 20 mM of phosphate buffer (pH 7) was monitored by measuring the absorbance at 240 nm. The activity was calculated by using a molar absorption coefficient, and the enzyme activity was defined as nanomoles of dissipating hydrogen peroxide per milligram protein per minute. Total GPx activity in cytosol was determined as previously reported⁽²³⁾. The enzyme solution was added to a mixture containing hydrogen peroxide and glutathione in 0.1 mM of Tris buffer (pH 7.2) and the absorbance at 340 nm was measured. Activity was evaluated from a calibration curve and the enzyme activity was defined as nanomoles of NADPH oxidized per milligram protein per minute. The protein concentration of the tissue was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA, USA).

XIX. Histological Examination

For histological examination, biopsies of paws were taken following the interplanetary injection of Carr. The tissue slices were fixed in 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 H & E stain. All samples were observed and photographed with BH2 Olympus microscopy. Every 3-5 tissue slices were randomly chosen from Carr, Indo and AFLL-treated (500 mg/kg) groups. The number of neutrophils was counted in each scope (400x) and the average count from 5 scopes of every tissue slice was obtained.

XX. Western Blot Analysis of iNOS and COX-2 in Paw

Total protein was extracted with a radioimmuno-precipitation assay buffer (RIPA) solution at -20°C overnight. Protein samples (30 μ g) were resolved by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using standard methods, and then were transferred to PVDF membranes by electroblotting and blocking with 1% BSA. The membranes were probed with the primary antibodies (iNOS, COX-2 and β -actin) at 4°C overnight, washed three times with PBST, and incubated at 37°C for 1 h with horseradish peroxidase conjugated secondary antibodies. The membranes were washed three times and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) reagent. The results of Western blot analysis were quantified by measuring the relative intensity compared to the control using Kodak Molecular Imaging Software and represented in the relative intensities.

XXI. 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 $p < 0.05$, $p < 0.01$, and $p < 0.001$.

RESULTS

I. Antioxidant Activities of AFLL

The total phenol contents in the AFLL were determined in gallic acid equivalents (Table 1). The results showed that AFLL with total phenols was equal to 24.3 mg gallic acid/mL. Table 1 also shows ABTS scavenging, reducing power, chelating activity and liposome protection of the AFLL. AFLL showed 61.9-88.7% scavenging activity on ABTS radicals in the range of 50-200 μ g/mL. Reducing activity of natural products can usually be achieved by terminating the radicals' chain reaction. The reducing activity of AFLL

Table 1. Effects of the aqueous extract of Fructus *Ligustri Lucidi* (AFLL) on the total polyphenols content, radical-scavenging, reducing activity, chelating activity and liposome protection

Test	AFLL (μ g/mL)		
	50	100	200
Total polyphenols (μ g/mg)	5.8 \pm 0.1 ^a	12.3 \pm 0.2 ^b	24.3 \pm 0.7 ^c
ABTS inhibition (%)	61.9 \pm 3.9 ^a	86.8 \pm 2.9 ^b	88.7 \pm 3.1 ^b
Reducing activity (OD ₇₀₀)	0.23 \pm 0.01 ^a	0.45 \pm 0.01 ^b	0.68 \pm 0.02 ^c
Chelating activity (%)	6.1 \pm 0.2 ^a	17.4 \pm 0.5 ^b	38.8 \pm 1.7 ^c
Liposome protection (%)	5.6 \pm 0.1 ^a	13.8 \pm 0.5 ^b	29.3 \pm 2.4 ^c

All values are expressed as mean \pm SEM of triplicate tests ($n = 3$). Values with different superscripts in each row are significantly different ($p < 0.05$).

occurred in a concentration-dependent manner and increased to 3.0 fold in the range of 50-200 $\mu\text{g/mL}$. The chelating activity of natural products can serve as an important factor in oxidation prevention; thus, we determined the metal ions chelating capacity of AFLL. As shown in Table 1, AFLL in the range of 50-200 $\mu\text{g/mL}$ exhibited 6.1-38.8% chelating activity on ferrous ions. Lipid peroxidation is a harmful process which produces toxic aldehyde. As shown in Table 1, liposome protection was used as an index to assay the protective action of the AFLL on lipid molecules. AFLL in the range of 50-200 $\mu\text{g/mL}$ exhibited a dose-dependent protective effect (5.6-29.3%) on the lipid damage induced by the $\text{Fe}^{3+}/\text{H}_2\text{O}_2$ reaction. These data imply that the antioxidant properties of AFLL could protect lipid molecules against oxidative damage.

II. Effects of AFLL and its Reference Compounds on DPPH Radical Scavenging and LPS Induced NO Production in RAW264.7 Macrophages

The DPPH radical scavenging and anti-inflammatory activity of AFLL and its reference compounds were determined. Previous studies suggested the antioxidants observed in AFLL were recognized as phenolic components. As shown in Table 2, AFLL and its reference compounds including quercetin, myricetin, rutin, epicatechin, caffeic acid, gallic acid, ferulic acid and ellagic acid showed DPPH radical scavenging activity with an EC_{50} value of 89.34, 6.52, 7.63, 10.65, 18.21, 5.21, 2.52, 19.78 and 2.21 $\mu\text{g/mL}$. Oleanolic acid and ursolic acid had weak antioxidant activity on DPPH radical scavenging. In a cellular model of inflammation, the NO inhibitory activity was determined by using the LPS activated macrophages to produce NO radicals

Table 2. Effects of the aqueous extract of *Fructus Ligustri Lucidi* (AFLL) and its reference compounds on DPPH radical-scavenging and lipopolysaccharide (LPS)-induced nitric oxide (NO) production in macrophages

Sample	DPPH scavenging (EC_{50} , mg/mL)	NO inhibition (IC_{50} , mg/mL)
AFLL	89.34 \pm 1.56	265.48 \pm 2.32
Quercetin	6.52 \pm 0.17	12.35 \pm 0.27
Myricetin	7.63 \pm 0.24	19.73 \pm 0.17
Rutin	10.65 \pm 0.05	> 100
Epicatechin	18.21 \pm 0.23	> 100
Caffeic acid	5.21 \pm 0.13	8.45 \pm 0.14
Gallic acid	2.52 \pm 0.23	18.85 \pm 0.37
Ferulic acid	19.78 \pm 0.28	> 100
Ellagic acid	2.21 \pm 0.11	3.21 \pm 0.13
Oleanolic acid	> 100	18.75 \pm 0.76
Ursolic acid	> 100	14.74 \pm 0.23
Indomethacin	(-)	53.86 \pm 0.43

All values are expressed as mean \pm SEM of triplicate tests ($n = 3$). (-), not determined.

that were measured as nitrites in the culture medium by the Griess reaction. As shown in Table 2, AFLL reduced the NO production of activated macrophages with an IC_{50} value of 265.48 $\mu\text{g/mL}$, suggesting that AFLL could be a potential inhibitor of NO related inflammation pathway. In addition, no cell toxicity was observed with AFLL, as measured by the MTT cell viability test. The reference compounds of quercetin, myricetin, caffeic acid, gallic acid, ellagic acid, oleanolic acid and ursolic acid present in AFLL also showed NO inhibitory activity with an IC_{50} value of 12.35, 19.73, 8.45, 18.85, 3.21, 18.75 and 14.74 $\mu\text{g/mL}$. Rutin, epicatechin and ferulic acid had weak NO inhibitory activity induced by LPS in RAW264.7 macrophages. The reference compounds in AFLL, including quercetin, myricetin, caffeic acid, gallic acid and ellagic acid, exhibited the pharmacological activities by radical scavenging and decreasing LPS-induced NO production.

III. Acetic Acid-Induced Writhing Response

The cumulative amount of abdominal stretching correlated with the level of acetic acid-induced pain (Figure 1A). AFLL treatment (125, 250 and 500 mg/kg) significantly inhibited the number of writhing in comparison with the control. The inhibition rates of the number of writhing compared with the control are 12.34, 42.23 and 62.23% respectively. This inhibiting effect of acetic acid-induced writhing by AFLL (500 mg/kg) was similar to that produced by the positive control, Indo (10 mg/kg, $p < 0.001$).

IV. Formalin Test

AFLL significantly inhibited formalin-induced pain in the late phase. However, there was no inhibition in the early phase (Figure 1B). AFLL treatment (125, 250 and 500 mg/kg) significantly inhibited the formalin-induced pain (late phase) in comparison with the controls. The inhibition rates of formalin-induced licking compared with the control are 29.75, 48.51 and 63.96% respectively. The inhibiting effect of formalin-induced licking time by AFLL (500 mg/kg; $p < 0.001$) was better than the positive control, Indo (10 mg/kg, $p < 0.01$).

V. λ -Carrageenan (Carr)-Induced Edema

Figure 2A shows the effect of AFLL on Carr-induced paw edema in mice. Indomethacin (Indo) is an anti-inflammatory drug used to reduce acute inflammatory response, such as swelling. According to Figure 2A, Indo reduced the edema volumes by 0-44% in comparison to the Carr alone group during Carr treatment. Further, in the range of 125-500 mg/kg, AFLL showed a concentration-dependent inhibition of edema development. For AFLL at the concentration of 500 mg/kg, the levels of edema volume were decreased to 52% of that observed in the Carr alone group after 5th h treatment. There was no significant difference between Indo (10 mg/kg) and AFLL (500 mg/kg) groups after 3-5 h of Carr treatment.

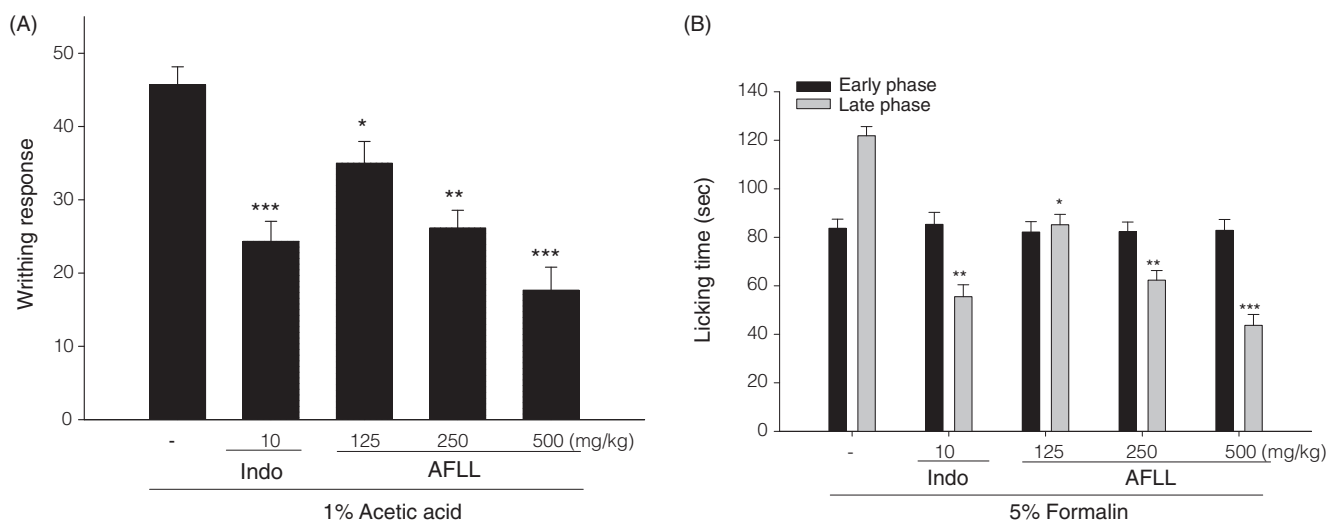


Figure 1. Analgesic effects of the aqueous extract of *Fructus Ligustri Lucidi* (AFLL) and indomethacin (Indo) were studied on acetic acid-induced writhing response (A) and on the early phase and late phase in formalin test (B) in mice. The values were averaged and obtained in individual animals ($n = 6$). Each value is expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared with the pathological model group.

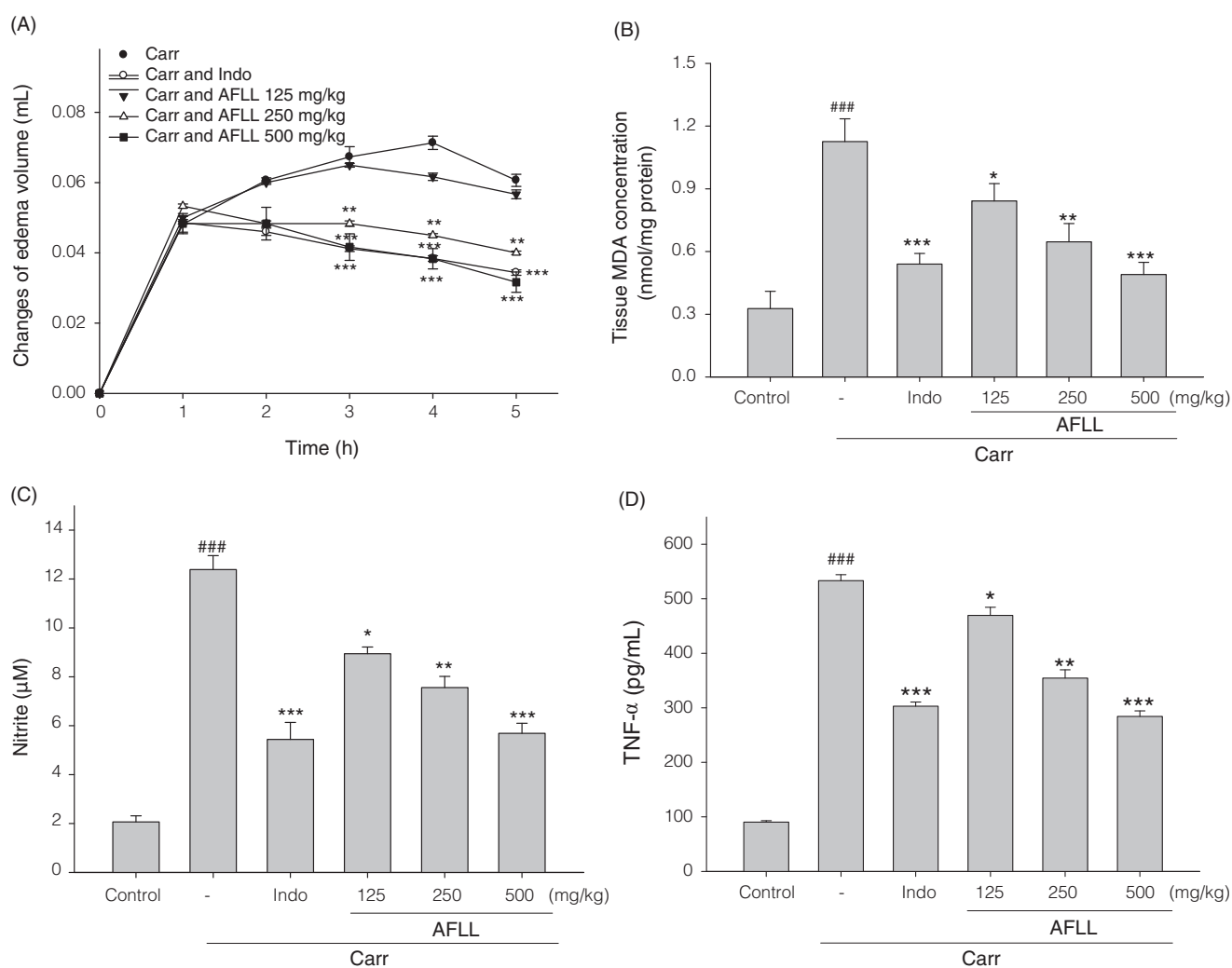


Figure 2. Effects of the aqueous extract of *Fructus Ligustri Lucidi* (AFLL) and Indo on hind paw edema induced by Carr (A), Carr-induced tissue MDA concentration of foot (B), Carr-induced NO (C) and TNF- α (D) concentrations of serum at 5th h in mice. The values were average from individual animals ($n = 6$). Each value is expressed as mean \pm SEM. ### $p < 0.001$ as compared with the control group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared with the Carr group.

These data imply that AFLL can act as an inhibitor of edema in acute inflammatory processes.

VI. Effects of AFLL on MDA, NO and TNF- α Levels

Lipid oxidation serves as a marker of cellular damage and has been recognized as a marker of inflammatory damage. As shown in Figure 2B, Carr increased the level of lipid oxidation by 2 folds in comparison with the control group. Meanwhile, Indo decreased the level of lipid oxidation to 48% of that observed in the Carr alone group. In fact, in the range of 125-500 mg/kg, AFLL inhibited the level of lipid oxidation down to 75-44% of that observed in the Carr alone group. These data imply that AFLL can protect against tissue lipid oxidation in Carr-induced inflammatory processes.

Previous studies have demonstrated evidence of increased NO, enhanced iNOS expression and elevated TNF- α production in Carr-induced inflammatory processes. The level of nitrite in serum is a regular index for intracellular NO and iNOS production *in vivo*. As shown in Figure 2C, Carr increased the level of nitrite in serum by 4.9 folds in comparison to the control group. Meanwhile, Indo decreased the level of serum nitrite to 44% of that observed in the Carr alone group. In fact, in the range of 125-500 mg/kg, AFLL reduced the level of nitrite to 72-46% of that observed in the Carr alone group. In addition, Carr increased the level of TNF- α in the serum by 4.9 folds in comparison to the control group (Figure 2D). Indo decreased the level of serum TNF- α to 57% of that observed in the Carr alone group. AFLL also inhibited the production of TNF- α to 88-53% of that observed in the Carr alone group. These data imply that AFLL acts as an inhibitor of Carr-induced tissue inflammation by decreasing NO and TNF- α production *in vivo*.

VII. Effects of AFLL on the Activities of Antioxidant Enzymes

Table 3 shows the activities of CAT, SOD and GPx in the livers of treated mice. As shown in Table 3, Carr decreased the activities of CAT, SOD and GPx in the livers by 45, 26 and 43%, respectively, in comparison to the control group. In the range of 125-500 mg/kg, AFLL increased the activities of CAT to 128-162%, SOD to 102-120%, and GPx to

106-148%, respectively, compared to that observed in the Carr alone group. Indo also increased effects in the activities of CAT (116%), SOD (118%) and GPx (141%) in comparison to the Carr alone group. These data imply that the anti-inflammatory effects of AFLL *in vivo* might be attributed to its elevation in the antioxidant enzymes activities of Carr induced mice.

VIII. Effects of AFLL on Carr-Induced iNOS and COX-2 Protein Expressions

The results showed that the injection of AFLL (500 mg/kg) inhibited iNOS and COX-2 protein expression in mouse paw (Figure 3A). The intensity of protein bands was analyzed and showed an average of 59.6 and 76.4% down-regulation of iNOS and COX-2 protein, respectively, after the treatment with AFLL at 500 mg/kg compared with the Carr alone group (Figure 3B). In addition, the protein expression showed an average of 57.6 and 59.5% down-regulation of iNOS and COX-2 protein, respectively, after treatment with Indo at 10 mg/kg compared with the Carr alone group.

IX. Histological Examination

Paw biopsies of animals treated with the AFLL showed a reduction in Carr-induced inflammatory response. Actually, inflammatory cells were reduced in number and confined to near 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 4A). Neutrophils were observed to increase with Carr treatment ($p < 0.001$). Indo and AFLL (500 mg/kg) could significantly decrease the neutrophil numbers as compared to the Carr alone group ($p < 0.001$)(Figure 4B).

DISCUSSION

Free radicals could induce biological damage and pathological events, such as inflammation, aging and carcinogenesis. Natural components, such as the polyphenols of plant

Table 3. Effects of the aqueous extract of Fructus *Ligustri Lucidi* (AFLL) on the hepatic catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities in mice

Groups	CAT (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)
Control	4.62 \pm 0.18	25.38 \pm 0.24	3.26 \pm 0.22
Carr	2.53 \pm 0.12 ^{###}	18.83 \pm 0.35 ^{###}	1.87 \pm 0.16 ^{###}
Carr + Indo (10 mg/kg)	4.06 \pm 0.30 ^{**}	22.17 \pm 0.15 ^{**}	2.64 \pm 0.13 [*]
Carr + AFLL (125 mg/kg)	3.24 \pm 0.21	19.13 \pm 0.32	1.98 \pm 0.23
Carr + AFLL (250 mg/kg)	3.86 \pm 0.04 [*]	21.39 \pm 0.16 [*]	2.45 \pm 0.13 [*]
Carr + AFLL (500 mg/kg)	4.12 \pm 0.10 ^{**}	22.67 \pm 0.21 ^{**}	2.76 \pm 0.16 ^{**}

The values are averaged and obtained from individual animals (n = 6). Each value is expressed as mean \pm SEM. ^{###} $p < 0.001$ as compared with the control group. ^{*} $p < 0.05$ and ^{**} $p < 0.01$ as compared with the Carr (carrageenan) group.

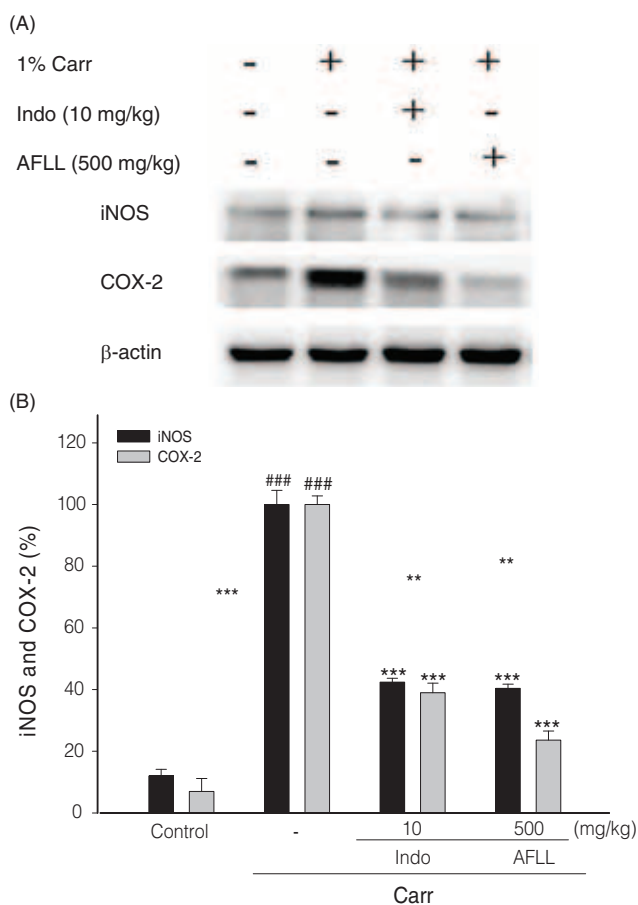


Figure 3. Inhibition of iNOS and COX-2 protein expression by the aqueous extract of *Fructus Ligustri Lucidi* (AFLL) was induced by Carr of foot at 5th h in mice. Tissue suspended was prepared and subjected to Western blotting using an antibody specific for iNOS and COX-2. β-actin was used as an internal control. (A) A representative Western blot from two separate experiments was shown. (B) Relative iNOS and COX-2 protein levels were calculated with reference to a Carr-injected mouse. The data were presented as mean±SEM for three different experiments performed in triplicate. ### $p < 0.001$ as compared with the control group. *** $p < 0.001$ as compared with the Carr group.

extracts can exhibit antioxidants and regular cellular redox states. The higher radical scavenging activity of AFLL seems to be closely correlated with its polyphenolic constituents though active components could play important roles in its antioxidative effect. In this study, AFLL showed significant antioxidant activities. In order to reduce the pollution of organic solvents and insure safety, we only used water as the extraction solvent.

Three common *Ligustrum* plant spp. were collected to assess their analgesic/anti-inflammatory properties on carrageenan-induced inflammation. *Ligustrum lucidum* Ait. had the highest content of oleanolic acid and ursolic acid⁽⁸⁾. Consequently, it is possible that the total phenolic constituents may contribute to the anti-inflammatory activity of AFLL. These protective activities of AFLL have contributed to their phenolic and triterpenoid components present, including quercetin, myricetin, rutin, epicatechin, caffeic acid, gallic acid, ferulic acid, ellagic acid, oleanolic acid and ursolic

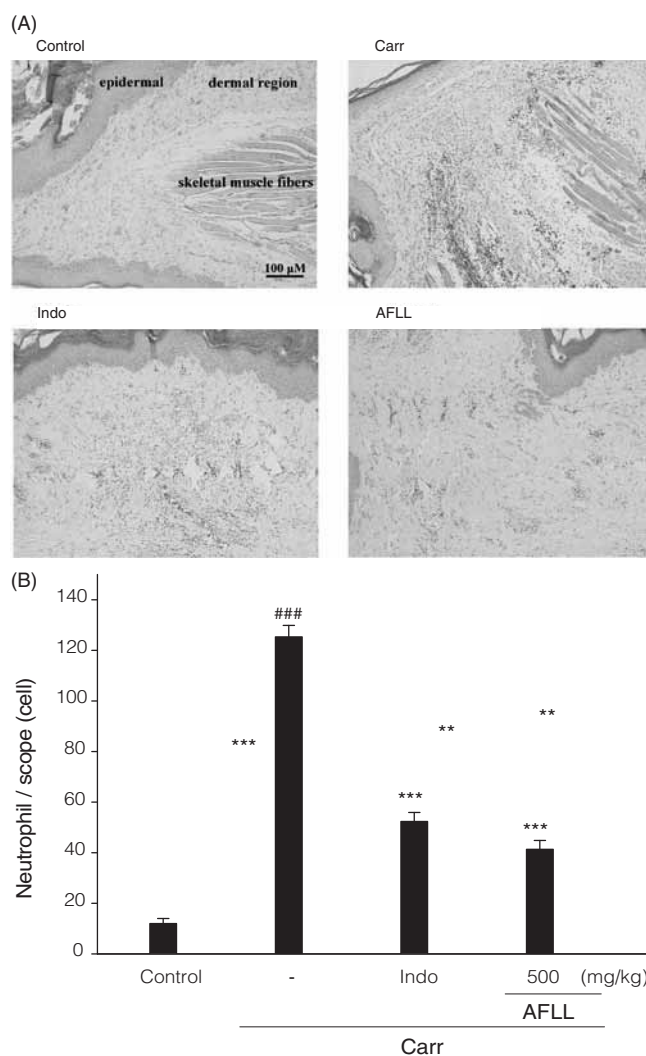


Figure 4. Histological appearance of the mouse hind footpad after a subcutaneous injection with Carr stained with H&E stain at 5th h revealed hemorrhage, edema and inflammatory cell infiltration in control mice. Carr-treated mice demonstrated hemorrhage with moderately extravascular red blood cells and a large amount of inflammatory leukocyte mainly neutrophils infiltration in the subdermis interstitial tissue of mice, and mice given Indo (10 mg/kg) before Carr. The aqueous extract of *Fructus Ligustri Lucidi* (AFLL; 500 mg/kg) showed significant morphological alterations compared to injection of Carr only (100x) and the number of neutrophils in each scope (400x) compared to subcutaneous injection of Carr only (B). ### $p < 0.001$ as compared with the control group. *** $p < 0.001$ as compared with the Carr group.

acid⁽²⁴⁾. In this paper, we demonstrated that the reference compounds in AFLL with antioxidant and anti-inflammatory activities were quercetin, myricetin, caffeic acid, gallic acid and ellagic acid (Table 2).

In accordance with the finding, previous studies have suggested that plant materials containing triterpenoid, flavonoids and phenolic acids possess analgesic and anti-inflammatory effects on experimental animals and the pharmacological effects are resulted from these contents⁽²⁵⁾. Some triterpene compounds such as amyryn, betulin, betulinic acid and lupeol have also been proven to possess analgesic and

anti-inflammatory activities⁽²⁶⁾. Hence, this work suggested that active components of AFLL with analgesic and anti-inflammatory activities may be related to its phenolic and triterpenoid compounds.

Two different analgesic testing methods were employed with the objective of identifying possible peripheral and central effects of the test substances. The acetic acid writhing test is normally used to study the peripheral analgesic effects of drugs⁽²⁷⁾. Acetic acid can indirectly induce the release of endogenous mediators of pain that stimulate the nociceptive neurons, which are sensitive to nonsteroidal anti-inflammatory drugs⁽²⁸⁾. When comparing antinociceptive activities, AFLL was relatively potential in the acetic acid writhing test. Further, AFLL (500 mg/kg) exhibited an action in similar magnitude with Indo (10 mg/kg), a reference drug for peripheral antinociception (Figure 1A).

Formalin-induced paw pain has been established as a model for analgesic study. It is well-known that the formalin test produces a distinct biphasic nociception, a first phase (lasting the first 5 min) corresponding to acute neurogenic pain, and a second phase (lasting from 15 to 30 min after the injection of formalin) corresponding to inflammatory pain responses⁽²⁹⁾. Therefore, the test can be used to clarify the possible mechanism of an antinociceptive effect of a proposed analgesic. The inhibitory effect of AFLL on the nociceptive response in the late phase of the formalin test suggested that the anti-nociceptive effect of AFLL could be due to its peripheral action (Figure 1B).

The Carr-induced edema test is a model to determine the anti-inflammatory activities of natural products⁽³⁰⁾. This edema model involves the synthesis and release of inflammatory mediators at the site of injury. The degree of paw swelling was maximal at the third hour after the injection of Carr. However, a reduction in paw swelling size is a good index in determining the protective action of anti-inflammatory agents. According to Figure 2A, statistical analysis revealed that AFLL (500 mg/kg) inhibited the development of edema at the third hour after treatment ($p < 0.01$). In fact, no significant difference existed between AFLL (500 mg/kg) and Indo groups at 3-5 h after Carr treatment, which suggests that AFLL have potential anti-inflammatory effects *in vivo*. In addition, the anti-inflammatory activity of AFLL (500 mg/kg) is superior to methanol extracts from three *Ligustrum* plants leaves (0.1, 0.25, 1 g/kg) on carrageenan-induced paw edema in mice⁽⁸⁾.

In the process of inflammation, a burst of NO is synthesized from L-arginine by iNOS in activated macrophages. In fact, the overproduction of NO could induce cell damage as well as inflammation. The inhibitory effects of AFLL on NO production could contribute to the decrease in inflammation development in tissues. NO produced by activated macrophages also acts as an important mediator in the cytotoxic/cytostatic mechanism of non-specific immunity. Therefore, AFLL decreased NO production *in vitro* (Table 2) and *in vivo* (Figure 2C), which could further lead to a reduction in the edema response in inflammation.

During inflammatory processes, large amounts of the

proinflammatory mediators, NO and PGE₂, are generated by inducible iNOS and COX-2 respectively. In resting cells, iNOS is generally not present but is induced by various stimuli, which include bacterial LPS, TNF- α , IL-1 β and interferon- γ ⁽³¹⁾. However, COX-2 is induced by pro-inflammatory stimuli, including LPS and cytokines in cells *in vitro* and in inflamed sites *in vivo*. In this study, there was a significant decrease in iNOS and COX-2 activities with AFLL treatment (Figure 3A). The suppression of NO production is probably due to the decrease in iNOS and COX-2 activities. AFLL acts as herbal antioxidants and its antioxidative action may partly be responsible for the inhibition of NO production. Therefore, the inhibitory effect of AFLL on NO production could contribute to its total polyphenols' inhibition to iNOS protein expression.

Lipids, the main components of cell membranes, are susceptible to the oxidation of free radicals. Lipid oxidation has not only been served as a marker of cellular damage *in vivo*, but has been recognized to be the inducer of inflammatory processes, atherosclerosis and cancer. In this study, AFLL exhibited radical-scavenging capacity and could decrease Carr-induced lipid damage *in vivo* (Figure 2B). The pretreatment with oleanolic and ursolic acid significantly retained GSH, and reversed H₂O₂-induced impairment in CAT and SOD activities and decreased lipid oxidation⁽²⁴⁾.

The Carr-induced inflammatory response has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as superoxide and hydroxyl radicals⁽³²⁾. However, these destructive species can be counteracted by intracellular antioxidant enzymes and non-enzymatic antioxidant systems in tissues. According to Table 2, AFLL seemed not to have good non-enzymatic antioxidant activity compared to most of the other samples listed. As shown in Table 3, there was a significant increase in CAT, SOD and GPx activities with AFLL treatment *in vivo*. This data implied that the anti-inflammatory effect of AFLL could be due at least in part to elevate intracellular antioxidant enzyme activities and decrease inflammatory stress in tissues. Further, a significant decrease in the tissue lipid oxidation was found with AFLL treatment in Carr-induced mice. This decrease in lipid oxidation is probably due to the increase of CAT, SOD and GPx activities. Consequently, AFLL positively increased cellular antioxidative activities against inflammatory oxidation and antioxidant system defects *in vivo*. However, AFLL decreased radical production and lipid oxidation *in vitro* as well as *in vivo*. These data suggest that AFLL could serve as a natural antioxidant to protect cells against inflammatory damage.

In conclusion, our data suggest that AFLL shows anti-inflammatory effects *in vitro* and *in vivo*. The anti-inflammatory effects of AFLL may be related to iNOS and COX-2 reduction and reduce excess TNF- α generation in physiological systems. The antioxidant effects of AFLL can be due to the increase in the activities of antioxidant enzymes (e.g. CAT, SOD and GPx) and its effects on radical scavenging. Therefore, we suggest that AFLL contain herbal antioxidants and exhibit analgesic and anti-inflammatory activities *in vivo*.

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