Analgesic and Anti-inflammatory Activities of the Extract from *Plectranthus amboinicus* (Lour.) Spreng. both *in vitro* and *in vivo* animal models

Yung-Jia Chiu [a], Tai-Hung Huang [b], Chuan-Sung Chiu [a,c], Tsung-Chun Lu [a], Ya-Wen Chen [d,e], Wen-Huang Peng [a]*, Chiu-Yuan Chen [f]*

[a] *Graduate Institute of Chinese Pharmaceutical Sciences, College of Pharmacy, China Medical University, Taichung, Taiwan*
[b] *School of Pharmacy, College of Pharmacy, China Medical University, Taichung, Taiwan*
[c] *Hsin Sheng College of Medical Care and Management,* Taoyuan, *Taiwan, R.O.C.*
[d] *Graduate Program in Life Science,* [e] *Greenebaum Cancer Center, University of Maryland, Baltimore, Maryland 21201*
[f] *Graduate Institute of Natural Healing Sciences, Nanhua University, Chiayi, Taiwan,*

* Corresponding authors:
Graduate Institute of Chinese Pharmaceutical Sciences, College of Pharmacy, China Medical University, 91, Hsueh-Shih Road, Taichung, Taiwan, ROC
TEL: 886 (4) 2205-3366 ext 5505
E-mail address: whpeng@mail.cmu.edu.tw

Abstract

The H_2O extract of *Plectranthus amboinicus* (Lour.) Spreng. (PA) inhibited pain induced by acetic acid and formalin, and inflammation induced by carrageenan. The anti-inflammatory effect of PA was related to modulating anti-oxidant enzymes' activities in the liver and decreasing the MDA level and the production of TNF-α and COX-2 in edema-paw tissue in mice. *In vitro* studies show that PA (0.1 - 0.5 mg/ml) inhibited the proinflammatory mediators in RAW 264.7 cells stimulated with LPS. PA blocked the degradation of IκB-α and nuclear translocation of NF-κB p65 subunit. Finally, the amount of carvacrol in the PA was 1.88 mg/g extract.

Our findings suggest that PA has analgesic and anti-inflammatory activities. These effects were mediated by inhibiting the proinflammatory mediators through blocking NF-κB activation. Meanwhile, the effects observed in this study provide evidence for folkloric uses of PA in relieving pain and inflammation.

1. Introduction

Inflammation is the result of host response to tissue injuries or pathogenic challenges, and ultimately leads to the restoration of a normal tissue structure and function. Acute inflammation is a limited beneficial process, particularly in response to infectious pathogens, whereas chronic inflammation is an undesirable persistent phenomenon that can lead to the developments of inflammatory diseases [1]. Prolonged inflammation contributes to the pathogenesis of many inflammatory diseases, such as, metabolic disease [2], atherosclerosis [3], obesity, cardiovascular disease [4], rheumatoid arthritis [5], and cancer [6].

Acute inflammation, which is typically characterized by redness, swelling, pain, and heat, is one of the most important host defense mechanisms against invading pathogens. Lipopolysaccharide (LPS) from Gram-negative bacteria is well known to cause bacterial sepsis mediated through activation of monocytes, neutrophils and macrophages [7]. Sometimes activation of these cells may induce over secretion of various proinflammatory and toxicity mediating molecules such as TNF-α, IL-6, eicosanoids, and nitric oxide (NO) [8]. However, excessive inflammatory response has damaging effects, such as septic shock, which can lead to multiple organ dysfunction syndrome and death. PGs and NO are two important proinflammatory mediators and inhibition of productions of both PGs and NO via inhibition of their synthases, cyclooxygenase2 (COX-2) and inducible nitric oxide synthase (iNOS) respectively, has been demonstrated beneficial in treating inflammatory disease [9]. Anti-inflammatory drugs such as steroids or nonsteroidal anti-inflammatory drugs (NSAIDs) have a number of adverse side effects, such as gastrointestinal discomfort, inhibition of platelet aggregation and liver and kidney toxicity [10]. Thus, there is considerable research interest in the identification of new anti-inflammatory agents from plants used in traditional medicine.

Plectranthus amboinicus is native Labiatae plant of Taiwan. The plants are commonly used in Chinese folk medicine for the treatment of cough, fever, sore-throats, mumps, and mosquito bite [11, 12]. Previous study showed that the ethanol extract of *Plectranthus amboinicus* possess nephroprotective and antioxidant effects against acetaminophen-induced nephrotoxicity and strong

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diuretics effect in rats [13]. *Plectranthus amboinicus* also showed the ability to treat collagen-induced arthitis in rats [14]. However, the therapeutic potential of *Plectranthus amboinicus* for inflammatory diseases remains fully unclear. The purpose of this study is to examine the analgesic, antioxidant, and anti-inflammatory effects of PA in *in vivo* models and the anti-inflammatory mechanisms of PA in *in vitro* models. The peripheral analgesic activity of PA was determined by the acetic acid induced writhing response and formalin test. We also analyzed the levels of the antioxidant enzymes in the liver and several proinflammatory markers in the paw tissue of carrageenan-induced edema models. The anti-inflammatory mechanisms of PA were revealed using LPS-induced RAW 264.7 macrophages model. Our results demonstrate that PA has the analgesic and anti-inflammatory abilities and suggest that PA has the therapeutic potential to be used as an alternative medicine for inflammatory diseases.

2. Materials and Methods

2.1 Preparation of plant extract

The plants of *Plectranthus amboinicus* were collected in Taichung of Taiwan in July 2008 and were identified by Dr. Chao-Lin Kuo, leader of the School of Chinese Medicine Resources (SCMR). Fresh *Plectranthus amboinicus* (6 kg) was minced using a mixer grinder with 10 L double distilled water (ddH_2O) at room temperature. The juice was filtered, concentrated, and freeze-dried to obtain PA with a yield ratio of 0.954% (w/w, with reference to fresh material). The PA was then analyzed with High-performance liquid chromatography (HPLC) analyses. The peaks of PA were identified by comparison with the standard solutions (carvacrol).

2.2 Animals

Male ICR mice (18-22 g) were obtained from the animal center of school of medicine in National Taiwan University. Animals used in this study were housed and cared in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Committee on Animal Research, China Medical University, under the code 2006-14-N. Mice were housed in standard cages at a constant temperature of 22 ± 1 °C, relative humidity 55 ± 5% with 12 h light-dark cycle for at least 1 week before the experiments. All tests were conducted under the guidelines of the International Association for the Study of Pain [15]. Each experiment was performed in five groups of ten rats. The animals received only inducing drug, inducing drug with PA (0.1, 0.5 and 1.0 g/kg, po, daily) or indomethacin (Sigma; 10 mg/kg, po, daily).

2.3 Chemicals

Carrageenan, indomethacin, carvacrol, Griess reagent, Lipopolysaccharide (LPS), MTT and other chemicals were purchased from Sigma-Aldrich Chemical Co (St Louis, MO, USA). Formalin was purchased from Nihon Shiyaku Industry Ltd (Japan). Murine TNF-α enzyme-link immunosorbent assay (ELISA) Development Kit (900-k54) was purchased from Peprotech EC Ltd. Antibodies of iNOS, COX-2 and IκB-α were purchased from AbCam. Indomethacin was suspended in 0.5% (w/v) carboxymethylcellulose sodium (CMC) and administered intraperitoneally (i.p.) to animals.

Carrageenan, acetic acid and formalin were diluted separately in normal saline.

2.4 *Preliminary phytochemical analysis*

Chromatographic separation was carried out on Synergi™ 4 µ Fusion-RP 80 column（4µm，4.60 × 250 mm）Phenomenex Inc. with a injection of 10μl using an elution of 0.2% formic acid: methanol (45:55) solvent at a flow rate of 1.0 ml/min. Peaks were detected at 274 nm with SPD -M10AVP (shimadzu) detector. The sample was injected of. The peaks of PA samples were identified by comparison with the standard solutions (carvacrol).The PA solutions were quantified by spiking with a known amount of standard and also by comparing the area under curve. The repeatability of the method was evaluated by injecting the solution of PA and standard solution three times, and the relative standard deviation (RSD) percentage was calculated.

2.5 Acetic acid-induced writhing test

The writhing test in mice was conducted as described in the previous study [16]. Mice were administered orally with PA (0.1, 0.5, and 1.0 g/kg) 60 min before the induction of writhes. The writhes were induced by intraperitoneal injection of 1.0% acetic acid (v/v, 0.1 ml/10 g body weight). Indomethacin (10 mg/kg, i.p.) was used as therapeutic control and administered 30 min before acetic acid injection. Mice were placed in an observation box separately and the number of writhing responses was counted within 10 min.

2.6 Formalin test

The test was conducted according to the method described in the previous study [16]. Mice were administered orally with PA (0.1, 0.5, and 1.0 g/kg) 60 min before formalin treatment or intraperitoneal injection of indomethacin (10 mg/kg) 30 min before formalin treatment or the same volume of saline by oral administration as control[17]. Twenty microliter of 5% formalin in distilled water was then injected subcutaneously into the right hind paw of mice to cause pain. These mice were individually placed in a transparent Plexiglas cage (25×15×15 cm). The time spent licking and biting the injected paw as the index of pain was recorded separately from 0-5 min as early phase or neurogenic pain and from 20-30 min as late phase or inflammatory pain [18].

2.7 Carrageenan-induced mice paw edema

This method was previously described and was used with some modifications [19]. Male ICR mice (ten per group) were fasted for 24 h before the experiment with free access to water. The mice were injected subcutaneously with 50 l of 1% carrageenan solution in normal saline (0.9% w/v NaCl) into the sub-plantar region of the right hind paw. Paw volume was measured by using a plethysmometer immediately before injection and 1, 2, 3, and 4 h after the administration of the carrageenan. PA (0.1, 0.5, and 1.0 g/kg, p.o.) was administered at 120 min after carrageenan injection. Indomethacin (10 mg/kg, i.p.), a therapeutic control, was administered at 150 min after carrageenan injection. The percent increase in paw volume was calculated and compared with the vehicle control.

For the malondialdehyde(MDA), Tumor necrosis factor-α (TNF-α), and Cyclooxygenase-2 (COX-2) assays, the whole right hind paws were collected at the third hour after carrageenan injection. The right hind paw tissue was rinsed in ice-cold normal saline and immediately placed in cold normal saline four times their volume and finally homogenized at 4 °C. Then, the homogenate was centrifuged at 12,000 rpm for 5 min. The supernatant was obtained and stored at -80 °C for the MDA, TNF-α, and COX-2 assays.

For the antioxidant enzyme activity assays, liver tissues were collected at the third hour after carrageenan injection and rinsed in ice-cold normal saline and immediately placed in cold normal saline of the same volume and finally homogenized at 4 °C. Then, the homogenate was centrifuged at 12,000 rpm for 5 min. The supernatant was obtained and stored at -80 °C for the antioxidant enzyme (superoxide dismutase, glutathione peroxidase and glutathione reductase) activity assays.

2.8 Malondialdehyde Assay

Malondialdehyde (MDA) was evaluated by the thiobarbituric acid-reacting substance (TBARS) method [20]. Briefly, MDA can react with thiobarbituric acid (TBA) under the acidic and high temperature conditions. MDA and TBA then formed a red-complex TBARS, which can be measured colorimetrically. The absorbance of TBARS was determined 532 nm.

2.9 Anti-oxidant enzymes' activities

Liver tissue homogenates were collected for the estimation of superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GRx) enzyme to detect the antioxidant activities of PA [21].

2.10 Tissue COX-2 by Quartz Crystal Microbalance

The P-sensor 2000 designed by ANT (Asia New Technology, Taiwan) is based on the principle of piezoelectric biosensor. P-sensor 2000 based on quartz crystal microbalance (QCM) was used to monitor the antibody-antigen interaction in real time. It is made of three portions including electronic oscillation circuit, frequency counter, and piezoelectric quartz of fixed biosensor molecule (p-chip). The piezoelectric quartz crystal consists of a quartz crystal slab with a layer of gold electrode on each side. It is the signal conversion component of the piezoelectric sensor chip and can convert the result sensed by the sensor molecule into electronic signal to be amplified. The function of gold electrodes is mainly to introduce an oscillating electric field perpendicular to the surface of the chip so that the internal part of the chip generates mechanical oscillation because of the piezoelectric effect. If the thickness of the quartz crystal is fixed, the mechanical oscillation will be generated at a fixed frequency. Using a suitable electronic oscillation circuit, the resonant frequency can be measured. The PBS, a mobile carrier, would flow through the sensor cell with the antibody-immobilized chip in flow rate of 30μl/min and clean the fluid lines of QCM, alternating with the 1N NaOH and 1N HCl solution and ultra-pure water before the measurement. After the introduction of PBS to fill the sensor cell, the frequency shift of QCM reached a steady equilibrium (“_F” < 0.2 Hz/min) and was defined as a zero baseline, “F0”. Upon the injection of supernatant solution into the sensor cell, the dynamic interactions between antigens and immobilized antibodies were monitored, and the frequency shifts were recorded for the next steady equilibrium, “F”. Thus, the apparent frequency change of crystal oscillator, “_F”, can be measured by subtracting “F” from “F0”. All of PBS and diluted sera solutions were filtered with Millex GP filter unit (0.22 μm, PES membrane; Millipore, Ireland) and degassed before used. The sensor chips were disposable to ensure the sensitivity and reproducibility of each of the QCM experiments. With a temperature controller, the temperature of the sensor cell was controlled at constant temperature of 25 °C to suppress the fluctuations of kinetics by ambient environment.

2.11 Tissue and cells release TNF-α by ELISA

TNF-α level was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instruction. The absorbance at 450 and 540 nm was measured on a microplate reader (VersaMax, Massachusetts, USA).

2.12 Cell culture and Cell viability

RAW 264.7 macrophage cell line was obtained from Culture Collection and Research Center (Hsinchu, Taiwan). Cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 µg/ml) in a humidified 5% CO_2 atmosphere. Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to purple formazan. Cells were incubated with MTT (0.5%) for 4 h at 37 °C. The medium was removed by aspiration, and formazan crystals were dissolved in DMSO. The extent of the reduction of MTT was quantified by measurement of A550.

2.13 Nitrite measurement

The nitrite concentration in the medium was measured according to the Griess reaction, and the calculated concentration was taken as an indicator of NO production. The supernatant of cell cultures was mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). The optical density at 550 nm was measured and calculated against a sodium nitrite standard curve.

2.14 Western blot analysis

Cells were lysed at 4 °C in RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.25% Sodium deoxycholate, 5 mM EDTA (pH 8.0), and 1 mM EGTA and supplemented with protease and phosphatase inhibitors. After 20 min of lysis on ice, cell debriswas removed bymicrocentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined by the Bradford method. Equal amounts of proteinswere separated onto SDS-polyacrylamide gels and then electrophoretically transferred from the gel onto a PVDF membrane (Millipore, Bedford, MA). After blocking, the membrane was reacted with specific primary antibodies overnight at 4 °C and then incubated with horseradish peroxidaseconjugated secondary antibody for 1 h. The blots were visualized using the ECL-Plus detection kit (PerkinElmer Life Sciences, Inc. Boston,MA, USA).

2.15 Immunofluorescence

Cells were pretreated without or with PA (0.1~0.5 mg/ml) for 30 min and then treated with LPS (100 ng/ml) in the presence or absence of luteolin, and then fixed with 2% paraformaldehyde for 20 min. The cells were incubated with 0.1% Triton X-100 for 30 min then blocked with 1% BSA for 30 min. Cells were probed with mouse anti-p65 antibody (Santa Cruz Biochemicals, Santa Cruz, CA, USA, 1:500) overnight at 4 °C, followed by FITC-conjugated goat anti-mouse IgG antibody (Sigma, St. Louis, MO, USA, diluted 1:200) 1 h at 37 °C, washed with PBS three times and then stained with propidium iodide for 15 min. NF-κB p65 subunit was observed with a laser scanning confocal microscope.

2.16 Statistical analysis

All the data were expressed as mean ± S.E.M. Statistical analysis was carried out using one-way ANOVA, followed by Scheffe's multiple range test. The criterion for statistical significance was $p < 0.05$.

3. Results

3.1 Phytochemical study

The phytochemical study of PA showed the presence of carvacrol. The content and variety of carvacrol which has a maximum absorbance at 274 nm is 1.88±0.53 mg/g extract.

3.2 Analgesic effect of PA in mice

Effect of the PA in decreasing the acetic acid-induced writhing responses in mice which indicates the analgesic activity is presented in Figure 2. Treatment of PA at 1.0 and 0.5 g/kg and indomethacin at 10 mg/kg showed inhibition of writhing number compared to the control ($p<$ 0.01-0.001). Moreover, PA also showed a dose-dependent effect on the decrease of licking time in the late phase of formalin-induced pain (Figure 3B, $p<$ 0.05-0.001) though there are no significant inhibitions in the early phase (Figure 3A).

3.3 PA inhibited carrageenan-induced edema and inflammation in mice paw tissue

The carrageenan-induced mice paw edema is a biphasic process [22] . In the early hyperemia, 0-2 h after carrageenan injection, there is a release of histamine, serotonin, and bradykinin to increase vascular permeability. The inflammatory edema reached its maximum level at the third hour and after that it started declining. In our study, the paw edema was increased and reached maximally at 4 h after carrageenan injection. Treatment of PA (1.0 g/kg) significantly reduced the paw edema formation ($p<$ 0.001) as shown in Figure 4A. The inhibition rate at 4 h was shown as 41.2% and 62.3% with the treatment of PA (1.0 g/kg) and indomethacin, respectively.

Previous reports have demonstrated that accumulations of MDA, TNF-α, and COX-2 are indications of inflammation. Thus, we set out to measure the MDA level using TBARS method, the TNF-α level using ELISA, and the COX-2 level using QCM in paw tissues from carrageenan-induced edema model mice. As expected, the levels of MDA, TNF-α, and COX-2 were increased in the carrageenan-induced paw edema mice (Figure 4B, 4C, 4D). However, treatment of PA (1.0 g/kg) significantly decreased the levels of MDA, TNF-α, and COX-2 ($p<$0.001, Figure 4B, 4C, 4D, respectively). Moreover, treatment of PA at lower dose (0.5 g/kg) also decreased the levels of TNF-α and COX-2 (p< 0.001 and 0.05, respectively).

3.4 Antioxidant abilities of PA in mice

To investigate the antioxidant abilities of PA, the activities of antioxidant enzymes (SOD, GPx, and GRx) at the third hour after carrageenan injection were investigated. In our result, SOD activity increased significantly after treatment with indomethacin and PA (1.0 g/kg) (p< 0.01 and p<0.001, respectively)) (Figure 5A). GRx activities in the liver tissues increased significantly with the treatment of indomethacin and PA (1.0 g/kg) (p< 0.001 and p<0.05, respectively) (Figure 5B). There are no significant inhibitions of GPx activities when treatment with any dose of PA, which was comparable to the treatment of indomethacin (p< 0.001) (Figure 5C).

3.5 PA inhibited LPS-induced TNF-α and NO production in RAW 264.7 cell

Proinflammatory cytokines and mediators play important roles in the inflammatory process. To further validate the effect of PA on anti-inflammatory function *in vitro*, the levels of secreted TNF- and NO were measured in mouse peripheral macrophage RAW 264.7 cells when treated with LPS. As demonstrated in Figure 6., treatment of RAW 264.7 cells with LPS (100 ng/ml) caused a substantial increase in the production of TNF- and NO. However, pretreatment with PA before being incubated with LPS resulted in a dose-dependent inhibition of the LPS-induced TNF-αand NO production in RAW 264.7 cells (Figures 6A and 6B). To examine whether PA is cytotoxic to the cells, RAW 264.7 cells were incubated with 0.1-1.0 mg/ml of PA for 24 h. Within our tested concentrations, no cytotoxic effect of PA was observed (Fig. 6C).

3.6 The anti-inflammatory effects of PA are via down regulation the protein levels of iNOS and COX-2

To determine if the inhibitory effect of PA on these inflammatory mediators was related to the regulation of iNOS and COX-2, the levels of these two proteins were examined by Western blot analysis at 8 and 12 h after LPS treatment. As shown in Figure 7, the protein levels of iNOS and

COX-2 were markedly increased upon LPS treatment, and these inductions were drastically blocked by treatment with PA (0.5 mg/ml).

3.7 Prevention of LPS-induced NF-κB activation by PA

NF-κB is an important transcriptional regulator of inflammatory cytokines and it plays a crucial role in immune responses [7]. To determine if PA would inhibit the expression of the pro-inflammatory mediators through suppression of NF-κB activation, we examined the regulatory effect of PA on LPS-induced nuclear translocation of the cytosolic NF-κB p65 subunit by immunostaining (Figure 8A). As expected, treatment of LPS stimulated nuclear translocation of p65 (Figure 7A, LPS). However, treatment of PA markedly suppressed the LPS-induced NF-κB p65 nuclear translocation (Figure 7A, PA +LPS). Since nuclear translocation of NF-κB was preceded by the degradation of IκB [23], we next examined the effect of PA on LPS-induced IκB degradation by Western blot analyses. As demonstrated in Figure 8B, stimulation of RAW 264.7 macrophages with 100 ng/ml LPS induced a rapid degradation of cytosolic IκB protein within 10 to 20 min; this effect was drastically blocked by the treatment with PA (0.5 mg/ml).

4. Discussion

In Taiwan, *Plectranthus amboinicus* (PA) is a common folk medicine for summer cold, scald, wounds, and bites from bugs or mosquitoes. The PA extract (10 g/kg, po) did not produce any death or behavioral changes in the treated mice (data not shown). However, the scientific theories behind these therapeutic effects are still unclear. Here we report new insights into the functions and possible mechanisms of PA, including 1) its analgesic ability demonstrated by two different analgesic test methods: acetic acid-induced writhing response and formalin test, 2) its anti-inflammatory ability demonstrated by decreasing the swelling of carrageenan-induced mice paw edema and the levels of pro-inflammatory mediators (TNF-α, and COX-2), 3) its antioxidant ability demonstrated by increased SOD and GRx levels and decreased MDA level, (Notably, PA does not increase the level of GPx like the indomethacin does.) and 4) possible mechanisms of its anti-inflammatory activities.

The analgesic ability of PA was evaluated using two different animal models. Intraperitoneal injection of acetic acid causes an increase of prostaglandins in peritoneal fluids such as PGE_2 and $PGF_{2\alpha}$, serotonin, and histamine involved in part, which was a model commonly used for screening peripheral analgesics [24]. The formalin test is a tonic model of continuous pain resulting from formalin-induced tissue injury. It is a widely used model, particularly for the screening of novel compounds, since it encompasses inflammatory, neurogenic, and central mechanisms of nociception [25]. The results showed that the PA considerably inhibited acetic acid-induced writhing in mice (Figure 2) and the late-phase pain response, not the neurogenic (early-phase) pain, caused by intraplantar injection of formalin (Figure 2). Such results suggested that *Plectranthus amboinicus* possessed remarked analgesic activity.

Carrageenan-induced paw edema in mice has been accepted as a useful phlogistic tool for investigating anti-inflammatory agents. There are biphasic effects in carrageenan-induced edema [22]. The early hyperemia results from the release of histamine and serotonin and the delayed phase of carrageenan-induced edema results mainly from the potentiating effects of bradykinin on mediator release, and of prostaglandins producing edema after the mobilization of leukocytes. According to Figure 4A, PA showed effectively inhibitory activity on carrageenan-induced paw inflammation over a period of 4 h at the dose of 1.0 g/kg, which was comparable to that of indomethacin, which indicated its action against neutrophils migration and release of histamine, serotonin and kinins in early phase, and prostaglandin in later phase. Furthermore, considering the crucial role of COX-2 expression and cytokines production in the progress of inflammation in injury area, COX-2 and TNF-α content were also examined in this study. COX-2 is an inducible enzyme found in activated inflammatory cells that creates prostanoid mediators. Inhibition of COX-2 protein expression has also become the most popular and valid method for studying anti-inflammatory effects both in *in vivo* and *in vitro* models [8]. TNF-α a key mediator in inflammatory response, stimulates innate immune responses by activating T cells and macrophages that stimulate the release of other inflammatory cytokines. TNF-α is also a mediator of carrageenan-induced inflammation, and is able to enhance the further release of kinins and leukotrienes, which is suggested to have an important role in the maintenance of long-lasting nociceptive response [26]. The production of TNF-α in edema paw tissues induced by carrageenan was decreased by PA treatment (Figure 4C). PA also significantly restrained the protein expression of COX-2 in the edema paw tissues of mice (Figure 4D). Therefore, such results revealed that PA displayed significantly anti-inflammatory activities in the model of carrageenan-induced paw edema of mice, *via* inhibiting vascular permeability, which might be related to the reduction of COX-2 and TNF-α.

The carrageenan-induced inflammatory response has been linked to the neutrophil infiltration, the release of neutrophil-derived mediators, and as well as the production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide, and hydroxyl radicals, [27] and the production of MDA is due to the attack of plasma membranes by free radicals [28, 29]. Previous studies consider that endogenous glutathione plays an important role against carrageenan-induced local inflammation [30]. Glutathione is a known oxyradical scavenger and the enhancement of glutathione levels favor reducing MDA level [31]. In this study, significantly increase in SOD and GRx activities with PA treatment was found (Figure 5); contemporaneously, there was a significant decrease in MDA level with PA treatment (Figure 4B). We assume that the suppression of MDA production is probably due to the increase of SOD and GRx activities.

Finally, LPS-stimulated NO and TNF-α release from RAW 264.7 macrophages was used to evaluate the mechanism of the aqueous extract of PA *in vitro*. As shown in Figure 6, PA potently and dose-dependently inhibited the elevation of TNF-α and NO level induced by LPS in macrophages which further proved the anti-inflammatory activities of PA. Furthermore, we examined the levels of iNOS and COX-2, as shown in Figure 7, the protein levels of iNOS and COX-2 induced by LPS were drastically blocked by pre-treatment with PA.NF-κB 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. NF-κB subunits (p65 and/or p50) are normally sequestered in the cytosol as an inactive complex by binding to its inhibitory factor, IκB-in un-stimulated cells. Upon stimulation of pro-inflammatory signals including LPS, IκB is phosphorylated by IκB- kinase (IKK) and inactivated through ubiquitin-mediated degradation. The resulting free NF-κB is translocated into the nucleus and acts as a transcription factor. As shown in Figure 8, the treatment with PA effectively blocks the degradation of IκB and activation of NF-κB in RAW 264.7 macrophages stimulated by LPS. Therefore, these results suggest that PA inhibits the expression of iNOS and COX-2, and thus NO production through inactivation of NF-κB by reducing IκB- degradation. These *in vitro* findings were well correlated with the *in vivo* anti-inflammatory effects of PA.

Inhibition of inflammatory cytokine and mediator production or function serves as a key mechanism in the control of inflammation, and agents that suppress the expression of these inflammation-associated genes have therapeutic potential in the treatment of inflammatory diseases. However, the most common used non-steroidal anti-inflammatory drugs can cause gastric erosions, exacerbate asthma and cause kidney and liver damages. Therefore, natural products have attracted interest as potential therapeutic agents for the treatment of inflammation.

Separation and determination of active chemical constituents are generally recommended for standardization and quality control of herbal products[16]. Moreover, identification of the major compound in an herb or herbal extract could elucidate pharmacological activity of the herbal extract. There are a lot of essential oil in PA, such as Δ-3-carene, γ-terpinene, camphor, and carvacrol [32]. Phytochemical analysis shows the presence of carvacrol in the PA. The concentration of carvacrol in the PA was 1.87 mg/g extract. Previous studies demonstrated that carvacrol possess significant anti-inflammatory activities [33]. Thus, the anti-inflammatory activity of PA may relate to carvacrol in PA.

In this study, PA clearly showed its analgesic and anti-inflammatory activities. The mechanisms by which PA its analgesic as well as anti-inflammatory effect are correlated with the inhibition of iNOS and COX-2 expression via inactivation of NF-κB, and this serves as a possible rationale for the use of *Plectranthus amboinicus* (Lour.) Spreng. in traditional medicine for anti-inflammation.

Figure Legend

Figure 1. Representative HPLC chromatograms of carvacrol(R_t=15.060) and PA.

Figure 2. Analgesic effect of the aqueous extract of *Plectranthus amboinicus* (PA) on acetic acid-induced writhing response in mice. Indomethacin (Indo 10 mg/kg) was used as a positive control. The number of muscular contractions was evaluated as described in *Materials and Methods*. Each value represents as mean± SEM (n=10). **p< 0.01, ***p< 0.001 as compared with the acetic acid-treated only group.

Figure 3. Effect of the aqueous extract of *Plectranthus amboinicus* (PA) on the (A) early phase and (B) late phase in formalin test in mice. The index of pain (early phase and late phase) was evaluated as described in *Materials and Methods*. Each value represents as mean ± SEM (n=10). *p< 0.05, ***p< 0.001 as compared with the formalin-treated only group.

Figure 4. Inhibitory effects of the aqueous extract of *Plectranthus amboinicus* (PA) on carrageenan-induced mice paw edema and inflammation. (A) Inhibitory effects of PA on carrageenan-induced mice paw edema. Delta volume (ΔV) represents the degree of swelling of carrageenan-treated paw. The (B) MDA concentration and the levels of (C) TNF-α and (D) COX-2, showing as percentage, were presented as mean ± SEM (n=10). *p< 0.05, ***p< 0.001 as compared with the carrageenan-treaed only group.

Figure 5. Antioxidant abilities of the aqueous extract of *Plectranthus amboinicus* (PA) on carrageenan-induced mice. Liver tissues from carrageenan-treated mice were used to analyze the activities of (A) superoxide dismutase (SOD), (B) glutathione reductase (GRx), and (C) glutathione peroxidase (GPx). All values represent as means ± SEM (n=10). *p< 0.05, ***p< 0.001 as compared with the carrageenan-treaed only group.

Figure 6. The aqueous extract of *Plectranthus amboinicus* (PA) inhibited LPS-induced pro-inflammatory cytokine and mediator productions. RAW 264.7 cells were pretreated with PA (0.1-0.5 mg/ml) for 30 min, and then stimulated with LPS (100 ng/ml). Culture media were collected at 24 h for TNF-α and Nitrite analysis. (C) Cell viability in PA -treated cells was evaluated using the MTT assay. The results are displayed in percentage

of control samples. Data are presented as mean ± SEM (n=3) for three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001 as compared with the LPS treatment.

Figure 7. The aqueous extract of *Plectranthus amboinicus* (PA) decreased the protein levels of iNOS and COX-2 in LPS-stimulated macrophages. RAW 264.7 cells were pretreated with PA (0.25-0.5 mg/ml) for 30 min, and then stimulated with LPS (100 ng/ml) for 8-12 h. -actin was used as an internal loading control.

Figure 8. Prevention of LPS-induced NF-κB activation by aqueous extract of *Plectranthus amboinicus* (PA). (A) PA inhibits LPS-induced nuclear translocation of NF-κB p65 subunit. The subcellular localization of NF-κB p65 subunit was detected by immunofluorescence with an antibody specially against p65 as described in *Materials and methods*. The same fields were counter stained with DAPI for location of nuclei. (B) PA blocks LPS-induced IκB degradation. Protein extracts were separated by SDS-PAGE followed by Western blot analyses antibody specially against IκB. β-actin was used as an internal loading control.

Figure 1

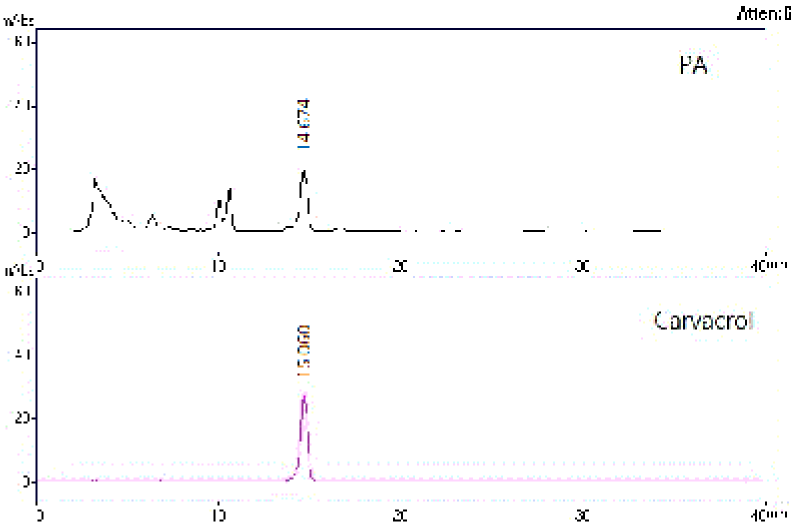


Figure 2

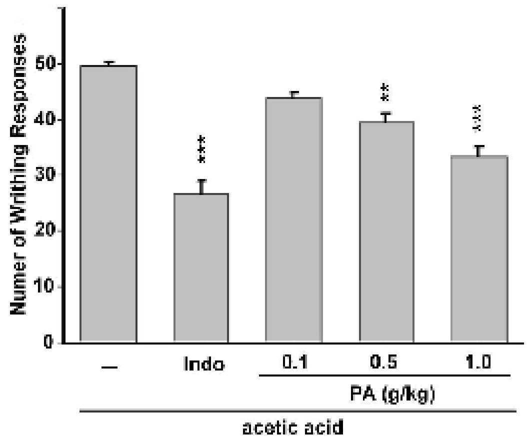

Numer of Writhing Responses
0
10
20
30
40
50
***
**
***
—
Indo
0.1
0.5
1.0
PA (g/kg)
acetic acid

Figure 3

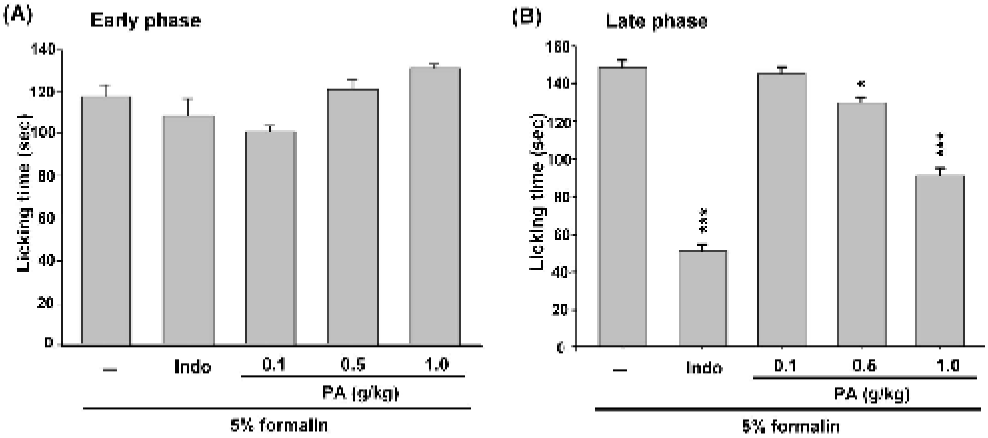
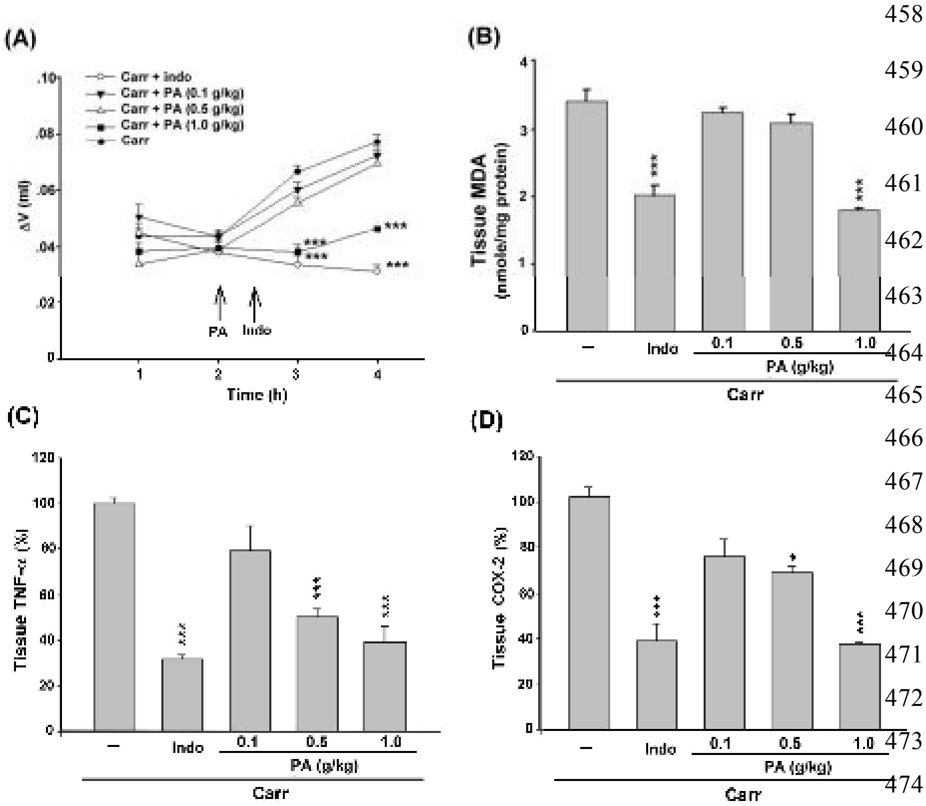

(A)
Early phase
Licking time (sec)
140
120
100
80
60
40
20
0
—
Indo
0.1
0.5
1.0
PA (g/kg)
5% formalin
(B)
Late phase
Licking time (sec)
160
140
120
100
80
60
40
20
0
***
*
***
—
Indo
0.1
0.5
1.0
PA (g/kg)
5% formalin

Figure 4



(A)
Carr + indo
Carr + PA (0.1 g/kg)
Carr + PA (0.5 g/kg)
Carr + PA (1.0 g/kg)
Carr
ΔV (ml)
***
PA
Indo
Time (h)
(B)
Tissue MDA
(nmole/mg protein)
Indo
0.1
0.5
1.0
PA (g/kg)
Carr
(C)
Tissue TNF-α (%)
Indo
0.1
0.5
1.0
PA (g/kg)
Carr
(D)
Tissue COX-2 (%)
Indo
0.1
0.5
1.0
PA (g/kg)
Carr

Figure 5

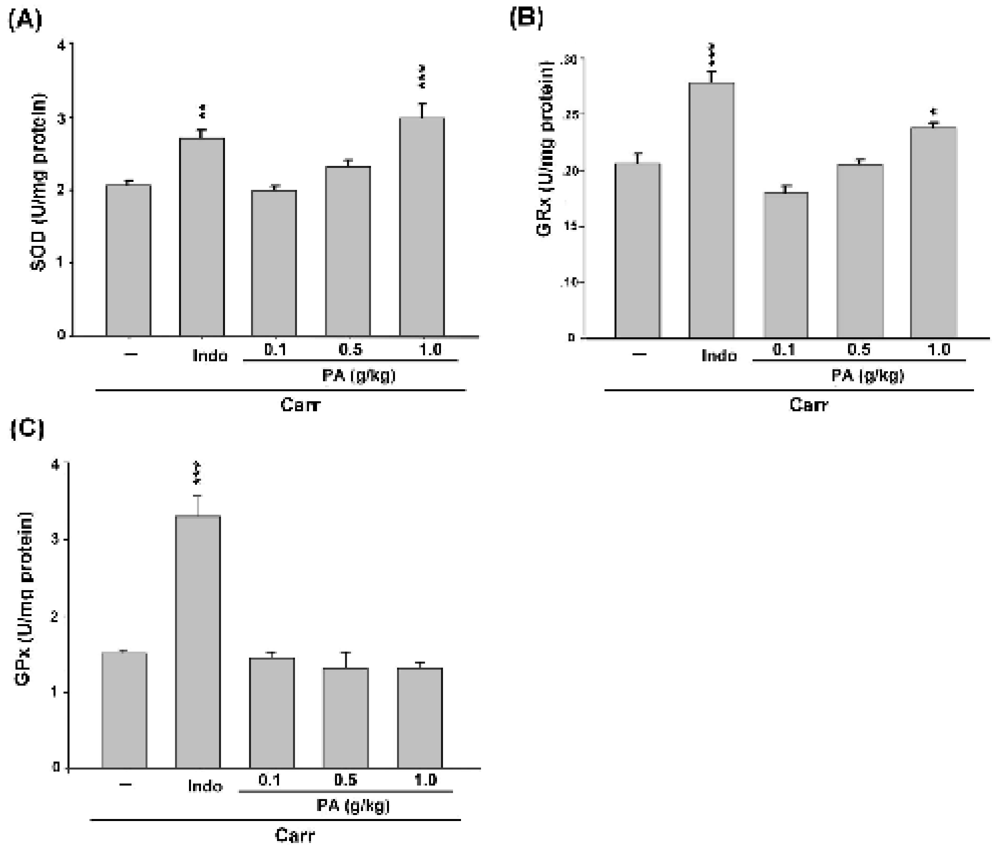
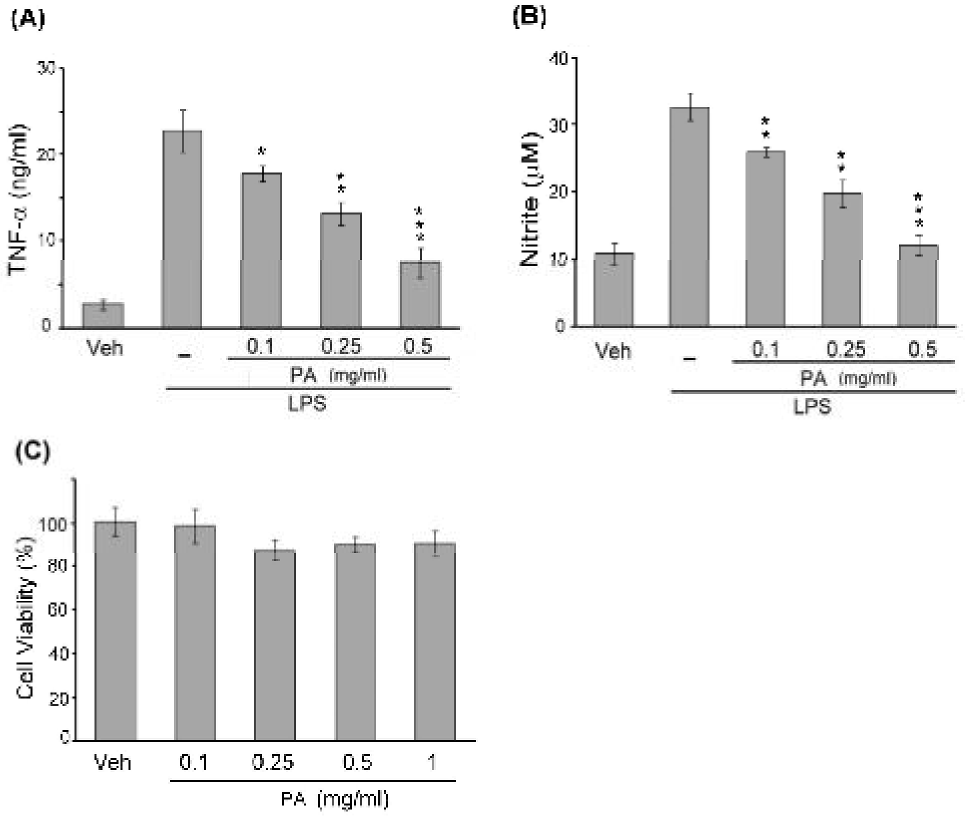

(A)
SOD (U/mg protein)
(B)
GRx (U/mg protein)
(C)
GPx (U/mg protein)
Indo
0.1
0.5
1.0
PA (g/kg)
Carr

Figure 6



(A)
TNF-α (ng/ml)
30
20
10
0
Veh
–
0.1
0.25
0.5
PA (mg/ml)
LPS
(B)
Nitrite (μM)
40
30
20
10
0
Veh
–
0.1
0.25
0.5
PA (mg/ml)
LPS
(C)
Cell Viability (%)
100
80
60
40
20
0
Veh
0.1
0.25
0.5
1
PA (mg/ml)

Figure 7

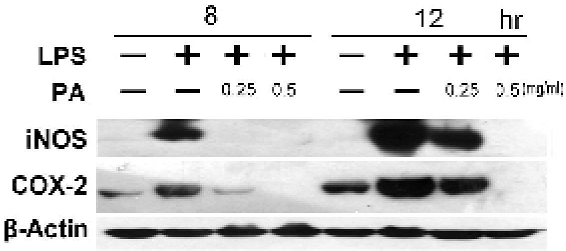
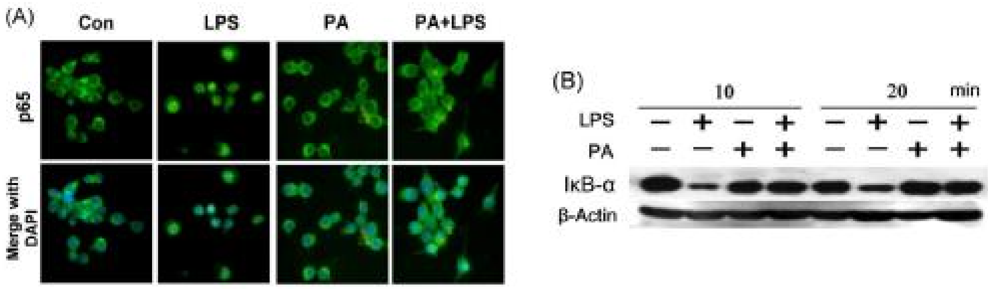


Figure 8


(A)
Con
LPS
PA
PA+LPS
p65
Merge with DAPI
(B)
10
20
min
LPS
PA
IκB-α
β-Actin

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