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Inhibition of Experimental Lung Inflammation and Bronchitis by Phytoformula Containing *Broussonetia papyrifera* and *Lonicera japonica*

Hyun Jeong Ko[1], Jeong Ho Jin[1], Oh Song Kwon[1], Jong Taek Kim[2], Kun Ho Son[3] and Hyun Pyo Kim[1,*]

[1]College of Pharmacy, [2]College of Veteniary Medicine, Kangwon National University, Chuncheon 200-701, [3]Deparment Food and Nutrition, Andong National University, Andong 760-749, Republic of Korea

Abstract

Broussonetia papyrifera and *Lonicera japonica* have long been used in the treatment of inflammatory disorders, especially respiratory inflammation, in Chinese medicine. Previously, phytoformula (BL) containing *B. papyrifera* and *L. japonica* was found to exert strong anti-inflammatory activity *in vitro* and *in vivo*. In this study, the effects of BL on lung inflammation including bronchitis were examined *in vitro* and *in vivo.* BL (10-100 μg/ml) inhibited nitric oxide (NO) production of lipopolysaccharide (LPS)-treated alveolar macrophages, MH-S cells, primarily by down-regulating inducible NO synthase. BL also inhibited production of the proinflammatory cytokines, TNF-α and IL-6. Against an animal model of pleural cavity inflammation, BL (200-400 mg/kg) significantly inhibited 5 h and 24 h carrageenan-induced pleurisy in rats when administered orally. Additionally, BL inhibited experimental bronchitis induced by intratracheal instillation of LPS to rats. Taken together, these results indicate that BL may be effective for the treatment of human lung inflammation as well as bronchitis.

Key Words: *Broussonetia papyrifera*, *Lonicera japonica*, Lung inflammation, Bronchitis

INTRODUCTION

Many patients suffer from lung inflammatory disorders including asthma, bronchitis and chronic obstructive pulmonary disorders (COPD). Several classes of drugs including bronchodilators, antitussives, steroids and leukotriene (LT) receptor antagonists have been used to treat these disorders. However, these drugs sometimes do not provide fundamental care, even though the major symptoms are alleviated (Jeffery, 2001). Thus, new effective drugs with novel action mechanism(s) are needed to treat lung inflammatory disorders. In this regard, natural products may be applied safely for treating human lung inflammation and bronchitis.

The root barks of *Broussonetia papyrifera* (L.) Vent. (Moraceae) distributed in China, Japan and Korea have been used as anti-inflammatory and antitussive agents in traditional Chinese medicine, especially for the treatment of older people having bronchitis (Editorial Committee, 1999). Recently, the 95% ethanol extracts of the radix, stems, leaves and fruits of *B. papyrifera* showed antinociceptive and anti-inflammatory activity in vivo, with radix having the strongest activity (Lin *et al.*, 2008). *Lonicera japonica* (Thunb.) (Caprifoliaceae) is a twining shrub that has long been used to treat urinary disorders, fevers, and headache (Shougakukan, 1985). Additionally, the whole plant of *L. japonica*, which is widely distributed widely in China and Korea, has frequently been used as an anti-inflammatory agent, especially for the treatment of respiratory tract inflammation (Lee *et al.*, 1998). Recently, it was also reported that highly purified fraction showed potent anti-inflammatory and analgesic activities (Ryu *et al.*, 2010). It has previously been reported that a 1:1 (w/w) mixture (BL) of the ethylacetate fraction from the root barks of *B. papyrifera* and the 70% aqueous ethanol extract from the whole plants of *L. japonica* exerted significant and considerable anti-inflammatory activity in vitro and in vivo (Jin *et al.*, 2010). BL exerted anti-inflammatory activity against λ-carrageenan-induced paw edema and arachidonic acid-induced ear edema. Moreover, BL also showed analgesic activity. The cellular mechanisms of these anti-inflammatory activities of BL include 5-lipoxygenase (5-LOX) inhibition and inhibition of proinflammatory enzyme induction such as inducible nitric oxide synthase (iNOS).

In the present study, inhibitory effects of BL against experimental lung inflammation and bronchitis were examined to establish its potential for use as a new therapeutic agent against

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***Corresponding Author**

E-mail: hpkim@kangwon.ac.kr
Tel: +82-33-250-6915, Fax: +82-33-255-7865

lung inflammatory disorders, especially for treating bronchitis.

MATERIALS AND METHODS

Chemicals

2-Amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT) was purchased from Tocris Cookson Ltd. (UK). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), indomethacin, prednisolone, λ-carrageenan (CGN) and LPS (*Escherichia coli* 0127:B8) were purchased from Sigma Chem. (St. Louis, MO). Zileuton (5-LOX inhibitor) and montelukast (LT receptor antagonist) were purchased from Tocris Bioscience and Hwail Pharm. Co., Ltd. DMEM and other cell culture reagents including FBS were products of Gibco BRL (Grand Island, NY). Protein assay kit was purchased from Bio-Rad Lab. (Hercules, CA).

Animals

Male Sprague-Dawley (SD) rats (4 weeks old, specific pathogen-free) were obtained from Orient-Bio Ltd. (Korea). Animals were fed with standard lab. chow and water ad libitum. The animals were maintained in an animal facility (KNU) at 20-22°C under 40-60% relative humidity and a 12 h/12 h (light/dark) cycle for at least 7 days prior to the experiment. The experimental design using the animals was approved by the local committee for animal experimentation, KNU (KIACUC-09-0012). In addition, the ethical guidelines described in the KFDA Guide for the Care and Use of Laboratory Animals were followed throughout the experiments.

Plant materials

B. papyrifera collected in southern China were obtained from Songlim Pharm. Co., Ltd. (Seoul, Korea) and the plant materials were authenticated by one of the authors, Dr. K. H. Son (Andong National University, Korea) and a voucher specimen was deposited in Andong National University. The dried root barks of *B. papyrifera* were chopped and extracted with ethanol. The ethanol extract was partitioned with ethylacetate and water. The ethylacetate fraction was then dried under vacuum and this dried fraction was used to prepare BL as below. The final yield of this ethylacetate fraction in one typical extraction and fractionation procedure was 7.77% (w/w). The major constituents, papayriflavonol A and broussochalcone A, were isolated according to the previously published procedures (Son *et al.*, 2001). For determination of the contents of major constituents, HPLC analysis was carried out. Alliance VWD separation module (Waters) and Inertsil ODS4 column (4.6×150 mm, 5 μm) were employed using the mobile phase of tetrahydrofuran:water, pH 3.0 (47:53). The flow rate was 0.6 ml/min and the peaks were detected at UV 390 nm. From the HPLC analysis, papyriflavonol A and broussochalcone A were found to be 1.35% and 2.58% (w/w), respectively as shown in Fig. 1.

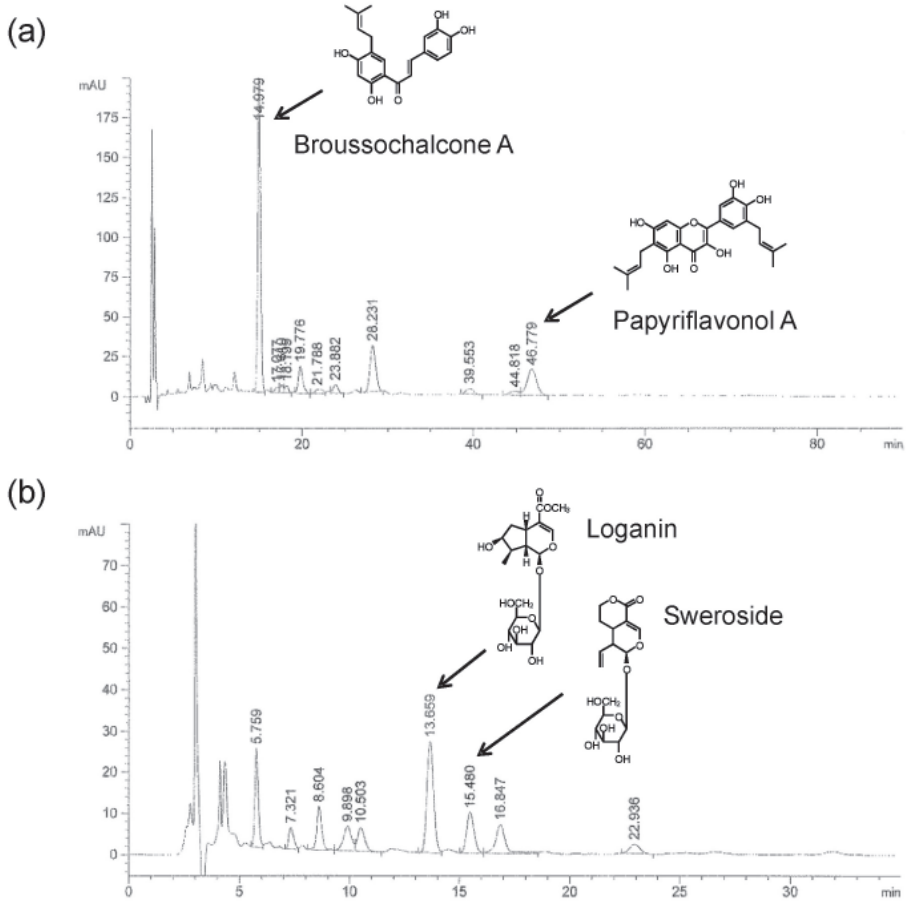

Fig. 1. Chemical structures of the major constituents in *B. papyrifera* and *L. japonica* and HPLC analysis of the extracts. (a) HPLC chromatogram of *B. papyrifera*, (b) HPLC chromatogram of *L. japonica*. All HPLC analytical conditions were described in Materials and methods.

L. japonica cultivated in southern China was purchased from Songlim Pharm. Co., Ltd. and this plant material was authenticated by Dr. K. H. Son and a voucher specimen was deposited in Andong National University. The dried whole plant was then chopped and extracted with 70% aqueous ethanol. The ethanol extract was then dried under vacuum and this dried extract was used to prepare BL. The yield of the ethanol extract in one typical extraction procedure was 9.74% (w/w). The major constituents, loganin and sweroside, were isolated as previously described (Kawai *et al.*, 1988). HPLC analysis using the mobile phase of acetonitrile:water, pH 3.0 (13:87) was performed. The flow rate was 0.6 ml/min and the peaks were detected at UV 239 nm. In this extract, loganin and sweroside were found to be 4.19% and 3.30%, respectively. BL is a (1:1, w/w) mixture of the ethylacetate fraction of *B. papyrifera* and the ethanol extract of *L. japonica* and used throughout this study.

MH-S cell culture and measurement of nitric oxide (NO), TNF-α and IL-6

MH-S cells, a mouse alveolar macrophage cell line, obtained from American type culture collection (ATCC, Rockville, VA) were cultured with DMEM supplemented with 10% FBS and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a 5% CO_2 atmosphere at 37°C. The cells were activated with lipopolysaccharide (LPS) as previously described (Chi *et al.*, 2001a). Briefly, cells were plated in 96-well plates (2×10^5 cells/well). After pre-incubation for 2 h, test compounds and LPS (1 mg/ml) were added and incubated for 24 h unless otherwise specified. Media was collected and NO, TNF-α and IL-6 concentrations were determined. To assess NO production, the stable conversion product of NO, nitrite (NO_2^-), was measured using Griess reagent and the optical density was determined at 550 nm. The concentrations of TNF-α and IL-6 were measured using ELISA kit (Assay Design Inc.) according to the manufacturer's recommendation. The cell viability was checked using an MTT bioassay as previously described (Mossman, 1983). At all concentrations tested, BL and AMT did not show cytotoxic effect on MH-S cells.

Western blot analysis

To measure the protein level of inducible NO synthase (iNOS), Western blotting technique was used (Chi *et al.*, 2001a). MH-S cells were cultured in 6-well plates (5×10^6 cells/well) in the presence or absence of LPS (1 μg/ml) with/without test compounds for 16-20 h. After cell homogenates were prepared, the supernatant was obtained by centrifugation at 15,000 g for 30 min. Using Tris-glycine gels (8%), electrophoresis was carried out and bands were blotted to PVDF membranes. The membranes were incubated with iNOS antibody (N32030, Transduction Lab.) and the bands were visualized by chemiluminescent reagent (Amersham, UK).

CGN-induced pleurisy

According to the procedure of Schrier *et al.* (1990), 0.2 ml of 1% CGN solution in sterile saline was injected intrapleurally to rats. The test compounds were orally administered 1 h prior CGN injection. The animals were sacrificed 5 or 24 h after CGN injection. The chest was opened by lateral incision. DMEM (3 ml) with 10% FCS was injected into pleural cavity and pleural fluid was aspirated using Pasteur pipette. This washing procedure was repeated again and exudates were combined. After the volumes of pleural exudates were recorded, cell pellets were obtained by centrifugation at 200 g for 5 min. Total cell numbers were counted after RBCs were lysed with hemolytic solution (0.85% NH_4Cl, 0.1 M Hepes).

Bronchitis induced by LPS instillation

For inducing bronchitis, the modified method of Starcher and Williams (1989) was employed. For intratracheal instillation, rats were anesthetized by tribromoethanol and LPS (*E. coli* 0127:B8, 2 mg/ml saline) was instilled directly to lung (100 μl/rat), using a microsprayer (Intratracheal aerosolizer, Penn-Century, Inc., USA). Rats were maintained in up-right position at least for 5 min. Sixteen hours later after LPS instillation, the animals were sacrificed and bronchoalveolar lavage fluid (BALF) was collected via intratracheal cannulation after 200 μl infusion of saline. BALF collected were approximately 150 μl/rat. For histology and other biochemical analysis, lungs were excised. Test compounds including BL were administered one hour prior to LPS instillation. For measuring proinflammatory cytokine concentration, reverse transcription-polymerase chain reaction (RT-PCR) analysis was carried out. In brief, total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) from lung tissue and cDNA was synthesized by Superscript II reverse transcriptase with oligo (dT) primer (Invitrogen). The forward and reverse primers used for PCR amplification of IL-1β and GAPDH from the lung of rats were as follows: IL-1β forward, 5'-GCA GCT ATG GCA ACT GTC C-3', and IL-1β reverse, 5'-GGT CAG ACA GCA CGA GGC-3', which amplified a 420 base pair sequence; COX-2 forward, 5'-GCA AAT CCT TGC TGT TCC AAT C-3', and COX-2 reverse, 5'-GGA GAA GGC TTC CCA GCT TTT G-3', which amplified a 335 base pair sequence, and GAPDH forward, 5'-GCC ATC AAC GAC CCC TTC AT-3', and GAPDH reverse, 5'-CGC CTG CTT CAC CAC CTT CT-3', which amplified a 702 base pair sequence of rat GAPDH. RT-PCR was performed using the MyCycler Thermal Cycler (Bio-Rad Lab., UK) and thermal cycler conditions were as follows: denaturation, annealing, and extension at 98, 55, and 72°C for 10 sec, 30 sec, and 1 min, respectively, for 30 cycles using Ex Taq (TaKaRa, Shiga, Japan). PCR products were electrophoresed on 1.2% agarose gel containing ethidium bromide and visualized using Gel Doc system (Bio-Rad Lab.).

Statistical analysis

Experimental values were represented as arithmetic mean ± SD. One way ANOVA followed by Dunnett's test was used to determine the statistical significance.

RESULTS

In vitro study

Macrophages induce iNOS when stimulated with LPS. LPS treatment (1 μg/ml) of MH-S cells produced high amounts of NO as well as the proinflammatory cytokines, TNF-α and IL-6. After 24 h of incubation, a significant amount of NO was produced (27.9 ± 1.2 μM) over the basal level of 2.7 ± 0.1 μM (n=3). Under this condition, BL reduced NO production in a concentration-dependent manner at 10-100 μg/ml (Fig. 2a). The IC_{50} value was calculated to be 102.5 μg/ml. The reference compound, AMT (NOS inhibitor), potently inhibited NO production (59.3% at 1 μM). BL also reduced TNF-α and IL-6

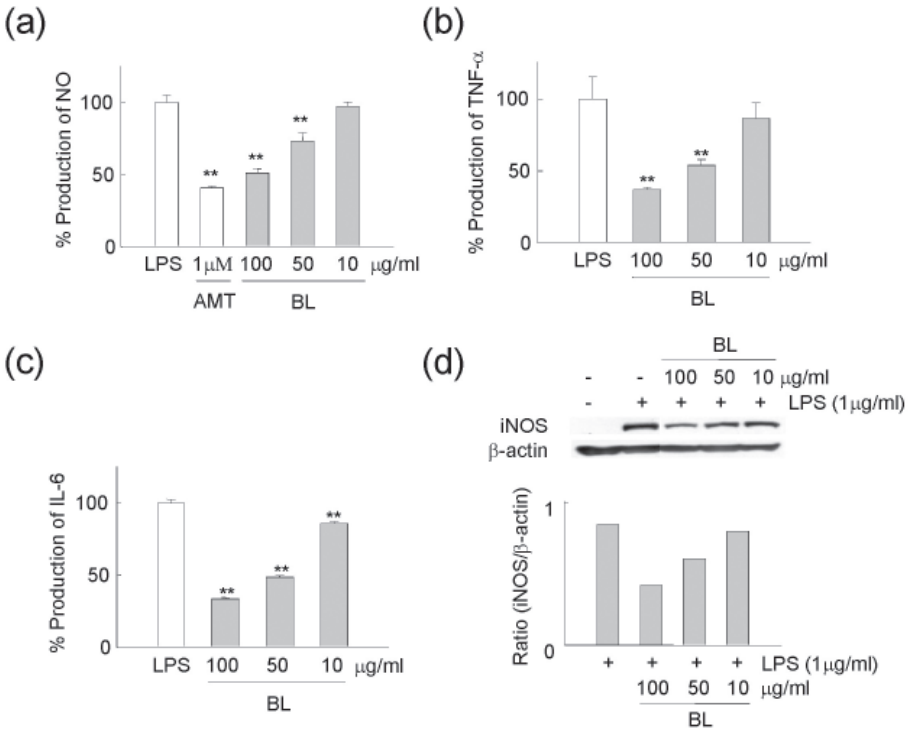

Fig. 2. Effects of BL on NO, TNF-α and IL-6 production from LPS-treated MH-S cells (a) Inhibition of NO production, (b) Inhibition of TNF-α production, (c) Inhibition of IL-6 production, (d) Western blotting analysis. **: $p<0.01$, Significantly different from the LPS-treated control group (n=3).

Table 1. Inhibition of CGN-induced pleurisy in rats (5 h)

Compounds	Dose (mg/kg)	Exudate volume (ml)	Total cell number (×10^7)
Vehicle	–	5.20 ± 0.05[a] (–)[b]	0.63 ± 0.01 (–)
CGN-treated	–	7.0 ± 0.12 (–)	9.85 ± 0.83 (–)
Indomethacin	20	6.38 ± 0.06[c] (34.5)	6.20 ± 0.78[d] (39.6)
BL	200	6.83 ± 0.17 (9.4)	7.33 ± 0.18[c] (27.3)
	400	6.40 ± 0.07 (33.3)	6.97 ± 1.04[d] (31.2)

All compounds dissolved in DMSO were administered orally. [a]n=5 (arithmetic mean ± SD), [b]% Inhibition based on the formula: 1−[(Test−vehicle control)/(CGN-treated−vehicle control)×100], [c]$p<0.05$, [d]$p<0.01$. Significantly different from the CGN-treated control group.

Table 2. Inhibition of CGN-induced pleurisy in rats (24 h)

Compounds	Dose (mg/kg)	Exudate volume (ml)	Total cell number (×10^7)
Experiment 1			
Vehicle	-	5.40 ± 0.24[a] (–)[b]	0.28 ± 0.30 (–)
CGN-treated	-	6.26 ± 0.28 (–)	15.66 ± 4.70 (–)
Prednisolone	20	5.87 ± 0.21[c] (45.3)	6.37 ± 0.71[d] (60.4)
Indomethacin	20	6.60 ± 0.99 (–53.5)	9.37 ± 1.20[c] (40.9)
BL	200	6.06 ± 0.17 (23.3)	13.50 ± 2.97 (14.0)
	400	5.94 ± 0.63 (37.2)	10.26 ± 6.83 (35.1)
Experiment 2			
Vehicle	-	5.50 ± 0.08 (–)	0.18 ± 0.21 (–)
CGN-treated	-	6.64 ± 0.55 (–)	17.44 ± 1.18 (–)
Zileuton	50	6.52 ± 0.42 (10.5)	13.48 ± 6.49 (23.0)
BL	400	6.44 ± 0.21 (17.5)	12.68 ± 1.84[d] (2.76)

All compounds dissolved in DMSO were administered orally. [a]n=5 (arithmetic mean ± SD), [b]% Inhibition based on the formula: 1−[(Test−vehicle control]/(CGN−treated-vehicle control)×100]. [c]$p<0.05$, [d]$p<0.01$. Significantly different from the CGN-−treated control group.

production at the same concentration ranges (Fig. 2b and 2c), with IC_{50} values of 61.7 and 48.4 μg/ml, respectively. To elucidate the inhibitory mechanism of iNOS-induced NO production, Western blotting analysis was carried out. As shown in Fig. 2d, BL clearly inhibited iNOS induction in LPS-treated MH-S cells, indicating that the NO inhibitory activity of BL may be mediated, at least in part, by iNOS down-regulation.

In vivo study

To elucidate the in vivo activity against animal models of lung inflammation, 5 h and 24 h CGN-induced pleurisy tests were employed. Injection of CGN into the pleural cavity resulted in the development of pleurisy as determined by increased exudate volume and recruitment of inflammatory cells including neutrophils and macrophages; neutrophils are the major cells recruited in the early time while macrophages are the major cells recruited in the later time (24 h) of CGN injection (Tomlinson *et al.*, 1994; Willis *et al.*, 1996). As expected, the exudate volumes of the pleural cavity increased and the cells in the exudates drastically increased in the 5 h model (acute lung inflammation) (Table 1). Under these conditions, BL (400 mg/kg) administered orally led to a considerable reduction in the exudate volume and blocked the recruitment of inflammatory cells. Indomethacin (20 mg/kg) used as a reference drug

also inhibited both parameters, showing slightly higher activity than BL.

Table 2 shows the results of 24 h CGN-induced pleurisy (subacute lung inflammation). BL (200-400 mg/kg) reduced the exudate volume and cell numbers in the exudates. Specifically, BL induced a reduction of 37.2 and 35.1% against these two parameters when administered at 400 mg/kg (experiment 1). Prednisolone used as a reference drug strongly reduced the exudate volume and cell numbers, while indomethacin reduced the total cell numbers but increased the exudate volume. In comparison, zileuton (5-LOX inhibitor) showed some inhibitory action at 50 mg/mg when administered orally, showing weaker inhibitory activity than BL (400 mg/kg) (experiment 2).

Bronchitis as an animal model was induced by LPS instillation in rats. When LPS was directly instilled intratracheally, bronchitis characterized by inflammatory cell infiltration was produced (Fig. 3a). Under these conditions, BL (200-400 mg/kg) significantly reduced cell numbers in BALF (41.2% and 66.7% reduction, respectively). The reference drug, montelukast (30 mg/kg, LT receptor antagonist), also reduced cell numbers (26.9%), but was weaker than BL. Moreover, IL-1β and COX-2 expressed in lung tissue was reduced by BL treatment when measured by RT-PCR analysis (Fig. 3b). Montelukast weakly reduced IL-1β and COX-2 expression. However, IL-6 and TNF-α genes were not detected in control or LPS instilled lung tissue (data not shown). Upon histological observation, a strong reduction of inflammatory response in the lung was observed in the BL-treated group (400 mg/kg) as well as the montelukast-treated group (Fig. 3c).

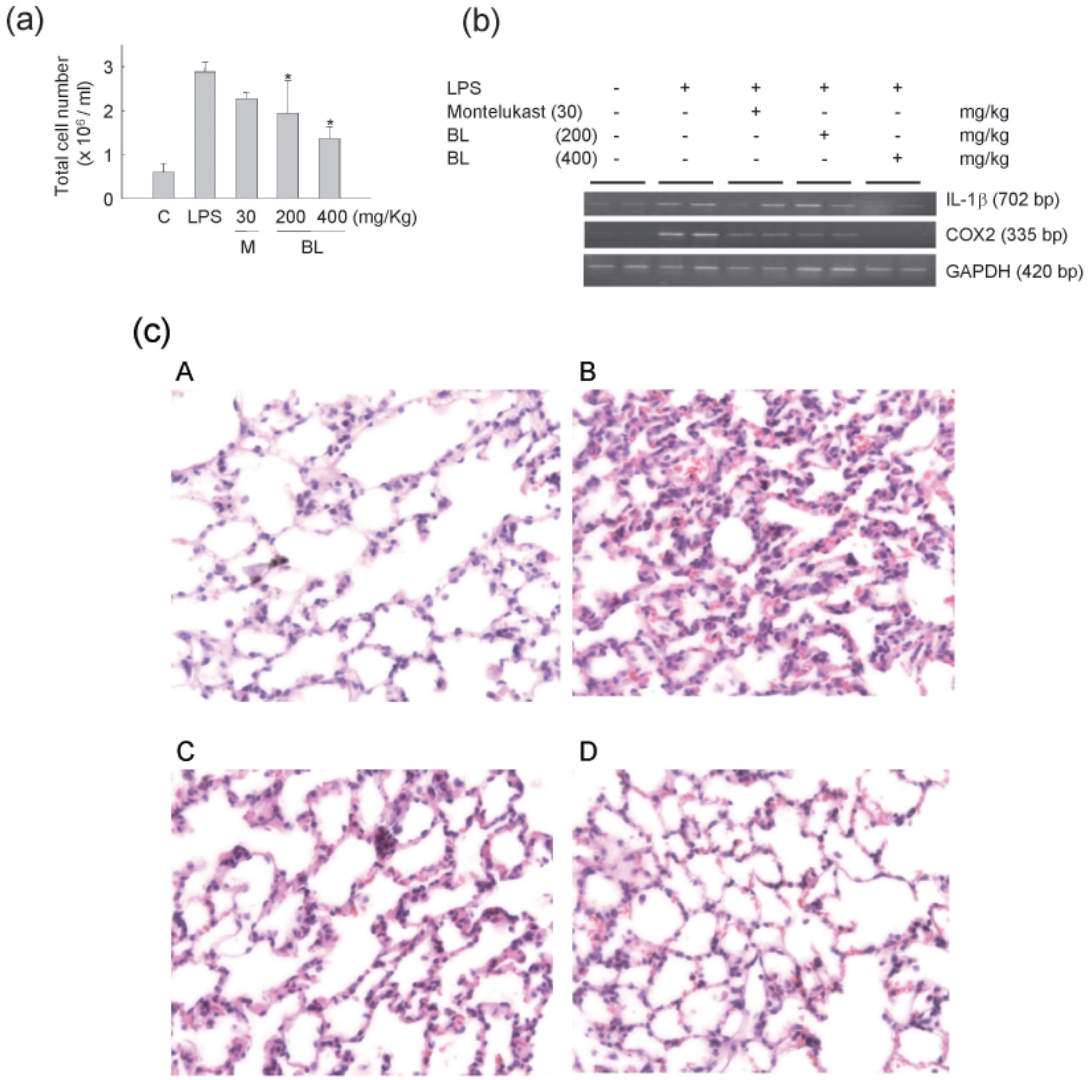

Fig. 3. Effects of BL on bronchitis induced by LPS instillation in rats. (a) Inhibition of cell infiltration, All compounds dissolved in DMSO were administered orally. The volume of BALF collected was approximately 0.15 ml in all groups. Inhibition (%) was calculated based on the formula: 1−[(test−vehicle control)/(LPS−instilled-vehicle control) ×100]. **p*<0.05, significantly different from the LPS-instilled control group (n=3). (b) RT-PCR analysis of IL-1β, (c) Histology, H&E staining (×400). Note: Saline-treated control group (A) showed normal appearance. In LPS-instilled airway, airway inflammation, mucous cell hyperplasia and inflammation-related cell recruitment were observed (B), while all these responses were markedly reduced in the montelukast-treated (30 mg/kg) (C) and BL-treated lungs (400 mg/kg) (D).

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DISCUSSION

The present investigation clearly demonstrated that BL possesses significant anti-inflammatory activity against in vitro and in vivo animal models of lung inflammation, especially bronchitis. BL showed anti-inflammatory activity comparable to currently used drugs. This study presents strong scientific data supporting the potential for the use of BL to treat human bronchitis.

Several prenylated flavonoids including papyriflavonol A and broussochalcone A were successfully isolated from the root barks of *B. papyrifera* as major constituents (Son *et al.*, 2001). Among these, papyriflavonol A is a relatively specific 5-LOX inhibitor (Chi *et al.*, 2001b). Papyriflavonol A has also been found to inhibit allergic responses in vivo (Kwak *et al.*, 2003). Because 5-LOX inhibitors have the potential to treat bronchial asthma as well as bronchitis, BL is thought to inhibit lung inflammation. In addition, broussochalcone A possesses iNOS down-regulating activity (Cheng *et al.*, 2001). Furthermore, *L. japonica* is a well-known anti-inflammatory drug (Lee *et al.*, 1998). This plant material is widely used to treat respiratory inflammation in Korea. Iridoids such as loganin have been isolated from these plant materials as major constituents. These compounds have also been reported to show anti-inflammatory activity (Lee *et al.*, 1995). All of this background information led us to examine the inhibitory activity of BL against lung inflammation, and the results of the present investigation demonstrated that BL is effective for the treatment of animal models of lung inflammation at doses of 200-400 mg/kg.

Our previous study (Jin *et al.*, 2010) showed that BL inhibits 5-LOX and down-regulates iNOS. In the present investigation, in vitro study demonstrated that BL inhibited NO, TNF-α and IL-6 production by alveolar macrophages and that the NO inhibitory action of BL was mediated in part by iNOS down-regulation. These results, in combination with the previous findings, may indicate that BL possesses multiple anti-inflammatory action mechanisms; 5-LOX inhibition, down-regulation of proinflammatory enzymes such as iNOS and inhibition of the production of inflammatory cytokines including TNF-α, IL-1β and IL-6. All these action mechanisms may contribute to the in vivo anti-inflammatory activity of BL.

One biomarker of inflammatory response is inflammation-related cell recruitment in the affected lesion. Although we did not differentially count the cell types, BL clearly inhibited infiltration of inflammatory cells in 5 h and 24 h CGN-induced pleurisy. These results may indicate that BL considerably inhibits lung inflammation of the pleural cavity, regardless of whether it is in the acute or subacute state. In addition, BL reduced cell numbers of BALF in a model of bronchitis. Since more than 90% of the cells in BALF were shown to be neutrophils (Starcher and Williams, 1989), BL is believed to reduce neutrophil infiltration in bronchitis induced by LPS instillation.

In the present investigation, we used indomethacin (COX inhibitor), prednisolone (steroidal anti-inflammatory drug), zileuton (5-LOX inhibitor) and montelukast (LT receptor antagonist) as the reference drugs depending on the in vivo experiments employed. In acute CGN-pleurisy (5 h), indomethacin strongly inhibited inflammatory responses based on cell numbers and pleurisy volume. Conversely, indomethacin increased the exudate volume in subacute CGN-pleurisy (24 h), while the 5-LOX inhibitors, zileuton and BL, inhibited this response. These results are well matched with the previous findings that 5-LOX is important to provoke inflammatory response in CGN-induced pleurisy (Cuzzocrea *et al.*, 2003) and 5-LOX inhibitors are active on this animal model (Batt, 1992). In contrast, indomethacin inhibits COXs; thus, a considerable amount of arachidonic acid may be converted to LTs by 5-LOX, leading to an increase in the exudate volume in the 24 h model. Montelukast (30 mg/kg) showed inhibitory activity against bronchitis induced by LPS instillation, being less active than BL (400 mg/kg) for blocking cell recruitment. In addition, montelukast clearly inhibited IL-1β and COX-2 m-RNA production and alleviated the lung inflammation shown by histological observation. These results are well correlated with the fact that LT receptor antagonist is effective for treating allergic asthma and bronchitis (Jeffery, 2001). When compared with the pharmacological actions of the reference drugs, BL showed broad inhibitory activity against these animal models, pleural cavity inflammation and bronchitis. Therefore, the results of the present study strongly suggest that BL has the potential for treatment of human lung inflammation as well as bronchitis.

In conclusion, the phytoformula, BL (mixture of *B. papyrifera* and *L. japonica*), showed in vitro and in vivo anti-inflammatory activity. BL exerted significant inhibitory activity in animal models of lung inflammation and against bronchitis. Moreover, BL has multiple action mechanisms. Specifically, it inhibits 5-LOX, down-regulates proinflammatory enzymes such as iNOS and inhibits the production of inflammatory cytokines such as TNF-α and IL-6. All of these action mechanisms may participate in the in vivo action of BL. Taken together, these findings indicate that BL has the potential for use in the treatment of human bronchitis.

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