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Inhibition of Experimental Systemic Inflammation (Septic Inflammation) and Chronic Bronchitis by New Phytoformula BL Containing *Broussonetia papyrifera* and *Lonicera japonica*

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Abstract

Broussonetia papyrifera and *Lonicera japonica* have long been used in the treatment of inflammatory disorders in Chinese medicine, especially respiratory inflammation. Previously, a new phytoformula (BL) containing *B. papyrifera* and *L. japonica* was found to exert strong anti-inflammatory activity against several animal models of inflammation, especially against an animal model of acute bronchitis. In the present investigation, the effects of BL on animal models of septic inflammation and chronic bronchitis are examined. Against lipopolysaccharide (LPS)-induced septic inflammation in mice, BL (200-400 mg/kg) reduced the induction of some important proinflammatory cytokines. At 1 h after LPS treatment, BL was found to considerably inhibit TNF-α production when measured by cytokine array. At 3 h after LPS treatment, BL inhibited the induction of several proinflammatory cytokines, including IFN-γ and IL-1β, although dexamethasone, which was used as a reference, showed a higher inhibitory action on these biomarkers. Against chronic bronchitis induced by LPS/elastase instillation in rats for 4 weeks, BL (200-400 mg/kg/day) significantly inhibited cell recruitment in bronchoalveolar lavage fluid. Furthermore, BL considerably reduced lung injury, as revealed by histological observation. Taken together, these results indicate that BL may have a potential to treat systemic septic inflammation as well as chronic bronchitis.

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

INTRODUCTION

Many patients suffer from lung inflammatory disorders, particularly from chronic obstructive pulmonary disorders (COPD). COPD is comprised of asthma, chronic bronchitis, and emphysema, in which several classes of drugs have been used clinically, including bronchodilators, antitussives, steroids and leukotriene (LT) receptor antagonists. However, these drugs sometimes do not provide fundamental care, even though major symptoms may be alleviated (Jeffery, 2001). Thus, there is a continuous and urgent demand for new effective drug(s) with novel mechanism(s) for the treatment of these disorders. Recently, several new candidate classes of drug have undergone clinical trials to validate their effectiveness in treating COPD, and roflumilast (phosphodiesterase 4 inhibitor) has been approved for the treatment of severe COPD (Reid and Pham, 2012). Being relatively safe in the vast majority of cases, natural products are preferable sources of new potential agents for the treatment of human acute and chronic bronchitis.

The root barks of *Broussonetia papyrifera* (L.) Vent. (Moraceae) and the whole plants of *Lonicera japonica* (Thunb.) (Caprifoliaceae) have been used as anti-inflammatory agents in traditional medicine (Lee *et al.*, 1998; Wu, 2012). Each plant material was found to possess anti-inflammatory activity as described previously (Lee *et al*., 1998; Lin *et al*., 2008). Furthermore, it has been proved 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 synergistic anti-inflammatory activity *in vitro* and *in vivo* (Jin *et al*., 2010). This combination has dual cellular mechanisms of 5-lipoxygenase inhibition and down-regulation of several proinflammatory cytokines.

BL is being developed as an anti-bronchitis drug, since leukotrienes, products of 5-lipoxygenase, and some proinflammatory cytokines such as TNF-α and IL-1 are deeply involved in human bronchitis as well as in COPD (Batt, 1992; Barnes,

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Table 1. Effects of BL on cytokine/chemokine induction in septic inflammation (1 h model)

Cytokines	Fold change (1 h model)				
	LPS	BL (200)	BL (400)	Dexamethasone	Montelukast
G-CSF	17.3[a)]	26.5	24.8	36.4*	38.2*
IL-6	98.4	103.8	83.3	62.8*	117.8
IL-10	62.4	75.2	64.5	57.1	76.2
KC	54.0	85.4	42.1	79.7	55.8
MCP-1	25.0	33.8	29.3	71.6*	75.7*
MIP-1α	15.8	16.3	13.7	20.3	28.9*
MIP-2	65.6	80.9	144.2	145.2	285.0*
TNF-α	72.5	58.8	44.1*	20.0*	95.1

Mice were sacrificed 1 h after LPS treatment. All cytokine/chemokine levels were measured using cytokine array (Millipore's MILLIPLEX™). [a)]Arithmatic mean, Here represented the cytokines/chemokines increased more than 25 fold compared to the basal level or those significant inhibited by the test compound(s) after LPS treatment among 32 cytokine/chemokine levels examined. BL: 200-400 mg/kg, Dexamethasone 10 mg/kg, Montelukast: 30 mg/kg. *Significantly increased or decreased compared to those of LPS-treated control group (n=6, p<0.05).

1999). Previously, it was also found that BL inhibited animal models of lung inflammation and acute bronchitis (Ko *et al*., 2011). In carrageenan-induced pleurisy, orally administered BL (200-400 mg/kg) significantly reduced pleurisy volume and inflammatory cell recruitment. In lipopolysaccharide (LPS)-induced acute lung injury, BL clearly inhibited bronchitis, as assessed by the reduction of inflammatory cell recruitment, cytokine expression and histopathological observation of the injured lung tissues. In this study, to further evaluate the therapeutic potential on lung inflammation, pharmacological effects of BL were examined using animal models of septic inflammation and chronic bronchitis.

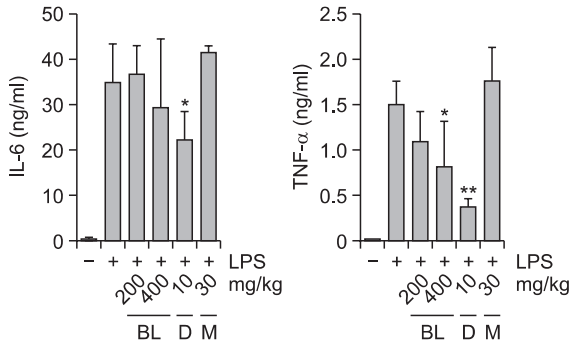

Fig. 1. Effects of BL on cytokine/chemokine induction in septic inflammation (1 h). Mice were sacrificed 1 h after LPS injection and the concentrations of cytokines were measured in the serum. Dexamethasone (D), montelukast (M), *p<0.05, **p<0.01, Significantly different from the LPS-treated control group (n=6).

MATERIALS AND METHODS

Chemicals

Lipopolysaccharide (LPS) from *Salmonella enteritidis* and *Escherichia coli* (026:B6), Dexamethasone and elastase (porcine pancreas) were purchased from Sigma Chem. (St. Louis, MO, USA). Montelukast (LT receptor antagonist) was obtained from Hwail Pharm. Co., Ltd. Protein assay kit was purchased from Bio-Rad Lab. (Hercules, CA, USA).

Animals

Male Sprague-Dawley (SD) rats and C57BL/6J mice (4 weeks old, specific pathogen-free) were obtained from Orient-Bio Ltd. (Korea). Animals were fed with standard 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 and handling of the animals was approved by the local committee for animal experimentation, KNU (KIACUC-09-0012). In addition, the ethical guideline described in the KFDA Guide for the Care and Use of Laboratory Animals was adhered to throughout the experiments.

Plant materials

B. papyrifera and *L. japonica* collected in southern China area were obtained from Songlim Pharm. Co. (Seoul, Korea). These plant materials were authenticated by Prof. K. H. Son (Andong National University, Korea), and voucher specimen were deposited in Andong National University. For preparation of new phytoformula, BL, the dried root barks of *B. papyrifera* were extracted with ethanol and the extract was dried *in vacuo*. The ethanol extract was partitioned with ethylacetate and water. The ethylacetate fraction was then dried and was used to prepare BL as below. By HPLC analysis, the ethylacetate fraction was revealed to contain 1.35% (w/w) papyriflavonol A and 2.58% broussochalcone A as major constituents (Ko *et al*., 2011). The dried whole plant of *L. japonica* was extracted with 70% aqueous ethanol. The ethanol extract was then dried and was used to prepare BL. In this extract, the contents of the major constituents, loganin and sweroside, were found to be 4.19% and 3.30% (Ko *et al*., 2011). BL is the mixture of above plant preparations (1:1, w/w) which was used throughout this study.

Systemic inflammation (Septic shock model) in mice

Systemic septic inflammation was induced in mice by peritoneal injection of a sub-lethal dose of LPS (*S. enteritidis*, 32 mg/kg) in 100 μl sterile saline (n=12). All test compounds

Table 2. Effects of BL on cytokine/chemokine induction in septic inflammation (3 h model)

Cytokines	Fold change (3 h model)				
	LPS	BL (200)	BL (400)	Dexamethasone	Montelukast
GM-CSF	2.5[a)]	2.4	2.4	1.7*	2.7
IFNγ	28.1	14.8	15.1*	0.9*	22.2
IL-1β	18.8	14.7	23.9	2.2*	12.7
IL-5	5.3	5.2	6.5	1.0*	8.9*
IL-6	155.3	133.1	146.2	117.3	145.9
IL-10	68.5	45.9	78.5	29.2*	54.0
IL-12(p40)	3.6	4.5	5.8*	1.6*	5.8*
IL-12(p70)	2.2	2.2	2.2	1.0*	1.6
IL-17	3.7	3.5	4.9	1.3*	5.7
KC	61.5	61.0	71.2	60.1	71.2
LIF	27.2	22.3	20.4	5.1*	26.1
MCP-1	112.9	109.7	116.6	89.6	117.0
MIG	800.3	152.6	345.3	41.6	332.3
MIP-1α	134.4	116.1	123.0	28.1	116.3
MIP-1β	1,232.5	1,037.7	1,232.5	669.8	1,232.5
MIP-2	241.3	217.2	288.7	84.1*	288.7
RANTES	43.5	56.1	39.4	26.2	73.2

Mice were sacrificed 3 h after LPS treatment. All cytokine/chemokine levels were measured using cytokine array (Millipore's MILLIPLEX™). [a)]Arithmatic mean, Here represented the cytokines/chemokines increased more than 25 fold compared to the basal level or those significant inhibited by the test compound(s) after LPS treatment among 32 cytokine/chemokine levels examined. BL: 200-400 mg/kg, Dexamethasone 10 mg/kg, Montelukast: 30 mg/kg. *Significantly increased or decreased compared to those of LPS-treated control group (n=6, $p<0.05$).

were orally administered on three consecutive days, and LPS was treated 1 h after final administration of the compounds. Mice were sacrificed 1 h (n=6) and 3 h (n=6) after LPS treatment. Blood was obtained by cardiac puncture. All cytokine levels in the serum were measured using cytokine array kit (MPXMCYTO-70K-32, MILLIPLEX™, Millipore) in which 32 cytokines/chemokines were quantitatively detected; eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, -1β, -2, -3, -4, -5, -6, -7, -9, -10, -12, -12(70), -13, -15, -17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, VEGF.

LPS/elastase-induced chronic bronchitis in rats

For intratracheal instillation, rats were anesthetized by tribromoethanol and LPS/elastase was administered directly to lung (n=6), using a microsprayer (Intratracheal aerosolizer, Penn-Century, Inc., USA) according to the modified method of Starcher and Williams (1989). Rats were maintained in an up-right position at least for 5 min. In brief, for inducing chronic bronchitis, 15 U of elastase dissolved in saline was administered directly to the lungs of rats (200 μl/rat) on day one and LPS (*E. coli* 026:B6) was administered (100 μg/200 μl/rat) on the fourth day of the week for 4 consecutive weeks, following the slightly modified method of Ganesan *et al*. (2010). After the final treatment of LPS, all test compounds were orally administered for 10 days. Rats were then sacrificed 1 h after the last oral administration of test compounds. After sacrifice, bronchoalveolar lavage fluid (BALF) was collected via intratracheal cannulation after 800 μl infusion of saline solution. The volume of BALF collected was approximately 450 μl/rat. Leukocytes were counted in the BALF after centrifugation (n=3). From the remaining rats (n=3), lung tissues were excised, and histology was carried out after routine fixing and staining.

Statistical analysis

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

RESULTS

Intraperitoneal injection of LPS to the mice resulted in systemic septic inflammation. In the present study, a sub-lethal dose of LPS was used to examine the effects on cytokine/chemokine induction. One hour after LPS treatment, several cytokine/chemokine levels, including G-CSF, IL-6, IL-10, KC, MCP-1, MIP-1α, MIP-2 and TNF-α in the serum, were highly increased among the 32 cytokines/chemokines examined. Under this condition, BL (400 mg/kg/day) and dexamethasone (10 mg/kg/day) significantly reduced the concentration of TNF-α, dexamethasone being more potent (Table 1, Fig. 1). BL did not significantly affect the levels of other cytokines/chemokines such as IL-6, while dexamethasone also significantly reduced IL-6 levels. Montelukast (LT receptor antagonist, 30 mg/kg) rather increased the cytokine/chemokine levels of G-CSF, MCP-1, MIP-1α and MIP-2.

On the other hand, three hours after LPS treatment many cytokine/chemokine levels (eotaxin, IL-1β, IFN-γ, IL-10, IL-12, MCP-1, RANTES, etc.) were significantly and considerably increased (Table 2), whereas the concentration of TNF-α decreased rapidly to non-significant level at 3 h, compared to the level of 1 h model. In this condition, BL treatment (200 mg/kg)

reduced the induction of several cytokines, such as IL-1β and IFN-γ, although this observation was not found to be statistically significant (Table 2 and Fig. 2). Especially, BL (400 mg/kg) significantly inhibited IFN-γ levels. Dexamethsone potently reduced the levels of cytokines/chemokines, including GM-CSF, IFN-γ, IL-1β, IL-10, IL-12, MIP-2, etc. On the other hand, montelukast (30 mg/kg/day) did not significantly reduce these cytokine/chemokine levels, but rather increased the levels of IL-5 and IL-12 secretion.

Chronic bronchitis as an animal model was induced by LPS/elastase instillation in rats. When LPS/elastase was directly instilled intratracheally for 4 weeks, bronchitis characterized by inflammatory cell infiltration and histological change was produced (Fig. 3). Under these conditions, BL significantly reduced inflammatory cell numbers in BALF (41.2% and 66.8% reduction at 200 and 400 mg/kg/day, respectively) (Fig. 3A). The reference drug, montelukast (30 mg/kg/day, LT receptor antagonist), also reduced cell numbers (26.9%), but the inhibition was weaker than that of BL. 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 in the montelukast-treated group (Fig. 3B). The BL treatment clearly inhibited lung damage judged by bronchial hyperplasia, inflammatory cell infiltration and airway narrowing. In this animal model, montelukast also resulted in a similar response.

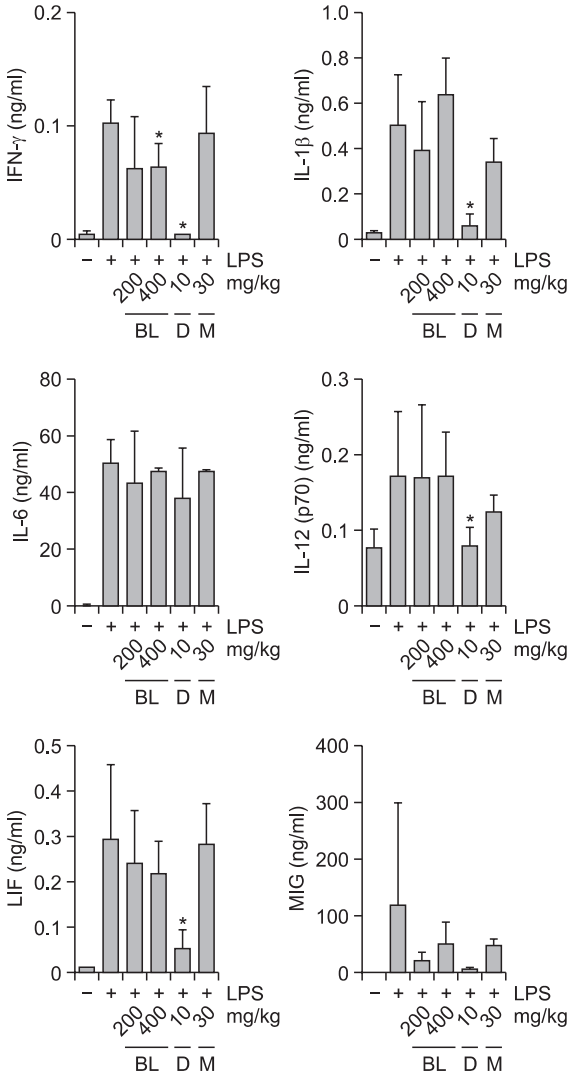

Fig. 2. Effects of BL on cytokine/chemokine induction in septic inflammation (3 h). Mice were sacrificed 3 h after LPS injection and the concentrations of cytokines were measured in the serum. Dexamethasone (D), montelukast (M), *p<0.05, Significantly different from the LPS-treated control group (n=6).

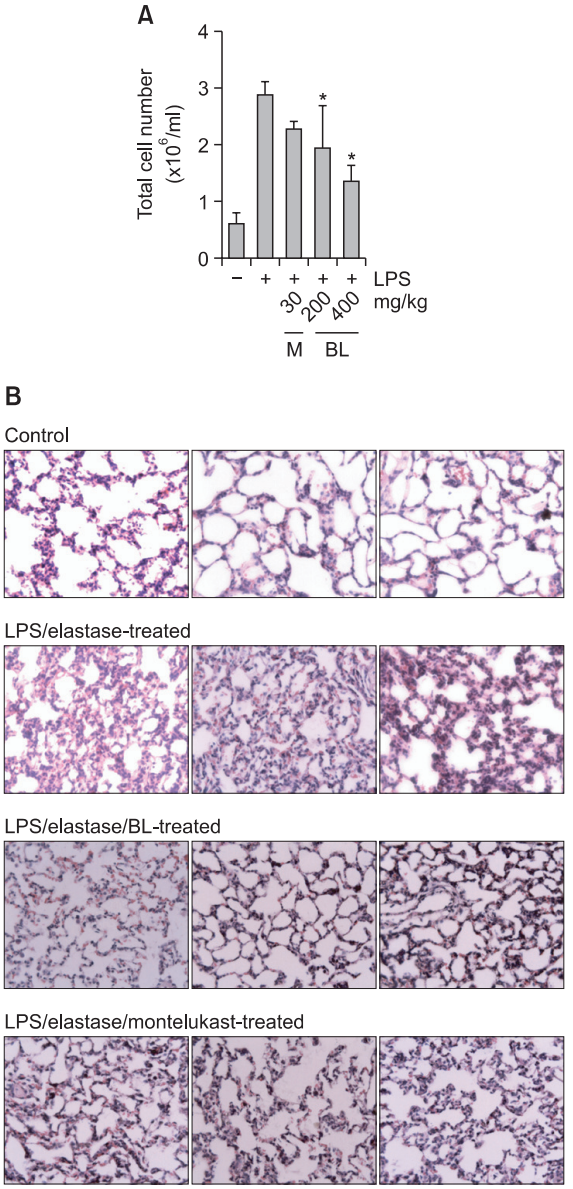

Fig. 3. Effects of BL on chronic bronchitis induced by LPS/elastase instillation in rats. (A) Inhibition of cell infiltration, The cells were counted in BALF (n=3). Montelukast (M), *p<0.05, significantly different from the LPS-instilled control group. (B) Histology, H&E staining (x400), Note: In LPS/elastase-instilled airway, airway inflammation, mucous cell hyperplasia and inflammation-related cell recruitment were observed, while all these responses were markedly reduced in the BL-treated (400 mg/kg/day) and montelukast-treated (30 mg/kg/day) lungs.

DISCUSSION

The present investigation clearly demonstrated that BL possesses significant inhibitory activity against animal models of systemic septic inflammation and chronic bronchitis. This study provides strong scientific data supporting the use of BL for the treatment of human chronic bronchitis.

Previously, BL was found to possess anti-inflammatory and analgesic activities on several animal models of inflammation (Jin *et al*., 2010). In particular, BL inhibits animal models of lung inflammation and acute bronchitis by oral administration (Ko *et al*., 2011). The anti-inflammatory potencies of BL were comparable to those of synthetic drugs which are currently in use. Previous studies have certainly exhibited the potential of BL as a new therapeutic agent against lung inflammatory disorders, especially for the treatment of bronchitis. The present study extends the potential use of BL for the treatment of chronic states of bronchitis.

To induce acute–type bronchitis-like symptoms, single instillation of LPS to the lung is enough (Ko *et al*., 2011). But to produce the chronic-type lung injury, the repeated instillation of LPS and elastase is required. Multiple treatments of LPS only did not provoke inflammatory response in the lung, possibly due to the adaptation process to LPS (data not shown). In chronic model of the present study, direct instillation of LPS and elastase to the lung was used. Following the treatment for 4 weeks, inflammatory responses including inflammatory cell recruitment and histological changes were observed. Against this model, BL was active.

Our previous study (Jin *et al*., 2010) showed that BL inhibited 5-lipoxygenase (5-LOX) and down-regulated inducible nitric oxide synthase (iNOS) expression. In addition, BL inhibited NO, TNF-α and IL-6 production by alveolar macrophages, and the NO inhibitory action of BL was mediated in part by iNOS down-regulation (Ko *et al*., 2011). These results 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. The present study also demonstrated that BL regulated cytokines/chemokines production. Especially, TNF-α, IFN-γ and IL-1 were down-regulated by BL treatment. All these mechanisms of action may contribute to the *in vivo* anti-inflammatory activity of BL. Since 5-LOX is deeply related to asthma, and proinflammatory cytokines/chemokines are involved in human bronchitis (Batt, 1992; Barnes, 1999), multiple mechanisms of action of drug candidates are favorable for treating lung inflammatory disorders.

Montelukast (30 mg/kg) showed inhibitory activity against acute bronchitis induced by LPS instillation, being less active than BL (400 mg/kg) for blocking cell recruitment (Ko *et al*., 2011). In addition, montelukast clearly inhibited IL-1β mRNA production and alleviated lung inflammation, as shown by histological observation. These previous results were in good agreement with previous reports that LT receptor antagonist is effective for the treatment of allergic asthma and bronchitis (Jeffery, 2001). In the present investigation, it was also found that montelukast is somewhat effective against animal models of chronic bronchitis. In contrast, montelukast did not exert significant inhibitory activity against septic inflammation, suggesting that 5-lipoxygenase products are not mainly involved in this animal model of sepsis. On the other hand, BL showed inhibitory activity towards septic inflammation as well as chronic bronchitis. It is suggested that the multiple anti-inflammatory mechanisms contribute to the inhibitory action of BL against these animal models.

The major constituents are the prenylated flavonoids, including papyriflavonol A and broussochalcone A in the root barks of *B. papyrifera* (Son *et al*., 2001). These compounds are found to be active constituents, in that papyriflavonol A is a relatively specific 5-LOX inhibitor (Chi *et al*., 2001) and papyriflavonol A has also been found to inhibit allergic responses *in vivo* (Kwak *et al*., 2003). Broussochalcone A possesses iNOS down-regulating activity (Cheng *et al*., 2001). In the whole plant of *L. japonica*, iridoids such as sweroside and loganin are major constituents in the whole plant of *L. japonica*. These compounds have also been reported to show anti-inflammatory activity (Lee *et al*., 1995). Therefore, it is strongly suggested that these constituents contribute to the *in vivo* activity and the multiple cellular mechanisms of BL.

In conclusion, the new phytoformula, BL (mixture of *B. papyrifera* and *L. japonica*), exerted the inhibitory activity in animal models of septic inflammation and chronic bronchitis. Especially, BL is effective in the treatment of chronic bronchitis at oral doses of 200-400 mg/kg/day. These findings indicate that BL has potential for the successful treatment of human chronic bronchitis.

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