Journal of Ethnopharmacology 149 (2013) 169–175

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

The root barks of *Morus alba* and the flavonoid constituents inhibit airway inflammation

Hun Jai Lim [a], Hong-Guang Jin [b], Eun-Rhan Woo [b], Sang Kook Lee [c], Hyun Pyo Kim [a,*]

[a] College of Pharmacy, Kangwon National University, Chunchon 200-701, South Korea
[b] College of Pharmacy, Chosun University, Kwangjoo 151-759, South Korea
[c] College of Pharmacy, Seoul National University, Seoul 151-742, South Korea

ARTICLE INFO

Article history:
Received 11 April 2013
Received in revised form
3 June 2013
Accepted 5 June 2013
Available online 24 June 2013

Keywords:
Morus alba
Prenylated Flavonoid
Lung inflammation
Bronchitis

ABSTRACT

Ethnopharmacological relevance: The root barks of *Morus alba* have been used in traditional medicine as an anti-inflammatory drug, especially for treating lung inflammatory disorders.
Aim of study: To find new alternative agents against airway inflammation and to establish the scientific rationale of the herbal medicine in clinical use, the root barks of *Morus alba* and its flavonoid constituents were examined for the first time for their pharmacological activity against lung inflammation.
Materials and methods: For *in vivo* evaluation, an animal model of lipopolysaccharide-induced airway inflammation in mice was used. An inhibitory action against the production of proinflammatory molecules in lung epithelial cells and lung macrophages was examined.
Results: Against lipopolysaccharide-induced airway inflammation, the ethanol extract of the root barks of *Morus alba* clearly inhibited bronchitis-like symptoms, as determined by TNF-α production, inflammatory cells infiltration and histological observation at 200–400 mg/kg/day by oral administration. In addition, *Morus alba* and their major flavonoid constituents including kuwanone E, kuwanone G and norartocarpanone significantly inhibited IL-6 production in lung epithelial cells (A549) and NO production in lung macrophages (MH-S).
Conclusions: Taken together, it is concluded that *Morus alba* and the major prenylated flavonoid constituents have a potential for new agents to control lung inflammation including bronchitis.

1. Introduction

Lung inflammatory disorders include acute and chronic bronchitis. Against these diseases, several classes of drugs such as anti-inflammatory steroids, antitussives, mucolytics and/or bronchodilators are prescribed in clinics to alleviate the various symptoms of airway inflammation. However, it is sometimes difficult to successfully treat these inflammatory disorders, especially chronic bronchitis (one form of chronic obstructive pulmonary diseases, COPD) with currently-available drugs (Jeffery, 2001). Thus, it is necessary to develop new agents in order to fundamentally control these diseases and to have alternative drug (s) showing different action mechanism(s). Among the many efforts to find new drug candidates, a search for active constituents from various plant products has been continued.

The root barks of *Morus alba* L. have been widely used in traditional medicine for treating various inflammatory conditions, including acute and chronic bronchitis in North-East Asia (Bae, 2000). Previous phytochemical studies on this plant reported the isolation of various flavonoids. Moreover, it is well-known that the main constituents of this plant material are the isoprene substituted flavonoids, prenylated flavonoids (Nomura, 2001). Previously, the methanol and ethylacetate extracts of the root barks of *Morus alba* were reported to show anti-inflammatory activity (Hong et al., 2002; Chao et al., 2009). The prenylated flavonoids such as morusin, kuwanon C and kuwanon G isolated from *Morus alba,* have also been shown to possess various anti-inflammatory activities (Cheon et al., 2000; Yang et al., 2011). However, despite the frequent use on lung inflammation-related disorders, the pharmacological evaluation of the total extract of *Morus alba* as well as its major constituents on airway inflammation has not been carried out to date. Therefore, in the present study, the inhibitory action of the ethanol extract of *Morus alba* was examined using a lipopolysaccharide (LPS)-induced airway inflammation model in mice to provide the scientific rationale for the clinical use. The main flavonoid constituents were isolated from the root barks of *Morus alba* and their effects on inflammatory responses of lung epithelial cells and alveolar macrophages were investigated to prove the potential for new therapeutic agents.

* Corresponding author. Tel.: +82 33 2506915; fax: +82 33 2557865.
E-mail address: hpkim@kangwon.ac.kr (H.P. Kim).

0378-8741/$ - see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jep.2013.06.017

2. Materials and methods

2.1. Chemicals

2-Amino-5,6-dihydro-6-methyl-4 H-1,3-thiazine hydrochloride (AMT) was purchased from Tocris Cookson Ltd. (UK). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dexamethasone, IL-1β and LPS (*Escherichia coli* 0127:B8) were purchased from Sigma Chem. (St. Louis, MO). MEM 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).

2.2. Animals

Male ICR mice (male, 18–22 g, specific pathogen-free) were obtained from Central Exp. Animal Ltd. (Korea). Animals were fed with standard lab. chow and water *ad libitum*. The animals were maintained in 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-12-0012). In addition, the ethical guideline described in the Korean Food and Drug Administration guide for the care and use of laboratory animals was followed throughout the experiments.

2.3. Plant materials

The root barks of *Morus alba* L. (Moraceae) were collected in Geongju, Geongbuk province, Korea, and authenticated by Prof. J. H. Lee (Dongguk University, Gyoungju, Korea). The voucher specimen (CSU-1048-17) were deposited in the Herbarium of the College of Pharmacy, Chosun University.

2.4. Extraction and isolation of kuwanon E, kuwanon G, and norartocarpanone

The dried root barks of *Morus alba* (1 kg) were extracted with 70% ethanol under reflux. Evaporation under reduced pressure gave the dried extract (MA, 170.3 g), which was used throughout the pharmacological study. In addition, for isolating major flavonoid constituents, the dried root barks of *Morus alba* (12 kg) were extracted three times with methanol under reflux and 1511.6 g of residue was produced. The methanol extract was suspended in water and was then partitioned sequentially with equal volumes of dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH). Each fraction was evaporated under reduced pressure to yield the residues of CH_2Cl_2 (318.2 g), EtOAc (192.2 g), *n*-BuOH (182.4 g), and water (534.3 g) extract. The EtOAc fraction (53 g) was chromatographed over a silica gel column using a gradient solvent system of *n*-hexane-EtOAc (2:1→1:8, EtOAc, MeOH) to give 5 subfractions (E1–E5). Subfraction E2 was subjected to MCI gel column chromatography (CC) eluting with a gradient solvent system of MeOH:H_2O (1:1 →3:1) to yield 16 subfractions (E21–E216). Subfraction E22 was purified by LiChroprep RP 18 CC (MeOH:H_2O, 2:3→1:1) to give norartocarpanone (3.42 mg). Subfraction E24 was subjected to silica gel CC ($CHCl_3$:MeOH:H_2O, 12:1:0.1→8:1:0.1) to give 6 fractions (E241–E246). Subfraction E245 was purified by LiChroprep RP 18 CC (MeOH:H_2O, 1:1) to yield kuwanon G (1011 mg). Subfraction E27 was purified repeated silica gel CC ($CHCl_3$:MeOH:H_2O, 25:1:0.1→10:1:0.1) and LiChroprep RP 18 CC (MeOH:H_2O, 2:1) to yield kuwanon E (43.7 mg) (Fig. 1). The physico-chemical data including ^{1}H NMR, ^{13}C NMR, and HSQC of these compounds were identical with those reported in the literature (Hano et al., 1984; Nomura et al., 1980; Nomura and Fukai, 1981).

Kuwanon E: ^{1}H–NMR (Acetone-d_6, 600 MHz): δ 7.20 (1H, s, H-6′), 6.50 (1H, s, H-3′), 5.95 (1H, d, *J*=1.8 Hz, H-6), 5.94 (1H, d, *J*=1.8 Hz, H-8), 5.70 (1H, dd, *J*=3.0, 13.8 Hz, H-2), 5.35 (1H, dt, *J*=1.2, 7.2 Hz, H-2″), 5.11 (1H, tt, *J*=1.2, 7.2 Hz, H-7″), 3.27 (2H, d, *J*=7.2 Hz, H-1″), 3.20 (1H, dd, *J*=13.8, 17.4 Hz, Ha-3), 2.69 (1H, dd, *J*=3.0, 17.4 Hz, Hb-3), 2.09 (2H, m, H-6″), 2.02 (2H, t, *J*=7.2 Hz, H-5″), 1.71 (3H, s, H-4″), 1.62 (3H, s, H-9″), 1.57 (3H, s, H-10″); ^{13}C–NMR (Acetone-d_6, 150 MHz): δ 197.8 (C-4), 167.3 (C-7), 165.4 (C-8a), 164.9 (C-5), 156.7 (C-4′), 154.2 (C-2′), 135.9 (C-3″), 131.7 (C-8″), 129.0 (C-6′), 125.2 (C-7″), 124.1 (C-2″), 120.2 (C-5′), 116.9 (C-1′), 103.4 (C-3′), 103.2 (C-4a), 96.7 (C-8), 95.9 (C-6), 75.5 (C-2), 42.7 (C-3), 40.5 (C-5″), 28.4 (C-1″), 27.5 (C-6″), 25.9 (C-9″), 17.8 (C-10″), 16.3 (C-4″).

Kuwanon G: ^{1}H–NMR (Acetone-d_6, 300 MHz): δ 7.41 (1H, d, *J*=8.0 Hz, H-6′or H-27), 7.29 (1H, d, *J*=8.0 Hz, H-6′or H-27), 6.78 (1H, d, *J*=8.0 Hz, H-33), 6.67 (1H, d, *J*=2.0 Hz, H-3′), 6.55 (1H, dd, *J*=2.0, 8.0 Hz, H-5′), 6.21 (1H, d, *J*=2.0 Hz, H-30), 6.08 (1H, dd, *J*=2.0, 8.0 Hz, H-32), 6.03 (1H, d, *J*=2.0 Hz, H-24), 5.98 (1H, s, H-6), 5.93 (1H, dd, *J*=2.0, 8.0 Hz, H-26), 4.95–5.40 (2H,m, H-10 and H-15), 4.30–4.70 (2H, m, H-14 and H-20), 3.30–3.90 (1H, m, H-19), 3.17 (2H, d, *J*=7.0 Hz, H-9), 1.80–2.20 (1H, m, H-18), 1.62 (3H, s, H-12), 1.52 (3H, s,, H-16), 1.48 (3H, s, H-13); ^{13}C –NMR (DMSO-d_6, 75.5 MHz): δ 208.1 (C-21), 181.7 (C-4), 164.2 (C-23), 164.2 (C-25), 161.3 (C-7), 160.83 (C-4′), 160.3 (C-8a), 159.2 (C-2), 156.3 (C-2′), 155.8 (C-29), 155.8 (C-31), 155.2 (C-5), 132.8 (C-16), 132.4 (C-33), 131.2 (C-11), 131.2 (C-6′), 130.8 (C-27), 123.2 (C-15), 121.8 (C-10), 120.7 (C-28), 119.7 (C-3), 114.0 (C-22), 111.4 (C-1′), 107.2 (C-26), 106.7 (C-8), 106.7 (C-32), 106.7 (C-5′), 103.7 (C-4a), 102.6 (C-24), 102.6 (C-3′), 101.9 (C-30), 97.5 (C-6), 45.8 (C-20), 38.3 (C-18), 38.3 (C-19), 25.4 (C-12), 23.5 (C-9), 22.9 (C-14), 22.5 (C-17), 17.3 (C-13).

Norartocarpanone: ^{1}H –NMR (Acetone-d_6, 600 MHz) δ: 7.31 (1H, d, *J*=8.4 Hz, H-6′), 6.47 (1H, d, *J*=1.8 Hz, H-3′), 6.43 (1H, dd, *J*=1.8, 8.4 Hz, H-5′), 5.96 (1H, d, *J*=2.4 Hz, H-8), 5.94 (1H, d, *J*=2.4 Hz, H-6), 5.70 (1H, dd, *J*=3.0, 13.2 Hz, H-2), 3.17 (1H, dd, *J*=13.2, 17.4 Hz, H-3), 2.71 (1H, dd, *J*=3.0, 17.4 Hz, H-3); ^{13}C –NMR (Acetone-d_6, 150 MHz) δ: 197.7 (C-4), 167.7 (C-7), 165.4 (C-8a), 164.9 (C-5), 159.6 (C-2′), 156.4 (C-4′), 129.1 (C-6′), 117.5 (C-1′), 107.8 (C-5′), 103.5 (C-3′), 103.1 (C-4a), 96.8 (C-6), 95.9 (C-8), 75.4 (C-2), 42.7 (C-3).

2.5. LPS-induced airway inflammation in mice

Mice were divided into five groups, including the control, LPS-treated, LPS/MA (200, 400 mg/kg/day)-treated and LPS/dexamethasone-treated (*n*=14). The test compounds, including the reference drug, were dissolved in 0.3% carboxymethylcellulose (CMC) and were orally administered for three consecutive days. The control and LPS treatment groups also received the CMC solution. For inducing bronchitis, LPS (*Escherichia coli* 0127:B8, 800 μg/ml, saline) was administered intranasally to mice (10 μl/mouse, 5 times) 1 h after the final oral treatment of the test compounds. At 4 h after LPS treatment, 5 mice per group were sacrificed and bronchoalveolar lavage fluid (BALF) was collected via intratracheal cannulation after 700 μl of saline was administered 3 times. BALFs collected were approximately 2000 μl/mouse. From BALF, TNF-α concentration was measured with an ELISA kit (eBioscience) according to the manufacturer's recommendation. At 16 h after LPS treatment, mice were sacrificed (*n*=7), and BALF was obtained. From BALF, the total cell number was counted with a haemocytometer, and the cells were differentially counted with FACS (BDbioscience). For histology and biochemical analysis, the remaining mice (*n*=2) were sacrificed and lung tissues were excised. Histology was carried out using conventional methods of H&E staining.