Anti-inflammatory compounds moracin O and P from *Morus alba* Linn. (Sohakuhi) target the NF-κB pathway

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Abstract. Accumulating evidence suggests that inflammation is linked to multiple pathological processes and induces cellular and molecular damage through the activation of inflammatory signaling pathways, including the NF-κB pathway. The aim of the present study was to identify natural anti-inflammatory products that can target NF-κB activity, in order to establish a novel therapeutic approach for inflammatory diseases. Using a 4T1 breast cancer cell line that expresses the firefly luciferase gene under the control of an NF-κB response element, 112 natural products were tested for their anti-inflammatory properties. Sohakuhi (*Morus alba* Linn. bark) extract was observed to strongly suppress NF-κB activity without affecting cell viability. To further examine the anti-inflammatory effect of Sohakuhi, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced cellular damage of human HaCaT keratinocytes was evaluated. While TRAIL triggered the phosphorylation of the p65 subunit of NF-κB, leading to cellular damage in HaCaT cells, treatment with Sohakuhi extract protected HaCaT cells against TRAIL-induced cellular damage. Moreover, Sohakuhi treatment also upregulated the anti-apoptotic proteins Bcl-xL and Bcl-2. Importantly, through chemical fractionation of Sohakuhi extract, moracin O and P were confirmed to mediate its anti-inflammatory effects. Collectively, the present results indicated that Sohakuhi and moracin may represent potential candidates for the development of novel anti-inflammatory drugs.

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Abbreviation: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

Key words: inflammation, NF-κB, cytoprotection, *Morus alba* Linn. bark, apoptosis

Introduction

Inflammation occurs in response to a variety of stimuli, such as tissue damage, infection, or cancer (1-3). While acute inflammation is of short duration and represents an early body reaction that resolves quickly, chronic inflammation is defined as a prolonged process whereby tissue destruction and inflammation occur simultaneously (4). In the early stages of inflammation, the vascular endothelium is activated by cytokines, leading to adhesion and transmigration of leukocytes into the site of inflammation. Some of the pro-inflammatory processes acquired at the endothelium and leukocytes are mediated by the transcription factor NF-κb (5). Failure to treat inflammation can lead to various diseases associated with chronic inflammation, including arthritis, atherosclerosis, and even cancer (6). For most of these conditions, no satisfactory treatment has been established.

NF-κB is a transcription factor that plays an important role in cellular stress responses, including the DNA damage response (7). NF-κB activation induces the expression of >200 genes that regulate inflammation, as well as cell death/apoptosis (8). The DNA damage response is known to play a pivotal role in ageing and carcinogenesis. It consists of a signal transduction cascade that is initiated by DNA double-strand breaks and leads to DNA repair, cell cycle arrest or programmed cell death (9). Evidence strongly suggests that inflammation is linked to multiple pathologies, such as cancer and obesity (10), by inducing cellular and molecular damage through the activation of several signaling pathways, including the NF-κB pathway.

The aim of the present study was to identify natural products with anti-inflammatory effects on NF-κB activity and further characterize active compounds from plant products for drug discovery. By screening 112 natural products for their anti-inflammatory properties, it was identified that Sohakuhi (*Morus alba* Linn. bark) extract markedly suppressed NF-κB-dependent luciferase reporter activity in murine 4T1 cells without affecting cell viability. The anti-inflammatory effect of Sohakuhi on tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced cellular damage was evaluated in human HaCaT keratinocytes. TRAIL triggered the phosphorylation of p65, a subunit of NF-κB, leading to cellular damage in HaCaT cells. However, treatment with Sohakuhi extract protected HaCaT

cells against TRAIL-induced damage. Moreover, Sohakuhi also upregulated the expression of the anti-apoptotic proteins Bcl-xL and Bcl-2. Importantly, through chemical fractionation of Sohakuhi extract, moracin O and P were determined to mediate its anti-inflammatory effect.

Materials and methods

Cells and reagents. The murine B16F10 and 4T1 cell lines were obtained from the American Type Culture Collection and maintained at 37˚C in Eagle's minimal essential medium or RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd.) containing 10% FBS (Nichirei Biosciences, Inc.), respectively. Human HaCaT keratinocytes (provided by Dr Takeda, Juntendo University, Tokyo, Japan) were maintained at 37˚C in culture medium consisting of DMEM (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% FBS, 100 U/l penicillin G and 100 mg/l streptomycin at 37˚C with 5% CO_2. Human recombinant TRAIL (rTRAIL) was purchased from PeproTech, Inc.

In vitro NF-κB luciferase reporter assay. B16F10NFκB and 4T1NFκB cells expressing firefly luciferase under the control of an NF-κB response element were established as previously described (11,12) and maintained at 37˚C in RPMI-1640 medium containing 10% FBS. Briefly, B16F10NFκB and 4T1NFκB cells were generated by transfecting the B16F10 and 4T1 cell lines with pGL4.32-*luc2P*/NF-κB-RE/Hygro vector (Promega Corporation) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were selected on hygromycin B and cloned by limiting dilution.

B16F10NFκB cells and 4T1NFκB cells in the exponential growth phase were seeded at a final concentration of $4x10^4$ cells/well in a 96-well plate. After 3-h incubation, the cells were co-cultured with 50 μg/ml extract from 112 natural products (Table SI) for 24 h. At the end of the assay, 900 μg/ml D-luciferin was added, and the plates were incubated for another 30 min. Luciferase activity was measured by the GloMax®-Multi Detection System (Promega Corporation).

Cell viability assay. Cell viability was quantified using the WST-1 cell proliferation reagent (Dojindo Molecular Technologies, Inc.). B16F10NFκB or 4T1NFκB cells were seeded on a 96-well plate and co-cultured with extracts from 112 natural products (Table SI) at 50 μg/ml for 24 h. In a separate experiment, HaCaT cells were seeded on a 96-well plate at a density of 10^4 cells/well and pre-treated with 20 ng/ml rTRAIL for 1 h. The cells were then cultured with or without Sohakuhi extract (6.25 to 50 μg/ml) for 24 h. After incubation, WST-1 solution was added and used according to the manufacturer's instructions, and absorbance was measured at 450 nm using a microplate reader. Cell viability was calculated as a percentage of the control.

Caspase-3 and -7 activity assay. For measurement of the activities of caspase-3 and -7, the Caspase-Glo® 3/7 assay system (Promega Corporation) was used according to the manufacturer's instructions. Briefly, HaCaT cells ($5x10^3$ cells/well in a 96-well plate) were pre-treated with 20 ng/ml rTRAIL for 1 h. The cells were then cultured with Sohakuhi extract for 24 h, and the Caspase-Glo® 3/7 reagent was then added. After 30-min incubation, caspase-3 and -7 activities were measured using the GloMax®-Multi Detection System (Promega Corporation).

Identification of the active components of Sohakuhi extract. To identify the bioactive components of Sohakuhi extract, 200 g root bark of *Morus alba* Linn. was decocted in 1 l water for 50 min. The water extract was fractionated by water-methanol gradient reverse-phase medium-pressure liquid chromatography (RP-MPLC) fractionation. Mass spectrometry and ^{1}H-nuclear magnetic resonance (NMR) analysis were used to identify compounds in the isolated fractions. Extraction and isolation of Sohakuhi was conducted using a water-methanol gradient MPLC fractionation system and high-performance liquid chromatography (Accela™ HPLC system; Thermo Fisher Scientific, Inc.) profiles of water extracts of Sohakuhi were determined at 254-nm ultraviolet wavelength. A Capcell Pak C18 MG III S-5 (4.5x250 mm, 5 μm; Shiseido Co., Ltd.) column was used for HPLC analysis at a flow rate of 1 ml/min at 40˚C. A gradient elution system composed of 5% CH_3CN (v/v) (A) and H_2O was used as follows: 0-4 min, 5% A; 4-8 min, 5-10% B; 8-12 min, 10-15% B; 12-15 min, 15-20% B; 15-18 min, 20-25% B; 18-21 min, 25-30% B; 21-25 min, 30-35% B; 25 min, 40% B. The active fraction was further analyzed using a $CHCl_3$-MeOH gradient RP-MPLC fractionation system with mass spectrometry and ^{1}H-NMR analysis to identify the active compounds of Sohakuhi. The MS conditions were set as follows: Negative ESI mode, spray voltage 4.5 kV, capillary voltage 40.0 kV, tube lens 150 V, capillary temperature 270˚C, sheath gas flow rate 50 units, aux gas flow rate 10 units, and scan range m/z 50-2,000. A polytyrosine solution was used for instrument calibration before each experiment.

Western blot analysis. HaCaT cells were seeded at a density of $5x10^5$ cells/well and cultured for 24 h in a 6-well plate. After treatment, the cells were washed in cold PBS, scraped and lysed in whole-cell lysis buffer (25 mmol/l HEPES, pH 7.7; 300 mmol/l NaCl; 1.5 mmol/l $MgCl_2$; 0.2 mmol/l EDTA; 0.1% Triton X-100; 20 mmol/l β-glycerophosphate; 1 mmol/l Na_3VO_4; 1 mmol/l phenylmethylsulfonylfluoride; 1 mmol/l dithiothreitol; 10 mg/ml aprotinin; 10 mg/ml leupeptin). Cell lysates (10 μl/lane) were resolved by SDS-PAGE on 7.5-15% gels, then transferred to an Immobilon-P nylon membrane (EMD Millipore). The membranes were treated with Block Ace (Dainippon Sumimoto Pharma Co., Ltd.) for at least 2 h at room temperature, then probed with primary antibodies at 4˚C overnight, followed by horseradish peroxidase-conjugated secondary antibodies (1:2,000; cat. nos. P0448 and P0260; Dako; Agilent Technologies, Inc.). Bands were visualized using ECL reagents (Amersham; Cytiva). The primary antibodies used were specific for Bcl-2 (clone no. D55G8; cat. no. 4223), Bcl-xL (clone no. 54H6; cat. no. 2764), p65 (clone no. L8F6; cat. no. 6956), phosphorylated-p65, (clone no. 93H1; cat. no. 3033; all Cell Signaling Technology, Inc.) and β-actin (clone no. C4; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.). All primary antibodies were used at 1:1,000 dilution.

Statistical analysis. All data are presented as the mean ± SEM of three independent experiments. SPSS versions 23 and 25

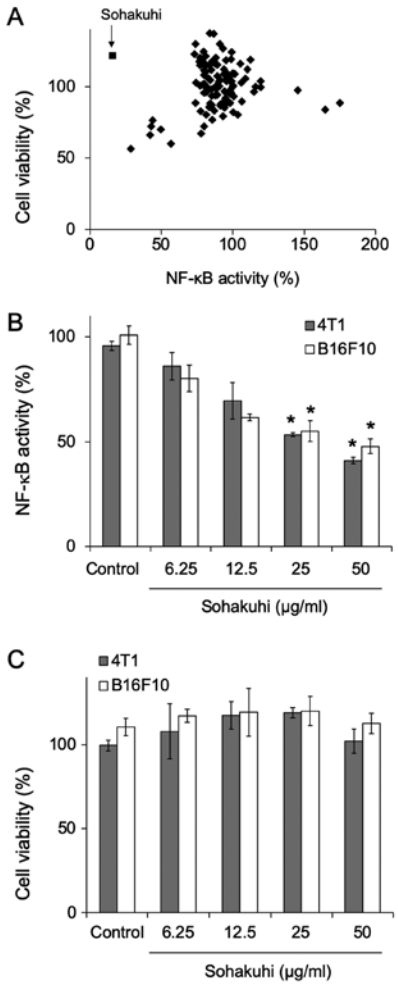

Figure 1. Sohakuhi extract suppresses NF-κB activity in murine cancer cell lines. (A) 4T1NFκB cells were co-cultured with extracts from 112 natural products (50 μg/ml) for 24 h. The inhibitory effect of each plant extract on NF-κB activation relative to untreated controls was determined. Cell viability was determined using a WST-1 assay and shown as a percentage of the untreated control. (B) NF-κB activation and (C) cell viability were determined in 4T1NFκB cells or B16F10NFκB cells treated with Sohakuhi extract at the indicated doses for 24 h. Data are normalized to the untreated controls and presented as the mean ± SEM; n=3. *P<0.05 vs. untreated control.

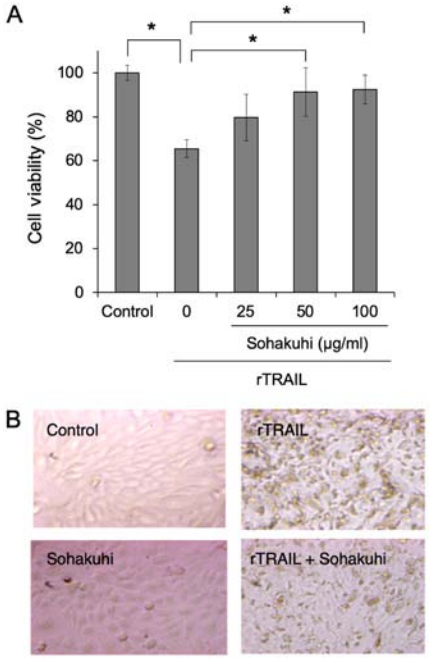

Figure 2. Cytoprotective effect of Sohakuhi extract against TRAIL-induced cellular damage in human keratinocytes. HaCaT cells were co-cultured with 20 ng/ml rTRAIL with or without Sohakuhi extract at the indicated doses. (A) Cell viability was determined using a WST-1 assay. Data are normalized to the untreated controls and presented as the mean ± SEM; n=3. *P<0.05 vs. 0 μg/ml Sohakuhi. (B) Representative images of the culture (magnification, x100). TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; r, recombinant.

software (IBM Corp.) were used to analyze data. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni correction. P<0.05 was considered to indicate a statistically significant difference.

Results

Sohakuhi suppresses NF-κB activity in murine cancer cell lines. To identify novel anti-inflammatory agents in plant products, 112 medicinal plant extracts (Table SI) were screened for their effect on NF-κB activity using cell lines that express luciferase under the control of an NF-κB response element. Among the tested extracts, Sohakuhi markedly suppressed NF-κB activity without affecting the viability of 4T1 cells (Fig. 1A). Furthermore, NF-κB activity was suppressed by Sohakuhi extract in 4T1 cells and B16F10 cells in a dose-dependent manner (Fig. 1B). However, Sohakuhi extract did not display any cytotoxic effect, even at doses reaching 50 μg/ml (Fig. 1C).

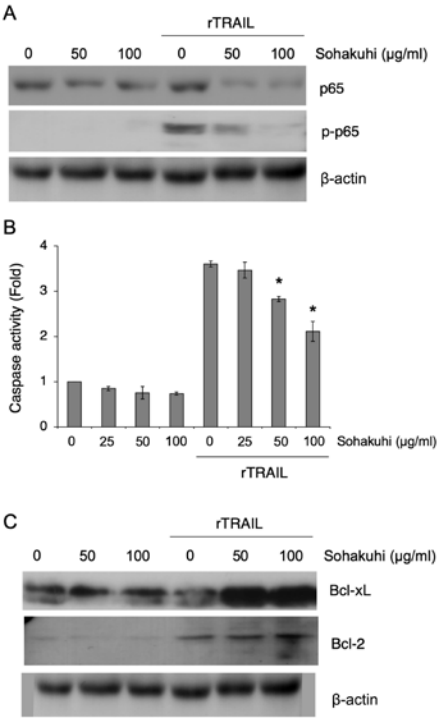

Figure 3. Anti-apoptotic effect of Sohakuhi extract on HaCaT cells. HaCaT cells were co-cultured with 20 ng/ml rTRAIL with or without Sohakuhi extract at the indicated doses. After 24-hr incubation, cell lysates were prepared for (A) western blot analysis of p65 and p-p65, and (B) measurement of caspase-3/7 activity using a commercial kit. (C) Protein levels of Bcl-xL, Bcl-2 and Bax were determined by western blot analysis. Data are presented as the mean ± SEM; n=3. *P<0.05 vs. untreated control. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; r, recombinant; p-, phosphorylated.

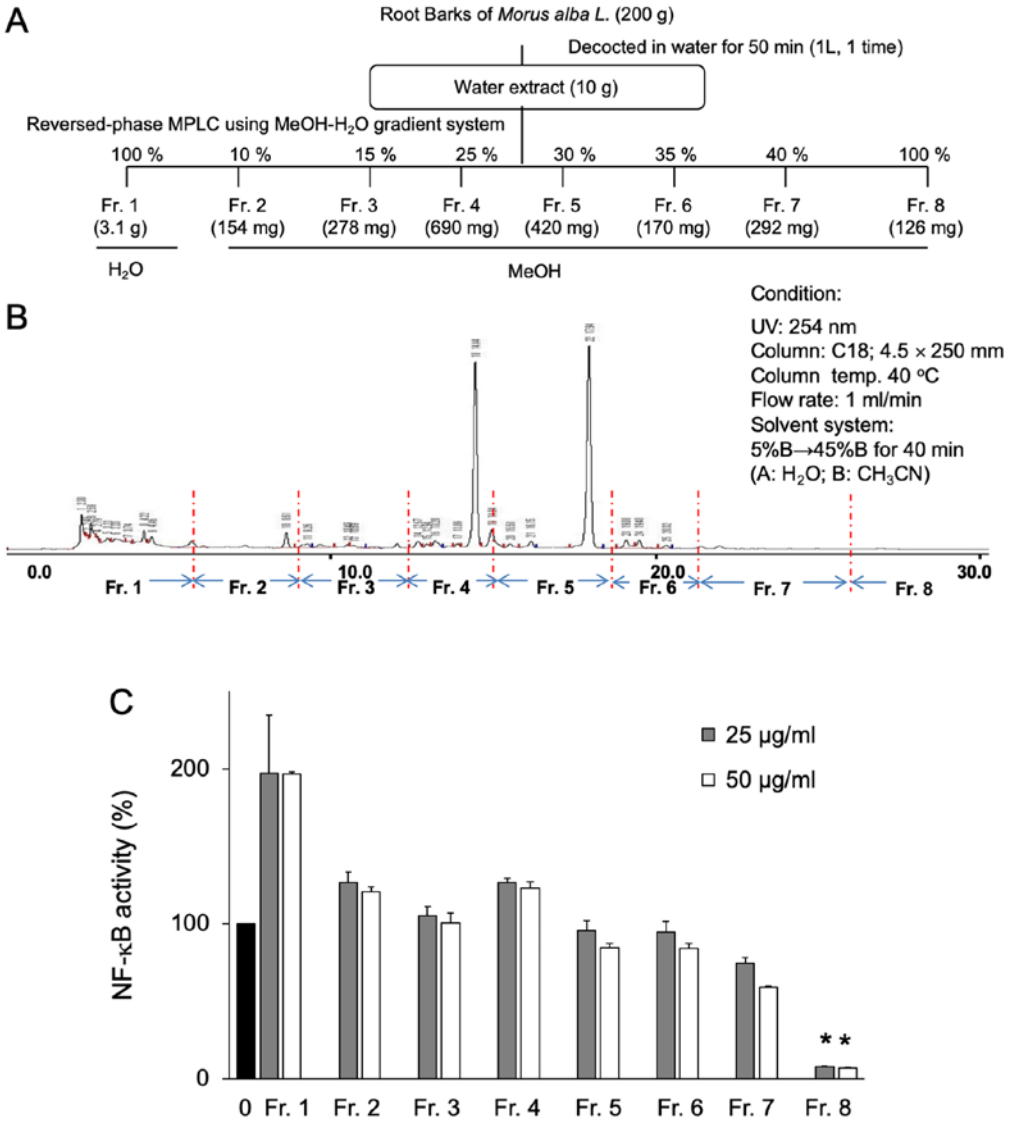

Figure 4. Identification of the active fraction of Sohakuhi extract that inhibit NF-κB activation. (A) Extraction and isolation flowchart for the identification of the active component of Sohakuhi extract using a water-methanol gradient MPLC fractionation. (B) High-performance liquid chromatography profile of water extracts of Sohakuhi at 254-nm UV wavelength. (C) 4T1NFκB cells were co-cultured with the indicated fraction of Sohakuhi at 25 or 50 μg/ml for 24 h. Inhibition of NF-κB activation was determined relative to the untreated control. Data are presented as the mean ± SEM; n=3. *P<0.05 vs. untreated control. UV, ultraviolet; MeOH, methanol; Fr, fraction; MPLC, medium-pressure liquid chromatography.

Cytoprotective effect of Sohakuhi extract on TRAIL-induced cellular damage in human keratinocytes. To examine the biological utility of Sohakuhi extract, human HaCaT keratinocytes were incubated with rTRAIL as an inflammatory stimulus to induce NF-κB activation and cytotoxicity. Compared with the control, rTRAIL treatment induced significant cytotoxicity in HaCaT cells. However, pre-treatment with Sohakuhi extract significantly protected HaCaT cells from TRAIL-induced cytotoxicity in a dose-dependent manner (Fig. 2A). The cytoprotective effect of Sohakuhi extract was evident from microscopic observation of cell morphology (Fig. 2B). These results indicated the cytoprotective effect of Sohakuhi extract against TRAIL-induced cellular damage in human keratinocytes.

Anti-apoptotic effect of Sohakuhi extract on HaCaT cells. To confirm whether Sohakuhi extract affects NF-κB activation in HaCaT cells, the expression of the p65 subunit of NF-κB and its phosphorylation status following rTRAIL stimulation was assessed in the presence or absence of Sohakuhi extract (Fig. 3A). Phosphorylation of p65 was detectable in HaCaT cells following rTRAIL stimulation, but suppressed in the presence of Sohakuhi extract. While Sohakuhi extract treatment reduced total p65 expression in rTRAIL-stimulated HaCaT cells, there was almost no effect on the basal expression of p65 in unstimulated HaCaT cells, suggesting that the effect of Sohakuhi extract might be specific to TRAIL-induced NF-κB activity. Treatment with the Sohakuhi extract also showed an inhibitory effect on TRAIL-induced apoptosis, as seen in the suppression of caspase-3/7 activation (Fig. 3B). Importantly, the anti-apoptotic proteins Bcl-xL and Bcl-2 were upregulated in Sohakuhi extract-treated HaCaT cells following TRAIL stimulation (Fig. 3C). These results indicated that the cytoprotective effect of Sohakuhi extract might result from inhibition of TRAIL-induced apoptosis and regulation of anti-apoptotic proteins.

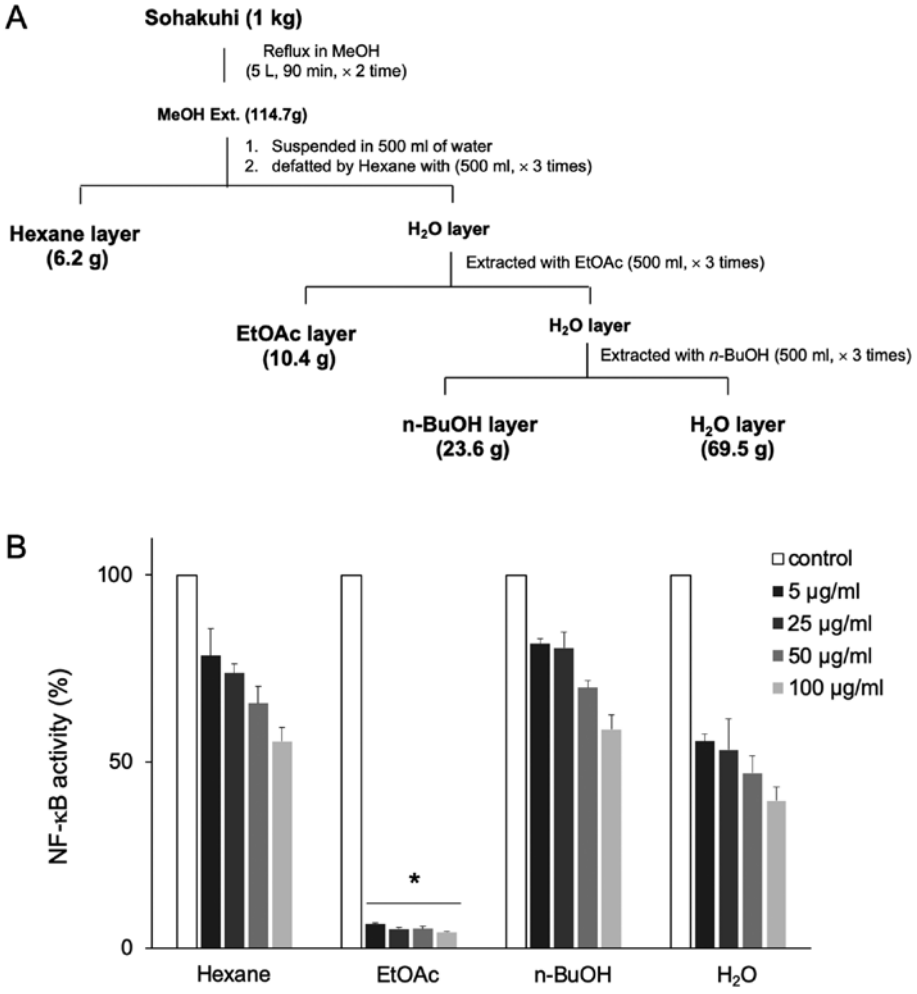

Figure 5. Identification of the active components of Sohakuhi that inhibit NF-κB activation. (A) Extraction flowchart for the identification of the active fraction of Sohakuhi. (B) 4T1NFκB cells were co-cultured with the indicated solvent fraction at the indicated dose for 24 h. Inhibition of NF-κB activation was determined relative to the untreated control. Data are presented as the mean ± SEM; n=3. *P<0.05 vs. untreated control. MeOH, methanol; EtOAc, ethyl acetate; n-BuOH, n-butanol.

Identification of the active component of Sohakuhi extract to inhibit NF-κB activation. As Sohakuhi extract significantly inhibited NF-κB activity and displayed a cytoprotective effect against inflammation-associated cellular damage of human keratinocytes, the bioactive components of Sohakuhi extract were then analyzed. A total of 8 yield fractions were isolated from a Sohakuhi water extract using RP-MPLC with methanol-water gradient (Fig. 4A). The preparative HPLC data for these 8 yield fractions are presented in Fig. 4B. Amongst those fractions, fraction 8 effectively and exclusively inhibited NF-κB activity (Fig. 4C). Considering that fraction 8 was eluted with 100% methanol in the water-methanol gradient, the methanol extract of Sohakuhi was then fractionated into hexane, ethyl acetate, n-butanol and a residual water layer (Fig. 5A) to test the activity of each layer with respect to NF-κB inhibition. Amongst those layers, the ethyl acetate layer displayed very potent inhibition of NF-κB activity in 4T1 cells, even at the lowest dose tested, 5 μg/ml (Fig. 5B). These results indicated that the active component of Sohakuhi was fractionated into an ethyl acetate layer from the methanol extract.

Isolation of moracin O and P as active compounds of Sohakuhi. Using a $CHCl_3$-MeOH gradient RP-MPLC fractionation system with mass spectrometry and ^{1}H-NMR analysis, two major compounds, moracin O and P, were identified in the ethyl acetate layer of Sohakuhi methanol extract (Fig. 6A). Importantly, both moracin O and P significantly inhibited NF-κB activity in 4T1 cells, starting at a dose of 3 nM (Fig. 6B). Additionally, both moracin O and P showed significant cytoprotective effects against TRAIL-induced cellular damage in HaCaT cells (Fig. 6C). Thus, moracin O and P were identified as active compounds of Sohakuhi that suppressed NF-κB activity and exerted a cytoprotective effect against TRAIL-induced damage in HaCaT cells.

Discussion

The aim of the present study was to identify a novel anti-inflammatory drug candidate that can target NF-κB activation. A total of 112 plant extracts were screened, identifying Sohakuhi as a promising anti-inflammatory compound. The white mulberry tree (*Morus alba* Linn.) is a deciduous tree originating from Asia, especially China, but currently cultivated in subtropical, tropical and mild environmental conditions (13,14). *Morus alba* Linn. tree bark, fruits and leaves contain proteins, carbohydrates, calcium, iron, ascorbic

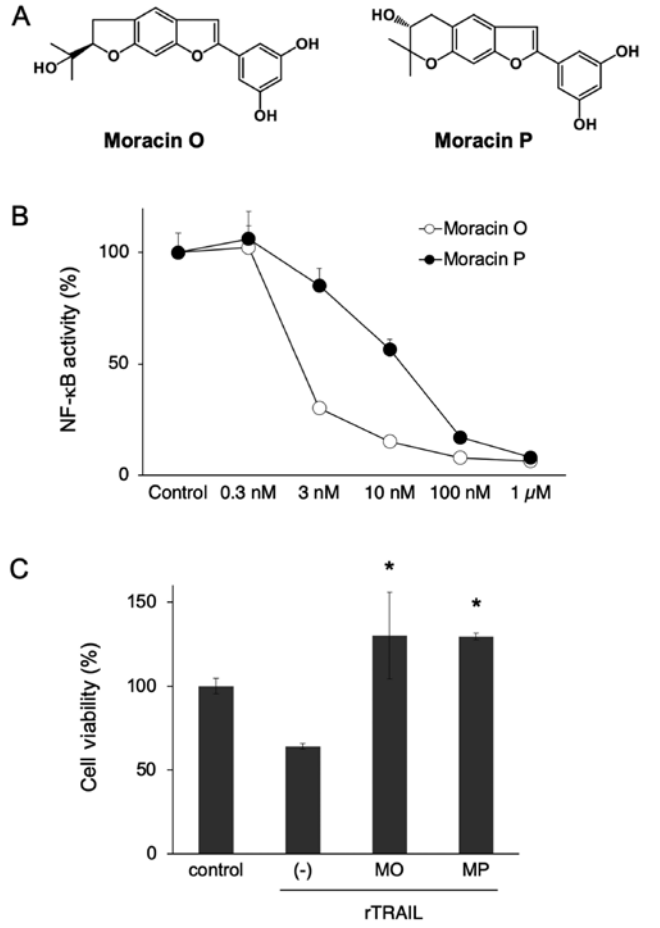

Figure 6. Isolation of moracin O and P as active compounds of Sohakuhi. (A) Chemical structures of moracin O and P. (B) 4T1NFκB cells were co-cultured with the indicated dose of moracin O or moracin P for 24 h. The inhibitory effect on NF-κB activation was determined relative to the untreated control. (C) HaCaT cells were pre-incubated with 10 μM of moracin O or moracin P for 1 h, then co-cultured with 20 ng/ml TRAIL for 24 h. Cell viability was determined using a WST-1 assay and normalized to the untreated control. Data are presented as the mean ± SEM; n=3. *P<0.05 vs. untreated control. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; r, recombinant

acid, thiamine, folic acid and vitamin D (15). It has been used in conventional and natural medicine to treat diabetes, atherosclerosis, hyperlipidemia, hypertension, neurodegenerative disease and cancer (13). Phytochemical studies have reported that alkaloids, flavonoids, flavones, flavanones, stilbenes, benzophenones, coumarin derivatives, terpenoid stilbenes, oxyresveratrol and resveratrol were present in *Morus* species (16-18). Moreover, oxyresveratrol can suppress inflammation by inhibiting nitric oxide (NO) production, inducible NO synthase expression, prostaglandin E2 production and NF-κB activation in macrophages (19). Moracin R, C, O, P and D belong to the 2-arylbenzofuran group, and display inhibitory activity against the differentiation of 3T3-L1 adipocytes and NO production in RAW264.7 cells (20). The 2-arylbenzofurans of *Morus* species, moracin O and P, markedly suppress hypoxia-inducible factor-1 (HIF-1) activity in the human Hep3B hepatocellular carcinoma cell line (21-23). Moracin-M-3'-O-β-D-glucopyranoside has been reported to suppress 12-O-tetradecanoylphorbol-13-acetate-induced tumor progression in mouse skin, and the underlying mechanism may involve the inhibition of leukocyte infiltration, epidermal hyper-proliferation, oxidative stress, and the endogenous tumor promoter TNF (24).

NF-κB plays a central role in inflammation, immunity and several other cellular responses (25-27). A variety of ligands and receptors can activate the NF-κB signaling pathway, including TNF and TNF receptor superfamily molecules that are key to inflammatory responses (24,28). Amongst these TNF superfamily members, TRAIL is known to be a potent inducer of apoptosis through activation of inflammatory signaling pathways (29,30). In the present study, TRAIL induced cellular damage of HaCaT keratinocytes through NF-κB activation. Moreover, both moracin O and P significantly protected HaCaT cells against TRAIL-induced cytotoxicity, possibly through the inhibition of apoptosis. Considering that NF-κB-mediated inflammation has been implicated in the regulation of cellular damage by balancing anti- and pro-apoptotic protein expression (31-34), moracin O and P may protect against HaCaT cell damage through their anti-inflammatory activity by targeting the NF-κB pathway. In the present study, moracin O and P as novel anti-inflammatory compounds inhibiting cellular damage induced by NF-κB activation.

The aforementioned effects of Sohakuhi extract and its active components, moracin O and P, suggested that these compounds may prove beneficial for the treatment of inflammatory diseases. Although the exact mechanism of action remains to be determined, the present study demonstrated that both moracin O and P represent promising phytochemicals that may act as novel anti-inflammatory or cytoprotective agents through the suppression of the NF-κB pathway.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BH, LU and FL performed the experiments and analyzed the data. SY and YH analyzed and interpreted the data. BH and YH wrote the manuscript and YH designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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