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Research Article

UHPLC/TOFHRMS analysis and anti-inflammatory effect of leaf extracts from *Zizyphus jujuba* in LPS-stimulated RAW264.7 cells

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Abstract *Zizyphus jujube* is a plant in the buckthorn family (Rhamnaceae) that has been the subject of research into antibacterial, antifungal and anti-inflammatory properties of its fruit and seed. However, few studies have investigated its leaves. In this study, the anti-inflammatory activity of ZJL (an extract of *Z. jujube* leaf) was evaluated to verify its potential as an anti-inflammatory agent and SARS-CoV-2 medicine, using nitric oxide (NO) assay, RT-PCR, SDS-PAGE, Western blotting, and UHPLC/TOFHRMS analysis. We found that ZJL suppresed pro-inflammatory mediators such as NO, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and tumor necrosis factor α (TNF-α) in lipopolysaccharide (LPS)-induced RAW264.7 cells. ZJL acted by inhibiting NF-KB and MAPK signaling pathway activity. We also confirmed that ZJL contains a phenol compound and flavonoids with anti-inflammatory activity such as trehalose, maleate, epigallocatechin, hyperoside, catechin, 3-O-coumaroylquinic acid, rhoifolin, gossypin, kaempferol 3-neohesperidoside, rutin, myricitrin, guaiaverin, quercitrin, quercetin, ursolic acid, and pheophorbide a. These findings suggest that ZJL may have great potential for the development of anti-inflammatory drugs and vaccines via inhibition of NF-κB and MAPK signaling in LPS-induced RAW264.7 cells.

Keywords Anti-inflammation, MAPK, NF-κB, UHPLC/TOFHRMS, *Ziziphus jujuba*

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) first emerged in Wuhan, China in December 2019. It is a type of highly pathogenic human coronavirus (HCoV) that poses a major threat to public health and causes disease in animals. Most coronavirus disease 2019 (COVID-19) patients had a good prognosis, but there were still some serious illnesses and deaths. One of the key features of COVID-19 is excessive inflammation observed in some patients, particularly those with severe illness. An exaggerated immune response mediated by a series of cytokines plays a key role in the development of disease. Studies have reported that several types of inflammatory mediators and immune cells are involved in the disease process (Saeedi-Boroujeni and Mahmoudian-Sani 2021; Zabetakis et al. 2020). The health status of our industrial population is at risk due to numerous inflammatory chronic diseases characterized by widespread and potentially low-grade inflammation. Therefore, researchers around the world have explored several anti-inflammatory and other pharmacological treatments to combat deadly inflammation (Tasinov et al. 2021; Tay et al. 2020).

Zizyphus jujuba Mill is one of the most important plants of the Rhamnaceae family (Ghani et al. 2022). *Z. jujuba* is a small deciduous tree that occurs naturally in Europe, East Asia and Australia. Many studies have been reported on jujube fruit and seed preparations that have been stimulated by their long-term use in Chinese and Korean traditional medicine for their antibacterial, antifungal, anti-inflammatory, antioxidant activity and anticancer properties (Damiano et al. 2017). *Z. jujuba* contains a wide range of constituents, including flavonoids such as quercetin, rutin, isoquercitrin, nicotiflorine and kaempferol, phenolic acid, triterepenoic acid and amino acids (Hong et al. 2015; Lee et al. 2004; Lee et al. 2016).

In this study, we evaluated the anti-inflammatory effect

of *Z. jujuba* leaf in LPS-induced RAW264.7 cells and identified the regulatory mechanisms of NF-κB (nuclear factor kappa B) and MAPK (mitogen activated protein kinase) as molecular targets. In addition, by analyzing the components of *Z. jujuba* leaf, we tried to evaluate the possibility of using them as anti-inflammatory agents.

Materials and Methods

Materials

As a medium for culturing mouse macrophages (RAW264.7 cells), Dulbecco's Modified Eagle's medium (DMEM) was purchased from Lonza (Walkersville, MD, USA). Griess reagent used for detection and quantification of nitrite and nitrite ion and Thiazolyl Blue Tetrazolium Blue (MTT) for cytotoxicity evaluation and lipopolysaccharide (LPS) for anti-inflammatory evaluation were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for western blot were purchased from Cell Signaling Technology (Danvers, MA, USA).

Preparation of extracts

Z. jujuba leaf (ZJL) was collected in June 2019 in Andong-si, Gyeongsangbuk-do, Korea, and were identified by Professor Jeong Kyu-young, Andong University (voucher number. FMRC-2190621A1). 20 g of freeze-dried ZJL was extracted with 100 mL of 70% ethanol (70% EtOH) for 48 hours while stirring at room temperature. After 48 hours, the 70% EtOH extract was filtered, concentrated using a vacuum evaporator (N-1000, EYELA, Tokyo, Japan), and then freeze-dried (recovery rate after sample extraction: 55.6 ± 4.1%). ZJL extract was dissolved in dimethyl sulfoxide (DMSO) and used in the experiments.

Cell culture

RAW264.7 cells were adopted from the American Type Culture Collection (ATCC, Virginia, USA). RAW264.7 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO2. Cells were treated with ZJL dissolved in DMSO used as a vehicle at a final concentration of less than 0.1% (v/v).

MTT assay

RAW264.7 cells (1×10^6 cells/well) cultured in a 12-well plate were treated with ZJL for 24 hours. After 24 hours, 200 μL of MTT solution (1 mg/mL) was added and incubated for 2 hours. After 2 hours, the medium was removed and DMSO was added to the cells. The absorbance of the solution dissolved by DMSO was measured at 570 nm using a UV/visible spectrophotometer (Perkin Elmer, Norwolk, CT, USA).

Nitric oxide (NO) assay

RAW264.7 cells cultured in a 12-well plate (1×10^6 cells/well) for 24 hours were pretreated with ZJL for 6 hours. After 6 hours, RAW264.7 cells were co-treated with LPS (1 μg/mL) for 18 hours to induce an inflammatory response. After 18 hours, 50 μL of cell medium and 50 μL of Griess reagent were mixed in a 1:1 ratio. After reacting at room temperature for 10 minutes, absorbance was measured with a UV/Visible spectrophotometer 540 nm (Perkin Elmer, Norwalk, Connecticut, USA).

Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) and Western blot

After the experiment, RAW264.7 cells were harvested after washing three times with cold 1X phosphate buffered saline (1XPBS). RAW264.7 cells were lysed in 1X radioimmunoprecipitation assay (RIPA) buffer (Cell signaling, Bervely, MA, USA) and centrifuged at 15,000 rpm for 10 minutes at 4°C. Cellular protein was quantified using BCA protein assay (Thermo Fisher Scientific, Waltham, MA USA). Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (Bio-Rad, CA, USA). After 1 hour of blocking (5% skim milk), the membrane was treated with primary antibody (1:1000) overnight. After overnight, they were washed three times with 0.05% Tween 20 and treated with secondary antibody (1:200) for 1 hour at room temperature. After ECL Western blotting was applied to the membrane, it was analyzed using the Chemi Doc MP Imaging system (Bio-rad, CA, USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNeasy Mini Kit (Qiagen, Valencia, CA, USA) was used to extract total RNA from RAW264.7 cells after the experiment. To synthesize cDNA, 1 μg of total RNA was processed with the Verso cDNA synthesis kit. After cDNA synthesis, PCR was performed using a PCR master mix kit (Promega Co., Madison, WI, USA). Primers used for PCR (mouse iNOS, mouse COX-2, mouse TNF-α and

mouse GAPDH) are listed in Table 1.

Ultra-high performance liquid-chromatography/time-of-flight high-resolution mass spectrometry (UHPLC/TOFHRMS) analysis

UHPLC instrument (Therom Dionex, USA) equipped with a Waters Cortect C18 (2.1 mm × 150 mm, 1.8 μm, Water, USA) column was used. 0.1% Formic acid in water (A) and 0.1% Formic acid in acetonitrile (B) were used as mobile phase at a flow rate of 0.3 mL/min. The system was run with the following gradient program: 1% B for 1 min, 1%-30% B for 1-13 min, 21%-100% B for 13-21 min, maintained until the 24th min, 100%-1% B for 24-25 min, and maintained until the 30 min. The temperature of the column was maintained at 45 °C, and the injection volume was 3 μL.

Statistical analysis

All results were expressed as mean ± standard deviation after repeated measurements three times, and significance between treatments was verified by Student's t-test. When p-value < 0.05, it was determined to be statistically significant.

Results and Discussion

The effect of ZJL on NO production and cytokines expression in LPS-stimulated RAW264.7 cells

COVID-19 infection is associated with an aggressive inflammatory response that releases large amounts of proinflammatory cytokines in an event known as a "cytokine storm." The host immune response to the SARS-CoV-2 virus is hyperactive, resulting in an excessive inflammatory response (Ragab et al. 2020). Therefore, suppression of excessive cytokines such as *iNOS*, *COX-2*, and *TNF-α* is the key to anti-inflammatory effect. The effect of ZJL on the inhibition of NO production in LPS-induced RAW264.7 cells was investigated. MTT assay showed that ZJL (0, 12.5, 25, 50 μg/ml) was not toxic to RAW264.7 cells (Fig. 1A). As shown in Fig. 1B, ZJL reduced LPS-induced excessive production of NO in RAW264.7 cells in a concentration dependent manner (0, 12.5, 25, 50 μg/ml). Therefore, macrophages are RAW264.7 cells stimulated with LPS to confirm the inhibitory activity of inflammatory mediator gene expression, such as *iNOS*, *COX-2*, and *TNF-α*, which are known to be involved in the pathogenesis of inflammatory diseases. In Fig. 1C, it was confirmed that ZJL decreased the expression of *iNOS*, *COX-2* and *TNF-α* in a concentration-dependent manner as affecting the expression of inflammatory derivatives. From these results, it was confirmed that ZJL can exhibit anti-inflammatory activity.

Effect of ZJL on NF-κB signaling activation in LPS-stimulated RAW264.7 cells

The basic mechanism of NF-κB activation is the inducible degradation of IκBα triggered by the multisubunit IκB kinase (IKK) complex. IKKs can be activated by cytokines, mitogens, microbial components, growth factors and infectious agents. Upon stimulation, NF-κB induces the expression of various pro-inflammatory cytokine genes. These pro-inflammatory cytokines further activate NF-κB signaling in an autocrine manner. SARS-CoV-2 appears to activate NF-κB and produce proinflammatory cytokines that correlate with mechanisms of COVID-19 pathogenesis. Because pro-inflammatory cytokines are elevated in patients with severe COVID-19. Indeed, NF-κB is activated in SARS-CoV-2 infected cells (Kircheis et al. 2020; Su et al. 2021). Therefore, in order to examine the effect of ZJL on the inhibition of NF-κB activity, it was confirmed that when LPS-derived RAW264.7 cells were treated with ZJL, IκB phosphorylation and degradation were inhibited in a

Table 1 Sequences of oligonucleotide primers used for RT-PCR

NO.	Gene name	Sequence
1	*iNOS*	Forward 5′-GTG CTG CCT CTG GTC TTG CAA GC-3′ Reverse 5′-AGG GGC AGG CTG GGA ATT CG-3′
2	*COX-2*	Forward 5′-GGA GAG ACT ATC AAG ATA GTG ATC -3′ Reverse 5′-ATG GTC AGT AGA CTT TTA CAG CTC -3′
3	*TNF-α*	Forward 5'-TAC TGA ACT TCG GGG TGA TTG GTC C-3' Reverse 5'-CAG CCT TGT CCC TTG AAG AGA ACC-3'
4	*GAPDH*	Forward 5′-CAG GAG CGA GAC CCC ACT AAC AT-3′ Reverse 5′-GTC AGA TCC ACG ACG GAC ACA TT-3′

concentration-dependent manner (Fig. 2A and B). As shown in 2C, it was confirmed that ZJL inhibits nuclear translocation of p65. These results suggest that ZJL has an anti-inflammatory effect by inhibiting NF-κB activity.

Effect of ZJL on MAPKs signaling activation in LPS-stimulated RAW264.7 cells

Several investigations have concluded that SARS-CoV-2 is a major factor in the increased cytokine inflammatory

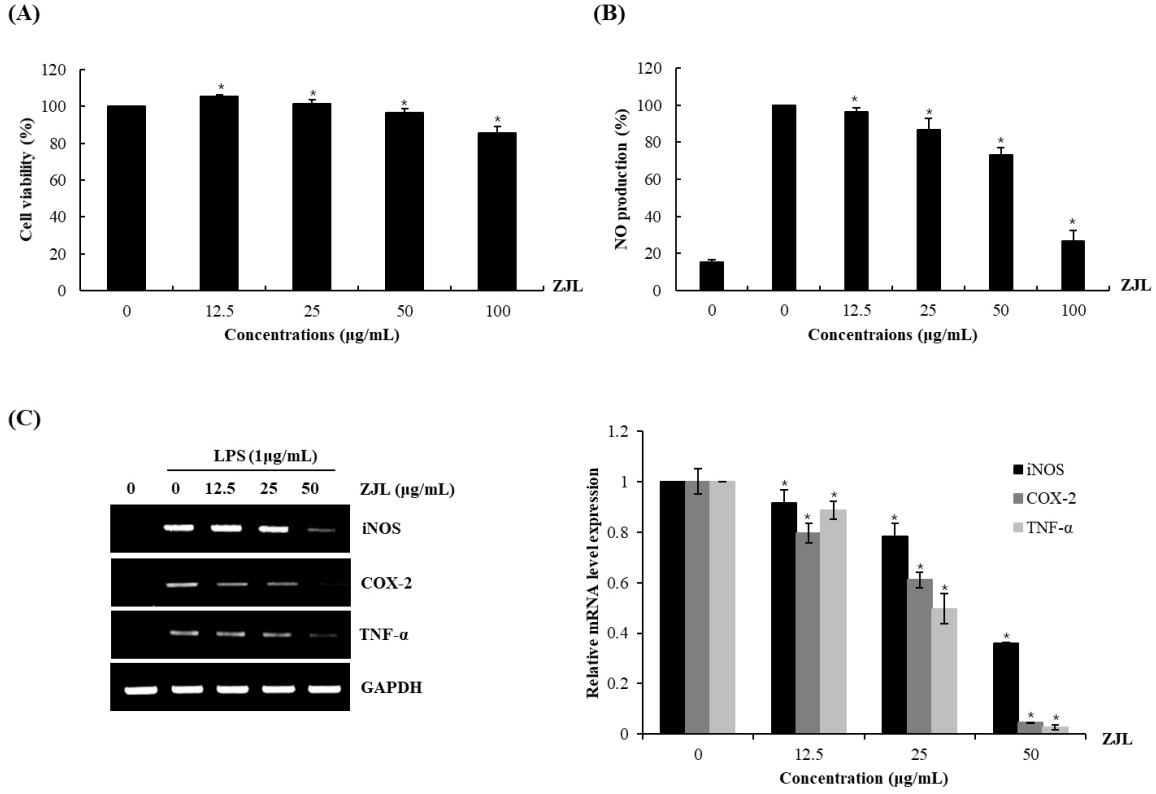

Fig 1 Effect of ZJL on the NO production of pro-inflammatory mediators such as *iNOS*, *COX-2* and *TNF-α* in LPS-stimulated RAW264.7 cells. (A) RAW264.7 cells were treated with 25, 50, 100 μg/ml of ZJL for 24 hours, then an MTT assay was performed. (B) RAW264.7 cells were pretreated with 25, 50, 100 μg/ml of ZJL for 6 hours, then co-treated with 1 μg/mL of LPS for 18 hours, and then a Griess assay was performed. (C) RT-PCR analysis of total RNA. GAPDH was used as an internal control. *: significant difference ($P < 0.05$) to cells without ZJL

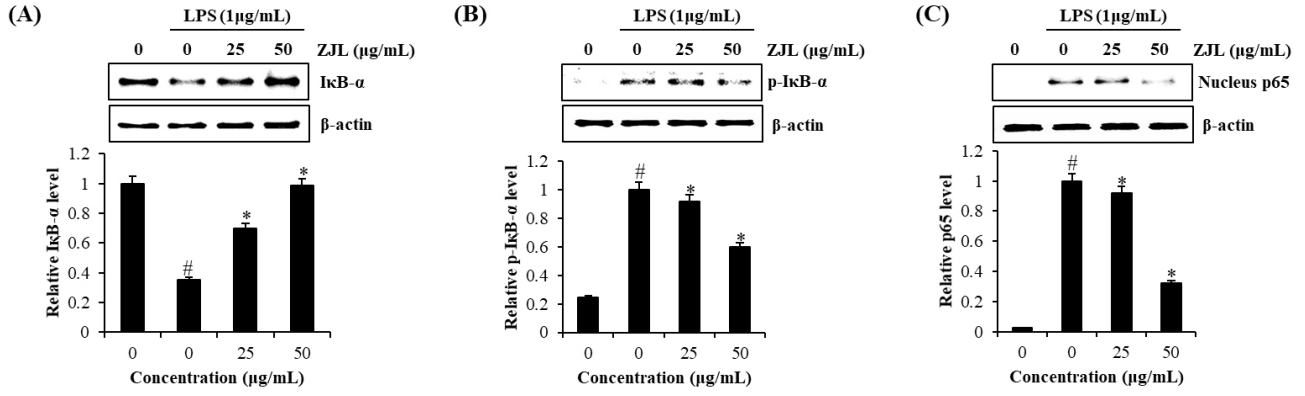

Fig 2 Effects of ZJL on NF-κB signaling activation in LPS-stimulated RAW264.7 cells. (A, B) RAW264.7 cells were pretreated with ZJL (0, 25, 50 μg/mL) for 6 hours and then co-treated with LPS (1 μg/mL) for 40 min. (C) RAW264.7 cells were pre-treated with ZJL (0, 25, 50 μg/mL) for 40 min. After the treatment, the cytosol and nucleus were prepared. For Western blot analysis, cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibodies against IκB-α, p-IκB-α and nucleus p65. β-actin was used as an internal control. #: signficant difference ($P < 0.05$) to cells without treatment; *: significant difference ($P < 0.05$) to cells treated with LPS only

response associated with COVID-19 through activation of MAPK/NF-κB signaling. Elevated MAPKs signaling pathways including proteins such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 control p65/NF-activation for cytokine production (Hariharan et al. 2021; Sharma et al. 2022; Ye et al. 2020). We investigated whether ZJL inhibits phosphorylation of ERK1/2, p38 or JNK, elucidating the inhibitory effect of ZJL on MAPKs signaling activation in LPS-induced RAW264.7 cells. Phosphorylation of ERK1/2 was inhibited in a concentration dependent manner (Fig. 3A). As shown in Fig. 3B, it was confirmed that ZJL inhibits p38 phosphorylation. JNK phosphorylation was inhibited in a concentration-dependent manner (12.5, 25, and 50 μg/ml). ZJL demonstrated anti-inflammatory potential in LPS-induced RAW264.7 cells by downregulating the MAPKs signaling pathway.

HUPLC/TOFHRMS analysis of anti-inflammatory compounds in ZJL

To analyze the bioactive compounds with anti-inflammatory activity from ZJL, we performed HUPLC/TOFHRMS analysis. As shown in Table 2, ZJL was analyzed to contain compounds such as trehalose (Echigo et al. 2012), maleate (Vijeesh et al. 2022), epigallocatechin (Wu et al. 2017), hyperoside (Kim et al. 2011), catechin (Fan et al. 2017; Kim and Heo, 2022), 3-O-coumaroylquinic acid, rhoifolin (Eldahshan and Azab, 2012; Peng et al. 2020), gossypin

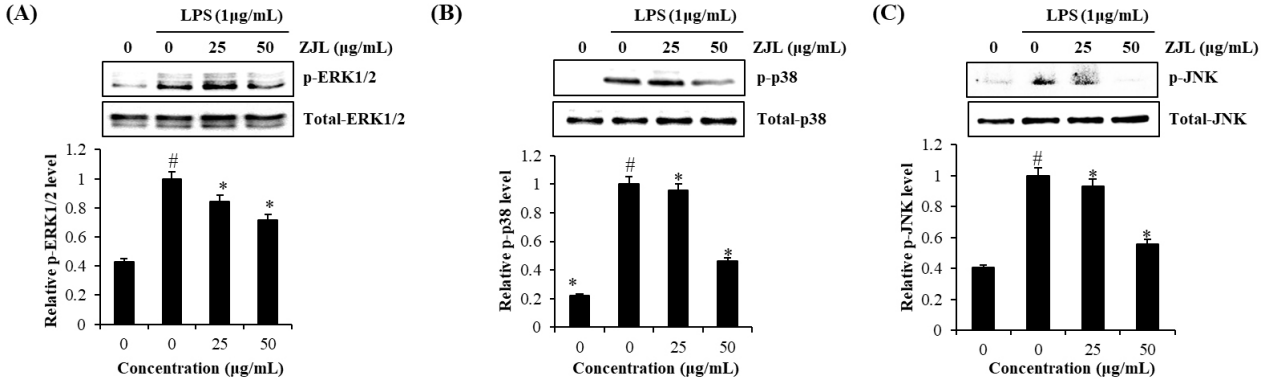

Fig 3 Effects of ZJL on MAPKs signaling activation in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pretreated with ZJL (0, 25, 50 μg/mL) for 6 h and then co-treated with LPS (1 μg/mL) for 40 min. For Western blot analysis, the cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibodies against p-p38, p-ERK1/2, and p-JNK. #: significant difference ($P < 0.05$) to cells without treatment; *: significant difference ($P < 0.05$) to cells treated with LPS only

Table 2 Results of UHPLC/HRMS analysis of 16 functional components in ZJL

NO.	Retention time (min)	Chemical	Molecular formula	Molecular weight
1	1.16	Trehalose	$C_{12}H_{14}O_6$	342.1
2	1.2	Maleate	$C_4H_4O_4$	116
3	6.85	Epigallocatechin	$C_{15}H_{14}O_7$	306.1
4	6.93	Hyperoside	$C_{21}H_{20}O_{12}$	464.1
5	7.14	Catechin	$C_{15}H_{14}O_6$	290.1
6	8.49	3-O-Coumaroylquinic acid	$C_{16}H_{18}O_8$	338.1
7	8.5	Rhoifolin	$C_{27}H_{30}O_{14}$	578.2
8	9.59	Gossypin	$C_{21}H_{20}O_{13}$	480.1
9	10.11	Kaempferol 3-neohesperidoside	$C_{27}H_{30}O_{15}$	10.11
10	10.37	Rutin	$C_{27}H_{30}O_{16}$	610.2
11	10.47	Myricitrin	$C_{21}H_{20}O_{12}$	464.1
12	11.22	Guaiaverin	$C_{20}H_{18}O_{11}$	434.1
13	11.67	Quercitrin	$C_{21}H_{20}O_{11}$	448.1
14	14.19	Quercetin	$C_{15}H_{10}O_7$	302
15	21.78	Ursolic acid	$C_{30}H_{48}O_3$	456.4
16	22.58	Pheophorbide a	$C_{35}H_{36}N_4$	592.3

(Cinar et al. 2019), kaempferol 3-neohesperidoside (Zanatta et al. 2008), rutin (Guardia et al. 2001), myricitrin (Domitrović et al. 2015), guaiaverin (Comalada et al. 2005; Tasinov et al. 2021), quercitrin (Lee et al. 2016), quercetin (Tang et al. 2019), ursolic acid (Checker et al. 2012) and phephorbide a (Nakamura et al. 1996) by UHPLC/MSMS. The compounds have been reported to anti-inflammatory activity.

In conclusion, these results suggest that ZJL can be used as an alternative supplement for anti-inflammatory and COVID-19 treatment (vaccine). Isolation of anti-inflammatory substances and identification of mechanisms are considered necessary in the future.

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