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Inhibition of LPS-induced expression of iNOS and COX-2 on extracts of *Acanthopanax leucorrhizus* (Oliv.) Harms stems

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Abstract

Acanthopanax leucorrhizus is an endemic medicinal plant growing abundantly in the northwest region of China. The roots and stem barks have been widely used to treat rheumatism, numbness, contracture, quadriplegia, hemiplegia, traumatic injury, edema, and itchy skin, although the anti-inflammatory effects of the extracts of *A. leucorrhizus* have not been assessed until now. To investigate the anti-inflammatory effects on macrophages of various extracts from *A. leucorrhizus* stems, alterations in the inducible nitric oxide synthase (iNOS)-mediated cyclooxygenase-2 (COX-2) expression and inflammatory cytokine production were measured in lipopolysaccharide (LPS)-activated RAW264.7 cells. Briefly, the dichloromethane extract prepared from stems of *A. leucorrhizus* (ALSDC) effectively inhibited NO production in LPS-stimulated cells and significantly reduced the production of pro-inflammatory cytokines IL-6 and TNF-α at a dose of 40 μg/mL. We also confirmed a dose-dependent and significant inhibition of iNOS and COX-2 protein expression. In conclusion, ALSDC exerted strong inhibitory effect on the expression of iNOS and COX-2 protein in LPS-induced RAW 264.7 macrophages and these results provide strong evidence to suggest that ALSDC may be considered as an important candidate for the treatment of inflammatory-related diseases in the future.

Keywords: *Acanthopanax leucorrhizus* (Oliv.) Harms; dichloromethane extract; anti-inflammatory activities; iNOS; COX-2; RAW 264.7 macrophages.

Practical Application: 1. Dichloromethane extract of *Acanthopanax leucorrhizus* stems (ALSDC) effectively inhibited NO production in LPS-stimulated cells and significantly reduced the production of pro-inflammatory cytokines IL-6 and TNF-α; 2. ALSDC exerted significant inhibition of iNOS and COX-2 protein expression; 3. *A. leucorrhizus* stems may be considered as an important candidate for the treatment of inflammatory-related diseases in the future; 4. This research provides evidence of the possible beneficial health advantages of *A. leucorrhizus* and furnished a reliable theoretical and practical basis for the developing and utilizing *Acanthopanax* species.

1 Introduction

Inflammation, a common but complex reaction after the immune system recognizes external pathogens or damaged cells, occurs in all types of human tissues and usually presents a protective effect (Wang et al., 2019; Lee et al., 2020a). However, abnormal inflammation has been reported to be related to several human chronic diseases, including rheumatoid arthritis, atherosclerosis, and diabetes (Hou et al., 2018). Lipopolysaccharide (LPS) is a potent activator of macrophage cells, which produce a variety of pro-inflammatory mediators such as nitric oxide (NO), and inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Bak et al., 2012). NO has been implicated in the pathogenesis of a variety of inflammatory diseases and is synthesized from L-arginine by inducible NO synthase (iNOS) (Lee et al., 2017). High levels of NO play a key role in disease pathophysiology as a free radical. Moreover, cyclooxygenase-2 (COX-2) is also involved in the pathology of chronic inflammation (Lee, et al., 2020b). Thus, the inhibition of these pro-inflammatory mediators may be an effective strategy for the treatment of inflammatory diseases (He et al., 2020; Je et al., 2021).

Acanthopanax spp., one of the important medicinal genera of Araliaceae, is a plant genus that embraces over 70 plant species that are widely distributed in Asian countries such as China, Korea, Thailand, and Japan (Yanchong, 2012). Their roots and stem barks have traditionally been used as a tonic, as well as in the treatment of rheumatism and diabetes, chronic, bronchitis and so on (Li et al., 2016; Zou, et al., 2017; Chinese Pharmacopoeia Commission, 2015). Moreover, their leaves and roots are popularly used as a health drink and drug in China and Korea (Yook et al., 1999; Hu et al., 2012; Hu et al., 2018a; Nie et al., 2021; Chen et al., 2021). *Acanthopanax leucorrhizus* (Oliv.) Harms is an endemic medicinal plant growing abundantly in the northwest region of China, and is often used as folk medicines (State Administration of Traditional Chinese Medicine, 1999). *A. leucorrhizus* possess multiple biological functions such as antibacterial, antioxidant, anti-tumor and so on (Hu et al., 2018b; Hu et al., 2020). Especially its stem barks have been used for a long time to treat rheumatism, numbness, contracture, quadriplegia, and so on (Hu et al., 2012). Although many plants of *Acanthopanax* species have been proved to improve the

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symptoms of the inflammation, such as *A. senticosus* (Fei et al., 2014; Fan et al., 2019; Jin et al., 2020), *A. gracilistylus* (Liu et al., 2017; Zou et al., 2017; Xu et al., 2018; Luo et al., 2021a), and *A. trifoliatus* (Wang et al., 2015; Chien et al., 2015; Luo et al., 2021b), the mechanism of *A. leucorrhizus* stems on its anti-inflammation activity is still not well known. For the purpose of developing and utilizing *Acanthopanax* species, we focused our study on the anti-inflammatory properties of various fractions of *A. leucorrhizus* stems through the effects on the production of NO, TNF-α, and IL-6, as well as on the expression of iNOS and COX-2 by LPS-induced RAW 264.7 macrophages.

2 Materials and methods

2.1 Plant materials

The stems of *A. leucorrhizus* were collected in October 2015 in Funiu mountain (China). The plant species was confirmed by Professor Xiang-Qian Liu (Hunan Key Laboratory of Traditional Chinese Medicine modernization, Hunan University of Chinese Medicine, Changsha, China), and the voucher specimen (no. 20151001) was deposited at the School of Pharmacy, Hunan University of Chinese Medicine (Changsha, China).

2.2 Preparation of sample

The air-dried stems of *A. leucorrhizus* were ground into a fine powder. The powder (30 g) was decocted with distilled water on reflux extraction for 2 h and the other powder (200 g) was decocted with methanol (MeOH, 2 × 2 h) on reflux extraction before filtration. The water extract of *A. leucorrhizus* stems (ALSW) was freeze-dried, while the methanol extract of *A. leucorrhizus* stems (ALSM) was vacuum dried, the methanol extract was partitioned into H_2O and extracted successively with petroleum ether (PE, 60-90), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAC), *n*-butanol (*n*-BuOH) to obtain a petroleum ether extract of *A. leucorrhizus* stems (ALSPE), CH_2Cl_2 extract of *A. leucorrhizus* stems (ALSDC), EtOAc extract of *A. leucorrhizus* stems (ALSEA), and *n*-BuOH extract of *A. leucorrhizus* stems (ALSBU). All fractions were stored at 4 °C until analyzed. Each fraction was dissolved in dimethyl sulfoxide (DMSO) for functional assays.

2.3 Cell viability

RAW264.7 cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Amresco, Solon, OH, USA) assay (Park et al., 2015). The cells [American Type Culture Collection (ATCC); Manassas, VA, USA] were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) with 100 μL of 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA) at 37 °C in 5% CO_2 atmosphere. All of the cells were seeded at a density of 1×10^5 cells/mL in 96-well plates. After seeding the cells for 5 h, samples with various concentrations (10, 20, and 40 μg/mL) were added. After another 20 h, 5μL of MTT solution (5 mg/mL) was added to each well and the cells were cultured for a further 4 h. Then the plates were removed and 100 μL of dimethyl sulfoxide were added in each well to dissolve the formazan crystals. The absorbance at 570 nm was read on a microplate reader (Spark10M, TECAN, Switzerland) as a measure of cell viability.

2.4 NO and inflammatory cytokine assays

The RAW 264.7 cells were seeded into a 96 well plate at a density of 2.5×10^4 cells/well and allowed to adhere for 4 h. Then, cells were pretreated with various concentrations of samples (10, 20, and 40 μg/mL) for 1 h and stimulated with 5 μL of lipopolysaccharide (LPS) at 0.5 μg/mL for 24 h. NO accumulation, the cell supernatant was harvested and reacted with the Griess reagent [0.1% N-(1-naphathyl)-ethylenediamine, 1% sulfanilamide in 5% phosphoric acid] for 10 min at room temperature in the dark. The absorbance at 540 nm was detected and the concentration was calculated using a nitrite standard solution.

The levels of IL-6 and TNF-α were measured using a commercial BD OptEIA™ ELISA kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocols.

2.5 Western blot analysis

Total protein (30 μg) was separated on a 10% SDS polyacrylamide gel and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Each membrane was then incubated for 1 h in 5% skim milk in TBS-T buffer (0.1 M Tris-HCl, pH 7.4, 0.9% NaCl, 0.1% Tween-20) to block non-specific binding and was then incubated with primary antibodies that recognized iNOS (Cat. no. ADI-905-431, 1:1,000; obtained from Enzo Life Sciences, Farmingdale, NY, USA), COX-2 (Cat. no. sc-1747, 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (Cat. no. MA5-15379, 1:2,000; Cell Signaling Technology, Danvers, MA, USA), Each protein was detected using a chemiluminescence detection system according to the instructions of the manufacturer (ECL; Amersham, Berkshire, UK).

2.6 Statistical analysis

The results are expressed as the means ± SEM of the sample size determinations. Statistical significance was determined using a two-tailed Student's t-test for independent means. The test results are reported as two-tailed *P*-values, where $P < 0.05$ was considered to indicate a statistically significant difference.

3 Results

3.1 Effect of extracts from ALS on the viability of RAW264.7 macrophages

As the potential food addictive or medicinal application, the component should be harmless, without undesirable cytotoxic side effects (Wang et al., 2019). To evaluate different extracts of *A. leucorrhizus* stems, the cytotoxicity on LPS-stimulated RAW264.7 macrophages was determined at concentration of 20 μg/mL using the MTT assay. As shown in Figure 1A, the results of this analysis demonstrated that four fractions of *A. leucorrhizus*, concluding water extract (ALSW), petroleum ether extract (ALSPE), CH_2Cl_2 extract (ALSDC), and *n*-BuOH extract (ALSBU), produced no significant change in cell viability

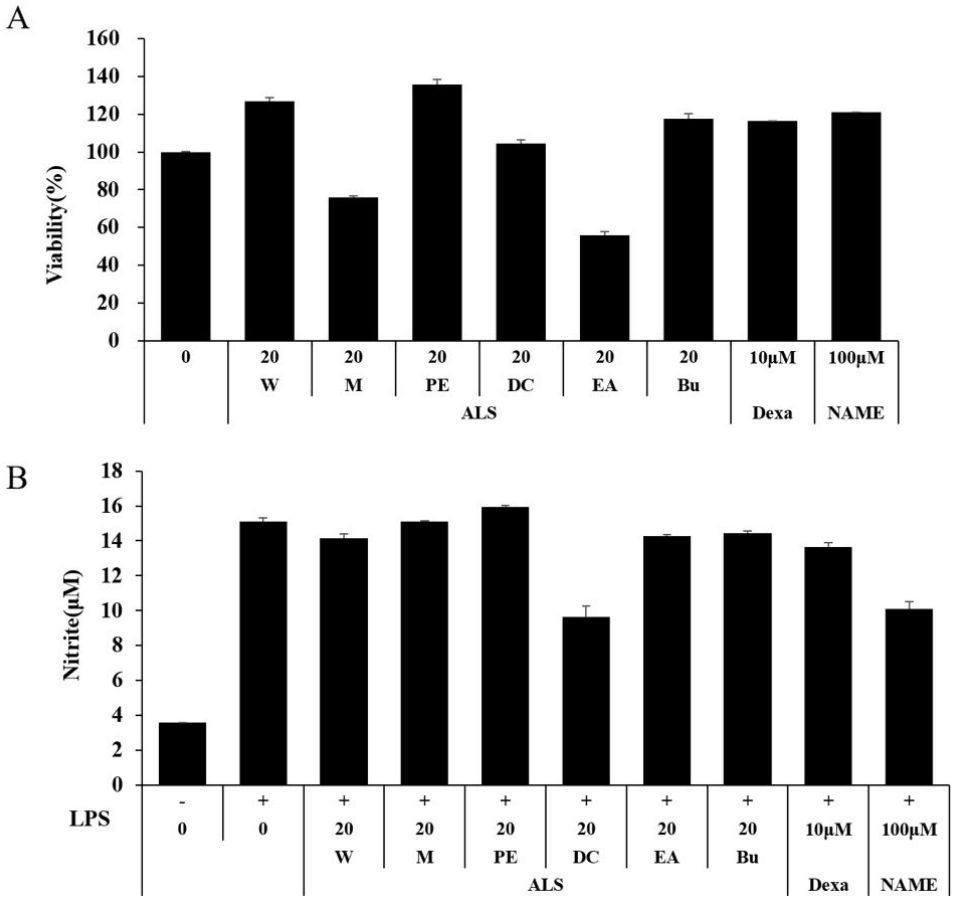

Figure 1. Effect of different solvent extracts from *A. leucorrhizus* stems on viability and NO production of LPS-induced RAW264.7 macrophages. Cells were pretreated for 1 h with six different fractions (20 μg/mL) and then treated with LPS (0.5 μg/mL) for 24 h. (A) Cell viability was measured using an MTT assay; (B) NO production was measured using a Griess reagent assay; Dexamethasone (Dexa) and L-NG-monomethyl arginine (L-NMMA) were used as a positive control. Data were denoted as the mean ± SEM (n = 3).

compared to that in an untreated control group. However, methanol extract (ALSM) and EtOAc extract (ALSEA) had cellular survival rates of 75.85 ± 0.68% and 55.73 ± 1.51%.

3.2 Effect of extracts from ALS on NO production of RAW264.7 macrophages

To examine whether extracts from ALS treatment could modulate NO production, we measured the NO secretion in LPS-induced RAW 264.7 cells after various extracts of *A. leucorrhizus* stems treatment separately at concentration of 20 μg/mL, using a Griess reagent assay. As shown in Figure 1B, LPS treatment significantly induced NO production compared to that in the untreated control, while cells pretreated with ALSDC demonstrated a significant inhibition of NO production.

Then, the further study determined the viability and NO production of ALSDC at different concentrations (10, 20, and 40 μg/mL) before the next inflammatory cytokine assays. As shown in Figure 2A-B, There was no significant change in cell survival at concentrations (10, 20, and 40 μg/mL) of ALSDC, which greatly reduced the production of NO in dose-dependent manner.

3.3 Effect of ALSDC on the expression of IL-6 and TNF-α in RAW264.7 macrophages

To determine whether the ability of ALSDC to inhibit inflammatory signaling corresponded to a reduction in the secretion of pro-inflammatory cytokines, we investigated cytokine secretion in LPS-activated macrophages using ELISA kits. As shown in Figure 2C-D, at a dose of 40 μg/mL, ALSDC treatment dramatically decreased the production of the pro-inflammatory IL-6 and TNF-α.

3.4 Effect of ALSDC on iNOS and COX-2 protein expression

We carried out Western blotting to analyze the inhibitory effects of ALSDC on expressions of iNOS and COX-2. The RAW264.7 macrophage were pretreated at 10, 20, and 40 μg/mL, and then stimulated with LPS (0.5 μg/mL) for 24 h. The inflammatory mediators, iNOS and COX-2, reflect the states of inflammation are often used to estimate the severities of the inflammation. The stimulations of LPS led the expression of three proteins upregulating, as shown in Figure 3. As we predicted, their expression levels were greatly down-regulated by ALSDC in a concentration-dependent manner. These results suggest that

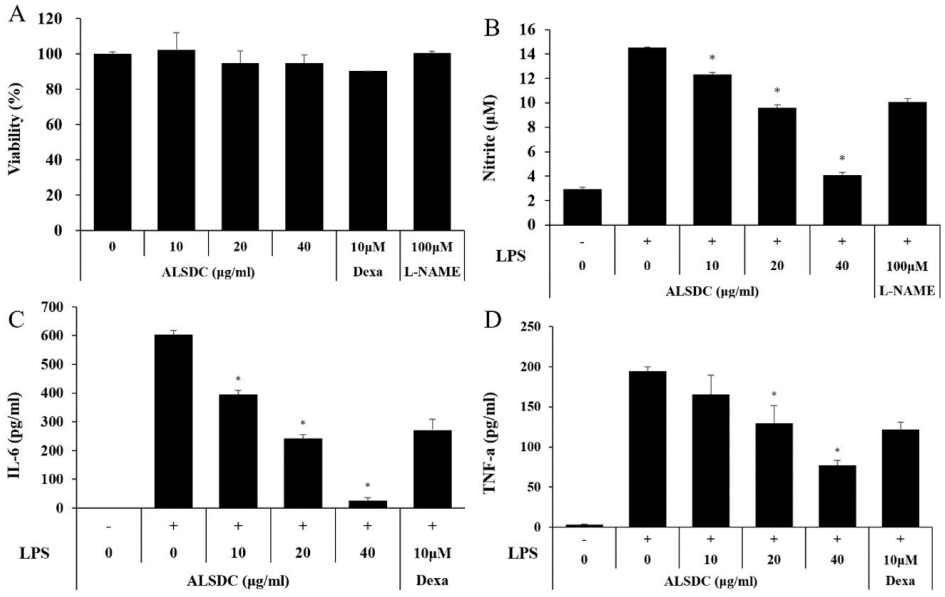

Figure 2. Effect of ALSDC on IL-6 and TNF-α production in LPS-induced RAW 264.7 macrophages. Cells were pretreated for 1 h with ALSDC (10, 20, and 40 μg/mL) and then treated with LPS (0.5 μg/mL) for 24 h. (A) Cell viability was measured using an MTT assay; (B) NO production was measured using a Griess reagent assay; Dexamethasone (Dexa) and L-NG-monomethyl arginine (L-NMMA) were used as a positive control. (C) IL-6 and (D) TNF-α contents in the culture medium were determined by ELISA kits. Dexamethasone (Dexa) was used as positive control. Data were denoted as the mean ± SEM (n = 3). $*P < 0.05$, relative to control group.

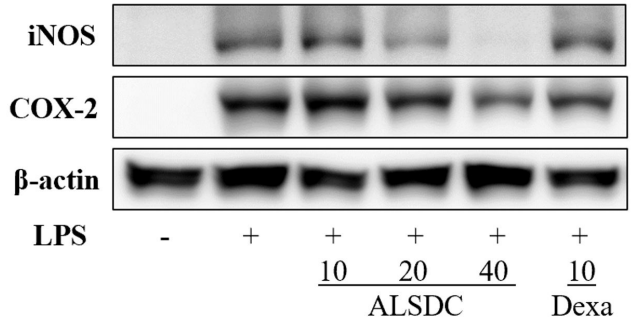

Figure 3. Effect of ALSDC on LPS-induced iNOS and COX-2 protein expression in RAW264.7 cells. Cells were pretreated for 1 h with ALSDC (10, 20, and 40 μg/mL) and then treated with LPS (0.5 μg/mL) for 24 h. iNOS and COX-2 and β-actin were detected by Western blot analysis.

the extract plays an anti-inflammatory role in LPS stimulated macrophage RAW 264.7 cells.

4 Discussion

Many previous studies on natural herbal medicines have been conducted to find the potential natural anti-inflammatory products through *in vitro* and *in vivo* systems (Liu et al., 2017). *A. leucorrhizus* is an important constituent of traditional Chinese medicine, which has been used since ancient times to treat various diseases, such as rheumatism, numbness, contracture, hemiplegia, traumatic injury, edema, and itchy skin (State Administration of Traditional Chinese Medicine, 1999; Hu et al., 2017). Plant extracts have been used for thousands of years to treat inflammatory diseases and cancer because of their bioactive properties (Ahn et al., 2018; Ghuman et al., 2019). Jiang & Wang (2015) showed that *A. senticosus* EtOAc fraction exhibited stronger antioxidant effects and CH_2Cl_2 fraction inhibited the mRNAs expression of iNOS, COX-2, TNF-α, and IL-6 and have antioxidant activity in macrophages (Jiang & Wang, 2015). Luo et al. (2021a) reported the inhibitory effects of two lupane-type triterpenes from the leaves of *A. gracilistylus* on LPS-induced TNF-α, IL-1β, and the HMGB1 release in RAW264.7 macrophages (Luo et al., 2021a). Ahn et al. (2018) investigated that *Acanthopanacis cortex*-gold nanoparticles (A-AuNPs) suppressed NF-κB and AP-1 through phosphorylation of MAPKs signaling in RAW264.7 cells and inhibited the LPS-induced production of iNOS and COX-2 (Ahn et al., 2018). Luo et al. (2021b) reported that the anti-inflammatory activities of dichloromethane extract from *A. trifoliatus* stems by inhibiting LPS-induced expression of iNOS and COX-2 (Luo et al., 2021b). Eun et al. (2021) investigated the Antiosteoporosis effects of *Eleutherococcus senticosus*, *Achyranthes japonica*, and *Atractylodes japonica* mixed extract fermented with nuruk and suggested that it could be a potential therapeutic candidate for the treatment of osteoclast-mediated inflammatory bone diseases (Eun et al., 2021). Although numerous pharmacological and biochemical pharmacokinetic studies of plants of *Acanthopanax* species have previously been conducted. The potential existence of anti-inflammatory properties of *A. leucorrhizus* stems extracts has not been explored. Here, this assay was aimed to evaluate the anti-inflammatory effects of various extracts of *A. leucorrhizus* stems on the production of NO, TNF-α, and IL-6, and the proteins expression of two inflammation-related mediators iNOS and COX-2.

Inflammation, a basic defense mechanism, plays an essential role in tissue injury and bacterial and viral infections (Antony et al., 2021). Stimulated inflammatory cells produce pro-inflammatory cytokines and inflammation-related mediators, such as NO, TNF-α, and IL-6 (Kang et al., 2016). NO and pro-inflammatory cytokines are important mediators of macrophage-mediated inflammation (Eslami et al., 2017). Various *in vitro* models have been used to measure the inhibitory effects of natural products on inflammatory cytokines TNF-α, and IL-6 and other inflammatory mediators (Lee et al., 2021). In the present study, the effects of the various extracts of *A. leucorrhizus* stems on NO production in LPS-simulated RAW 264.7 cells were measured by the Griess reaction as a cellular response to inflammation. Due to its greatest inhibition of NO and lack of cytotoxicity, the ALSDC was selected for future studies. Therefore, the further research had found that the pro-inflammatory cytokines, TNF-α and IL-6, were clearly inhibited by pre-treatment with ALSDC at a dose of 40 μg/mL in LPS-stimulated RAW264.7 cells.

In the other hand, blocking the expression and activity of COX-2 and iNOS restrains the production of high-output NO and prostaglandins (Jiang & Wang, 2015). Therefore, the inhibition of iNOS and COX-2 protein expression in inflammatory cells provides a novel therapeutic method for treatment of inflammation (Ma et al., 2020). We used LPS to irritate macrophages as an in vitro model of inflammation. ALSDC treatments extenuated LPS-induced inflammation. This study illustrated that iNOS, and COX-2 proteins levels increase significantly in LPS-induced cells, whereas, they were evidently decreased by treatment with ALSDC. It means that ALSDC prevent inflammation symptoms via decreasing the protein expressions of iNOS, COX-2.

5 Conclusion

This study is about anti-inflammatory effects of dichloromethane extract from *A. leucorrhizus* stems induced by LPS and provides evidence of the possible beneficial health advantages of this native Chinese plants. On the basis of the results from the anti-inflammatory experiments, we found that ALSDC showed excellent anti-inflammatory activity. LPS stimulation in RAW 264.7 cells enhanced the inflammatory activity on upregulations of inflammatory related proteins (iNOS and COX-2) expression. ALSDC reduced the cellular NO, IL-6 and TNF-α productions in a dose dependent manner from 0 to 40 μg/mL and decreased the above proteins. In a word, *A. leucorrhizus* showed good anti-inflammation properties to be useful as a functional food additive.

Conflict of interest

The authors declare that they have no conflict of interest.

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