Inhibitory effect of different *Dendrobium* species on LPS-induced inflammation in macrophages via suppression of MAPK pathways

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**ABSTRACT** Dendrobii Caulis (DC), named ‘Shihu’ in Chinese, is a precious herb in traditional Chinese medicine. It is widely used to nourish stomach, enhance body fluid production, tonify “Yin” and reduce heat. More than thirty *Dendrobium* species are used as folk medicine. Some compounds from DC exhibit inhibitory effects on macrophage inflammation. In the present study, we compared the anti-inflammatory effects among eight *Dendrobium* species. The results provided evidences to support *Dendrobium* as folk medicine, which exerted its medicinal function partially by its inhibitory effects on inflammation. To investigate the anti-inflammatory effect of *Dendrobium* species, mouse macrophage cell line RAW264.7 was activated by lipopolysaccharide. The nitric oxide (NO) level was measured using Griess reagent while the pro-inflammatory cytokines were tested by ELISA. The protein expressions of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2) and mitogen-activated protein kinases (MAPKs) phosphorylation were evaluated by Western blotting analysis. Among the eight *Dendrobium* species, both water extracts of *D. thyrsiflorum* B.S.Williams (DTW) and *D. chrysotoxum* Lindl (DCHW) showed most significant inhibitory effects on NO production in a concentration-dependent manner. DTW also significantly reduced TNF-α, MCP-1, and IL-6 production. Further investigations showed that DTW suppressed iNOS and COX-2 expression as well as ERK and JNK phosphorylation, suggesting that the inhibitory effects of DTW on LPS-induced macrophage inflammation was through the suppression of MAPK pathways. In conclusion, *D. thyrsiflorum* B.S.Williams was demonstrated to have potential to be used as alternative or adjuvant therapy for inflammation.

**KEY WORDS** *Dendrobium* species; Anti-inflammation; Macrophage; MAPK pathways


**Introduction**

Dendrobii Caulis (DC) belongs to a precious and medicinal orchid herb group. It has been used for thousands of years in traditional Chinese medicine (TCM). There are 1100 species all over the world \(^1\). More than 30 species are used as folk medicine to nourish stomach, enhance body fluid production, tonify “Yin” and reduce heat \(^2\). *Dendrobium* is served in diet or as medicinal herb to relieve symptoms such as thirst of throat and dryness with blurred vision \(^3\). Many active constituents have been isolated from *Dendrobium* species, like polysaccharides, bibenzyls, alkaloids and coumarins. Polysaccharides of *Dendrobium* exhibit immunomodulatory effect, hepatoprotective and neuroprotective effects, while bibenzyls of *Dendrobium* display anti-inflammatory, anti-tumor and antioxidant activities \(^4-8\). Literatures also report that some small molecular compounds of *Dendrobium* manifest anti-inflammatory and anti-tumor effects. The phenanthrene...
derivatives from *Dendrobium denneanum* and the alkaloids from *Dendrobium crepidatum* exhibit strong inhibitory effects on macrophage inflammation \[8-10\]. Ethyl acetate extract of *Dendrobium tosaense* inhibits mast cell infiltration in atopic dermatitis mouse \[11\]. These reports provide evidences that some ingredients isolated from *Dendrobium* species display inhibitory effect on inflammation.

Inflammation produces a series of events involved in many chronic diseases and attacking multi-organs. Intervention of inflammation is believed to be a potential therapy to prevent inflammatory conditions and improve treatment of diseases. Pathogens lead to activation of immune cells, such as macrophage, neutrophil, dendritic cells, ultimately triggering inflammatory responses \[12\]. Macrophages are one of the important immune cells which participate in the immune inflammatory process through production of various pro-inflammatory mediators \[13\]. Macrophages can be activated by lipopolysaccharide (LPS) which is an endotoxin and elicitor immune response to express nitric oxide (NO) and inflammatory cytokines, as well as some enzymes like inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2). The expressions of iNOS, COX-2, and inflammatory cytokines are regulated by different signaling pathways such as activation of mitogen-activated protein kinases (MAPKs) via phosphorylation \[14\]. Since macrophages participate in inflammation and produce a wide range of bioactive molecules which result in detrimental outcomes, therapy targeted on macrophages and their products as well as MAPKs phosphorylation may be a promising method in controlling inflammatory diseases \[13\].

In previous studies, phenanthrenes isolated from *Dendrobium denneanum* and *D. nobile* exhibited their inhibitory effects on LPS-induced macrophage inflammation \[15-16\]. However, there is no report about the anti-inflammatory effect of other species. Considering a multi-source for *Dendrobium*, in the present study, we investigated the inhibitory effects of water extracts of eight common *Dendrobium* species, including *Dendrobium aurantiacum* (F. Muell.) F. Muell (DAW), *Dendrobium chrysothamnus* Lindl. (DCHW), *Dendrobium crepidatum* Lindl. Et Paxton (DCRW), *Dendrobium devonianum* Paxton (DDW), *Dendrobium longicorne* Lindl. (DLW), *Dendrobium officinale* Kimura et Migo (DOW), *Dendrobium pendula* Roxb. (DPW) and *Dendrobium thyrsiflorum* B.S.Williams (DTW), on macrophage activation. We provided the evidences showing differences in pharmacological activity among different species of *Dendrobium*, shedding light on the usage of *Dendrobium* in selecting different bio sources. Our study also firstly indicated *D. thyrsiflorum* B. S. Williams had anti-inflammatory effect on macrophage activation via suppression of MAPK pathways.

**Materials and Methods**

**Chemical and reagents**

Raw herbs of eight *Dendrobium* species were collected from Yunnan Province (April, 2015) and authenticated by Prof. HU Jiang-Miao from Kunming Institute of Botany, Chinese Academy of Sciences, China. Griess reagent, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and indomethacin was purchased from Sigma-aldrich (St. Louis, MO, USA). TNF-α, IL-6, IL-1β, and MCP-1 ELISA kits were all from BD Biosciences (San Jose, CA, USA). Primary anti-bodies of iNOS and COX-2 were from Santa Cruz (Dallas, TX, USA) and antibodies of JNK, ERK, p38, phorsphorylated-JNK, phorsphorylated-ERK, phorsphorylated-p38, and β-actin were all purchased from Cell Signaling Technology (Danvers, MA, USA).

**Herbal extraction and characterization**

The raw herbs of different *Dendrobium* species were extracted under reflux in boiling water for 2 h and the extraction process was repeated twice. The water extracts were filtered and the filtrate was lyophilized into powder ready for use. The chemical characterization of DTW was analyzed by high performance liquid chromatography (HPLC). The chromatographic condition was modified according to a previous publication \[17\]. Ten microliter of DTW (20.6 mg mL\(^{-1}\)) was loaded onto Agilent USA SB-C\(_18\) column (4.6 mm × 250 mm, 5 μm) for chemical analysis. A linear gradient elution of 0.1% acetic acid and acetonitrile was used. The composition of the eluent was varied from 19% to 23% acetonitrile in the first 15 min, 23%–60% acetonitrile from 15 to 28 min, 60%–95% acetonitrile from 28 to 33 min. The flow rate was kept at 1.0 mL min\(^{-1}\) and the column temperature was kept constant at 30 °C. A diode array spectrophotometric detector (DAD) was used and the signal was detected at wavelength of 230 nm. Reference chemical markers gigantol and scoparone were dissolved separately in methanol at 1.25–20 and 17.5–280 μg mL\(^{-1}\), respectively.

**Cell culture**

Murine macrophage cell line (RAW264.7) was purchased from ATCC (Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, 1/1), 100 U·mL\(^{-1}\) of penicillin and 100 μg·mL\(^{-1}\) of streptomycin.

**Cell viability assay**

Cell viability was measured by MTT assay. Briefly, 2500 cells/well were seeded into 96-well plates and incubated overnight at 5% CO\(_2\), 37 °C. The cells were incubated with different species of *Dendrobium* extracts at gradient concentrations for 48 h. Then the cells were co-cultured with 5 mg·mL\(^{-1}\) MTT solution for 2 h. The supernatant was discarded and the formazan was dissolved in 100 μL of DMSO. Optical density (OD) values were read using a microplate reader (BioTek, Winooski, VT, USA) at a wavelength of 540 nm.

**Determination of NO and pro-inflammatory cytokines in culture medium**

The cells were seeded into 24-well plates at a density of 4 × 10\(^5\)/well and treated with different concentrations of *Dendrobium* species and 1 μg·mL\(^{-1}\) lipopolysaccharide (LPS) simultaneously for 18 h. The nitric oxide accumulation in
supernatant of culture medium was determined by Griess reagent. Indomethacin was used as positive control \cite{18}. Briefly, 50 \mu L of culture medium was reacted with equal volume of Griess reagent for 15 min, and the absorbance was measured at the wavelength of 540 nm. At the same time, culture medium was collected for proinflammatory cytokines determination. The levels of TNF-\alpha, IL-6, IL-1\beta, and MCP-1 were tested using commercial available ELISA kits, according to manufacturer’s instruction.

**Western blotting analysis**

The cells were treated with DTW and 1 \mu g\cdot mL^{-1} of LPS for 18 h or 2 h. Then cells were harvested with radioimmunoprecipitation (RIPA) buffer for analysis of iNOS and COX-2 protein levels (18 h) and MAPK signaling pathway protein levels (2 h), including ERK, pERK, JKN, pJNK, p38, and pp38. Total protein concentration was measured by BCA assay. The protein samples were loaded onto 8% resolving SDS-PAGE gel and 5% stacking gel. After the electrophoresis, the proteins were transferred to PVDF membrane. The membrane with proteins was blocked with 5% BSA for 1 h. Subsequently, the membranes were incubated with primary antibodies overnight at 4 \degree C. After washing thrice with Tris-buffered saline adding 0.1% Tween-20 (TBST), and the membranes were incubated with the secondary antibodies conjugated with the secondary antibodies were washed with TBST. The signal was detected using chemiluminescence ECL assay kit. Chemiluminescent bands were imaged using a Bio-Rad ChemiDoc™ XRS+ imaging system (Bio-Rad, Hercules, CA, USA). \beta-Actin was used as the internal control and protein expression levels were normalized to that of \beta-actin.

**Statistical analysis**

All the data were expressed as means ± SD and analyzed with Graphpad Prism 5. One-way ANOVA with Bonferroni test was used to examine differences among groups. The value of \(P < 0.05\) was considered statistically significant.

**Results**

**Identification of DTW by HPLC**

Scoparone and gigantol were used as the representative compounds for coumarins and bibenzyls, respectively. As shown in Fig. 1, HPLC analysis showed that scoparone was the major compound in the water extract of *D. thyrsiflorum* B.S. Williams, which was detected at 12.8 min. Gigantol was detected at 25.8 min. The contents of scoparone and gigantol in the water extracts of *D. thyrsiflorum* were 0.39\% (W/W) and 0.040\% (w/w), respectively.

**Effects of different Dendrobium species on cell viability**

After incubated with a series of gradient concentrations of water extracts from *Dendrobium* species for 48 h, the cell viability was measured by MTT assay. As shown in Fig. 2, DTW, DAW, DOW, and DDW had no apparent cytotoxicity on macrophages, up to 800 \mu g\cdot mL^{-1}. However, DCHW, DPW,
DLW, and DCRW showed cytotoxicity at various concentrations. Among them, DPW and DCRW exhibited the most significant inhibitory effects on cell viability over the concentration of 100 μg·mL⁻¹. Consequently, non-cytotoxic concentrations of Dendrobium species were used in the subsequent experiments.

![Fig. 2](image)

**Effects of different Dendrobium species on cell viability.** The cells were treated with different Dendrobium extracts for 48 h and then incubated with MTT solution for 2 h. DTW, DAW, DOW, and DDW had no apparent cytotoxicity on macrophage up to 800 μg·mL⁻¹. However, DCHW, DPW, DLW, and DCRW showed cytotoxicity at various concentrations. The data were expressed as means ± SD, n = 6. *P < 0.05, **P < 0.001 vs the blank control (0 μg·mL⁻¹).

**Effects of eight Dendrobium species on nitric oxide production**

The content of NO was determined using Griess reagent as an indicator of inflammatory reaction. The NO production in supernatant of RAW264.7 cells was significantly increased with LPS stimulation for 18 h. In order to evaluate inhibitory effects of Dendrobium species on LPS-induced macrophage activation, the water extracts of Dendrobium species within non-cytotoxic concentrations were co-cultured with 1 μg·mL⁻¹ of LPS for 18 h. Both DTW and DCHW showed significant inhibitory effects on NO production in a concentration-dependent manner. DTW at the concentrations of 400 and 800 μg·mL⁻¹ remarkably inhibited NO production by 35.3% and 67.9%, respectively, compared with LPS stimulation alone. Besides, DCHW at 400 μg·mL⁻¹ could also significantly reduce NO level by 48.0%. However, other species of Dendrobium used in our experiments did not show significant effects on inhibition of NO production in LPS-induced RAW264.7 cells (Fig. 3). Indomethacin was used as a positive control, which significantly inhibited about 50.9% of LPS-induced NO release at 100 μmol·L⁻¹.

**Effects of DTW on pro-inflammatory cytokines production**

Although both DTW and DCHW showed inhibitory effect on NO production in a concentration-dependent manner in RAW264.7 cells, DTW was more effective than DCHW. In addition, DTW was less toxic than DCHW based on the results from the MTT assay. Therefore, effects of DTW on pro-inflammatory cytokines and enzymes, as well as underlying pathways were further determined by ELISA or Western blot analysis. TNF-α, MCP-1, IL-6, and IL-1β were used as representative cytokines secreted by RAW264.7 cells. The concentrations of TNF-α, MCP-1, IL-6 and IL-1β in the culture medium were measured using commercial ELISA kits. The results showed that TNF-α, MCP-1, IL-6 and IL-1β in the culture medium were measured using commercial ELISA kits. The results showed that TNF-α, MCP-1, IL-6 and IL-1β in the culture medium were measured using commercial ELISA kits. The results showed that TNF-α, MCP-1, IL-6 and IL-1β in the culture medium were measured using commercial ELISA kits. The results showed that TNF-α, MCP-1, IL-6 and IL-1β in the culture medium were measured using commercial ELISA kits. The results showed that TNF-α, MCP-1, IL-6 and IL-1β in the culture medium were measured using commercial ELISA kits. The results showed that TNF-α, MCP-1, IL-6 and IL-1β in the culture medium were measured using commercial ELISA kits.
significant decrease in TNF-α production (Fig. 4A). For MCP-1, the cells treated with LPS and DTW showed reduced content in a concentration-dependent manner, especially with 800 μg·mL\(^{-1}\) of DTW treatment, compared with the cells in LPS alone group (Fig. 4B). As showed in Fig. 4C, DTW treatment at 100, 200, 400 and 800 μg·mL\(^{-1}\) exhibited significant and concentration-dependent inhibition in IL-6 secretion induced by LPS. However, IL-1β secreted by RAW264.7 was not detectable in our experiments, probably due to its low level.

**Fig. 3** Effects of different *Dendrobium* species on nitric oxide production. The cells were seeded in 24-well plate and treated with 1 μg·mL\(^{-1}\) of LPS and different *Dendrobium* extracts for 18 h. Then NO contents in supernatant were measured by Griess reagent. DTW and DCHW showed significant inhibitory effects on NO production in a concentration-dependent manner. But other species of *Dendrobium* used in our experiments did not show significant effects on inhibition of NO production. Indomethacin was used as a positive control. Three independent experiments were performed in duplicate. The data are expressed as means ± SD, \(n = 6\). \(**P < 0.01, ***P < 0.001\) vs the blank control (CTL). \(*P < 0.05, **P < 0.01, ***P < 0.001\) vs the LPS-stimulated group (LPS)

**Fig. 4** Effects of *D. thyrsiflorum* on TNF-α, MCP-1 and IL-6 secretion. The cells were treated with 1 μg·mL\(^{-1}\) of LPS and DTW at 100, 200, 400 or 800 μg·mL\(^{-1}\) for 18 h. Then supernatant was collected to test TNF-α (A), MCP-1 (B) and IL-6 (C) production by ELISA. Four independent experiments were performed in duplicate. The data are expressed as means ± SD, \(n = 4\). \(\ast P < 0.05, \ast\ast P < 0.01\) showed significant difference vs the blank control (CTL). \(\ast\ast\ast P < 0.001\) vs the LPS-stimulated group (LPS)
Effects of DTW on iNOS and COX-2 protein expression

iNOS and COX-2 are two enzymes related to inflammatory response. The expression of iNOS and COX-2 proteins were tested by Western blotting. As shown in Fig. 5, little iNOS and COX-2 protein expression in unstimulated RAW264.7 cells were observed. However, the levels of iNOS and COX-2 were remarkably upregulated with LPS exposure for 18 h. The treatment of DTW at 100, 200, 400, and 800 μg·mL⁻¹ inhibited the upregulation of iNOS and COX-2 in concentration-dependent manner. Particularly, DTW significantly reduced iNOS expression at 800 μg·mL⁻¹, while it significantly reduced COX-2 expression at 400 and 800 μg·mL⁻¹, compared with the LPS-treated alone group.

Effects of DTW on phosphorylation of MAPK pathways

MAPKs, including ERK, JNK and p38, are activated by environmental stress and regulate the activity of transcription factors, affecting expression of iNOS, COX-2 and pro-inflammatory cytokines. We evaluated effects of DTW on ERK, JNK and p38 phosphorylation in the present study. As shown in Fig. 6, the cells treated with LPS and different concentrations of DTW for 2 h showed equal level of inactivated ERK, JNK and p38 expression. On the other hand, the expression of phosphorylated ERK, JNK and p38 with LPS treatment was increased significantly. The cells treated with LPS and 800 μg·mL⁻¹ of DTW showed remarkably inhibition on ERK phosphorylation (Fig. 6B). Meanwhile, the cells treated with LPS and 400 or 800 μg·mL⁻¹ DTW exhibited remarkably inhibition on JNK phosphorylation (Fig. 6C). However, DTW treatment at different concentrations did not alter the phosphorylation of p38 in the LPS-stimulated cells (Fig. 6D).

Discussion

Macrophages are pleiotropic immune cells that exert a variety of functions. Activation of macrophage results in signal transduction and transcriptional regulation to eliminate the invading pathogen and coordinate responses to stresses such as infection as well as maintain homeostasis [19]. iNOS, which catalyzes the production of NO, is mainly located in macrophages and neutrophils [20]. COX-2 is responsible for initiating PGE2 synthesis which modulates production of inflammatory cytokines [21]. Excessive activation of macrophage results in a cascade of inflammation response which generates large amounts of inflammatory mediators, such as NO, PGE2, TNF-α, MCP-1, IL-6, and IL-1β, and is harmful to the host tissues [22]. In many previous studies, a bacterial endotoxin, LPS-mediated macrophage activation through toll-like receptor 4 (TLR4) raised secretion of NO, PGE2, TNF-α, MCP-1, IL-6, and IL-1β, indicating strong inflammation in macrophages [23-24]. In the present study, indomethacin, a cyclooxygenase inhibitor, was used as positive control and showed a
significant suppression in NO production. Our data demonstrated that the efficacy of DTW was comparable with that of indomethacin. The water extract of Chinese medicine, like DTW is generally considered safe, as demonstrated in our MTT assay. The contents of toxic compounds, or even possible active anti-inflammatory hydrophobic constituents, like scoparone and gigantol were at very low concentrations (3.12 and 0.32 μg·mL⁻¹, respectively, in 800 μg·mL⁻¹ of DTW). This was the partial reason for us to use relative high concentrations of DTW in the present study.

**Fig. 6** Effects of *D. thyrsiflorum* on phosphorylation of MAPK pathways. The cells were incubated with 100, 200, 400 or 800 μg·mL⁻¹ of DTW and 1 μg·mL⁻¹ of LPS for 2 h. Total cellular protein was harvested and analyzed by Western blotting analysis, and β-actin was used as an internal control. Relative expression of ERK, pERK, JNK, pJNK, p38 and pp38 were quantified by densitometry using ImageJ software and normalized to the β-actin level. The data from three independent experiments are expressed as means ± SD, n = 3. *P < 0.05, **P < 0.001 vs the blank control (CTL). #P < 0.05, ##P < 0.01, vs the LPS-stimulated group (LPS).

*Dendrobium* is a precious traditional medicine used for nourishing stomach, enhancing body fluid production and removing heat, according to the theory of traditional Chinese medicine. There are more than 30 species of *Dendrobium* used in folk medicine. Many chemical constituents have been isolated from *Dendrobium* species such as polysaccharides, alkaloids, bibenzyls, phenanthrenes, and coumarins. Recent studies have demonstrated chemical components among different species, which in turn gives diversity of its pharmacological activities [1]. For example, polysaccharides and bibenzyls are the main components of *D. officinale*, which show immunomodulatory and anti-tumor effects [25]. The alkaloids and phenanthrenes are the bioactive constituents of *D. nobile* and exhibit anti-tumor and anti-inflammation effects.
Recent studies have demonstrated that MAPK pathways play a crucial role in the regulation of inflammation. Cytokines, such as TNF-α, IL-1β, and IL-6, are known to activate MAPK pathways, leading to the phosphorylation of various MAPKs, including JNK, p38, and ERK. These pathways are involved in the expression of pro-inflammatory genes and the production of inflammatory mediators, such as NO, PGE2, and TNF-α.

Our results showed that the water extract of *Dendrobium thyrsiflorum* exhibited significant inhibitory effects on LPS-stimulated MAPKs phosphorylation, particularly on JNK and ERK1/2 phosphorylation at concentrations of 400 and 800 μg/mL. However, DTW had no significant effect on p38 phosphorylation. These results indicated that the suppression of NO production by DTW treatment might be mediated by the decreased expression of iNOS via inhibiting MAPK signaling activation.

To the best of our knowledge, the present study was the first time to compare anti-inflammatory effects among different *Dendrobium* species and carry out an in-depth study on anti-inflammatory effects of *D. thyrsiflorum* B.S. Williams through downregulation of MAPKs activation. Our current study also provided solid evidences that different pharmacological activities could be caused by various species of herbal medicines, which may guide us to compare the bioactive differences among *Dendrobium* species and to select the right bio-sources.

In conclusion, DTW inhibited NO production by reducing iNOS expression via downregulation of MAPKs activation and inhibited inflammatory cytokines, such as TNF-α, MCP-1, IL-6 levels in LPS-induced macrophage. Scoparone and gigantol may be two of the major active compounds in the water extract of *D. thyrsiflorum* B.S. Williams. The results of our study suggested that *D. thyrsiflorum* B.S. Williams represented a potential herbal item for alternative therapy or a food supplement for anti-inflammation treatment.

**References**


