

Immunopotentiating Activity of Dendrobium Species in Mouse Splenocytes

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Abstract

This study aimed to explore a pharmacological activity marker for quality assurance of Dendrobium species. The immunopotentiating activity in aqueous extracts prepared from four Dendrobium species, including *D. officinalis*, was assessed by an *in vitro* assay of concanavalin A (Con A)-stimulated proliferation of mouse splenocytes. Four samples of commercially available Dendrobii Caulis were also analyzed for comparison. The results indicated that the aqueous extract of *D. officinalis* produced immunopotentiating action, as evidenced by the increase in Con A-stimulated proliferation of mouse splenocytes, with the extent of stimulation being more prominent than those of other tested Dendrobium species and Dendrobii Caulis samples. In conclusion, an *in vitro* immunopotentiation assay may be used for assessing the pharmacological activity of Dendrobium species. The finding that *D. officinalis* produced a more potent immunopotentiating action is consistent with its “yin-nourishing” action in Chinese medicine, which is more effective than other Dendrobium species in clinical use.

Keywords: *Dendrobium officinalis*, Dendrobii Caulis, Splenocyte Proliferation, Concanavalin A

1. Introduction

Dendrobium species was regarded as a first-rate herb in Shen-nong Bencao Jing (Canon on Medicinal Herbs, 200 B.C.) and has a long history of usage in China. Over the past few decades, the dried stem of Dendrobium species (Dendrobii Caulis, also called Shi Hu in Chinese) has been used for clearing “heat” and nourishing “yin” in the practice of Chinese medicine [1]. A recent study showed that the administration of an aqueous extract of *Dendrobium candidum* ameliorated the dry-mouth symptom in patients suffering from Sjögren syndrome [2]. Pharmacological studies have demonstrated that the aqueous extract and/or polysaccharides of Dendrobii Caulis possess antioxidant [3,4], immuno-stimulating [5-7], anti-tumor [8,9], anti-microbial [9], anti-hyperlipidemia [10] and anti-hyperglycemic [11] activities. The widespread application of Dendrobii Caulis has raised a concern regarding the identity of Dendrobium species as herbal source. In this regard, the record of Dendrobii Caulis in Chinese Pharmacopoeia has been constantly amended over the past 20 years. While five Dendrobium species, including *D. loddigesii* Rolfe, *D. fimbriatum* Hook and *D. nobile* Lindl., were listed in the 1995 and 2000 editions,

a more extended list of Dendrobium species was generated in the 2005 edition. As such, up to 74 Dendrobium species can be used as Dendrobii Caulis in China. In the 2010 edition of Chinese Pharmacopoeia [12], *D. Officinalis* has been singled out as a distinct herb originated from *D. officinale* Kimura et Migo. Not surprisingly, *D. Officinalis* is relatively scarce in abundance and deemed to possess a stronger therapeutic potential than other Dendrobium species [13,14].

As more than 45 Dendrobium species have been identified to possess therapeutic potential [15-18], the development of chemical and DNA fingerprinting methods for species authentication, particularly for those recorded in Chinese Pharmacopoeia, has been an area of intensive research [19-26]. In addition to chemical markers, the measurement of pharmacological activity may offer a complementary and functionally relevant quality assessment for herbal materials [27]. In this connection, “yin-nourishing” tonic herbs (including Herba Dendrobii) have been found to produce immunopotentiating effect on mouse splenocytes both *in vivo* and *in vitro* [28]. In the present study, using an *in vitro* assay of concanavalin A (Con A)-stimulated proliferation of mouse splenocytes, we assessed the immunopotentiating activity of aqueous

extracts prepared from four *Dendrobium* species including *D. Officinalis*, with an objective of exploring a pharmacological activity marker for quality assurance. Four samples of commercially available *Dendrobii Caulis* were also analyzed for comparison.

2. Materials and Methods

2.1. Chemicals and Plant Materials

RPMI-1640 medium, heat inactivated fetal bovine serum (FBS) and Con A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)-based cell proliferation kit was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). All other chemicals were of analytical grade. Four *Dendrobium* species, namely, *D. loddigesii* Rolfe, *D. fimbriatum* Hook, *D. nobile* Lindl. and *D. officinalis* Kimura et Migo, were obtained from an ornamental flora shop in Hong Kong, and they were authenticated by floral structure with reference to China Flora. Four samples of *Dendrobium Caulis* were randomly bought from local herbal stores. Voucher specimens of *Dendrobium* plants and *Dendrobii Caulis* have been deposited in the Division of Life Science, The Hong Kong University of Science & Technology (HKUST), Hong Kong SAR, China.

2.2. Plant/Herbal Extraction

Fresh stems of *Dendrobium* plants or *Dendrobii Caulis* (*i.e.*, dried stems of *Dendrobium* species) were cut into small pieces and extracted by 10 volumes (w/v) of double distilled water at 60°C for 2 h. The extraction procedure was repeated once, and the pooled aqueous extracts were dried by lyophilization. The lyophilized powders (*i.e.* *Dendrobium* extracts) were stored at -20°C prior to use for experiment.

2.3. Measurement of *in Vitro* Immunopotentiating Activity in Con A-Stimulated Mouse Splenocytes

Procedures involved in isolation of mouse splenocytes were conducted under aseptic conditions. Splenic tissue obtained from adult female ICR mice (25 - 28 g) was teased with a plastic syringe in a culture dish (60 mm) containing 10 mL of RPMI-1640 medium, and it was gently strained through a 200 mesh stainless steel sieve to remove clumps to produce a cell suspension. The product was then left to stand on ice for 5 min to remove tissue fragments. The cell suspension was centrifuged at 600 × g for 10 min and washed 3 times with RPMI-1640

medium, and it was finally resuspended in RPMI-1640 medium supplemented with 10% FBS at a concentration of 1×10^7 viable cells/mL. The viability of isolated splenocytes, as determined by Trypan blue exclusion test, was found to be higher than 95%. Mouse splenocytes were cultured in medium with Con A in the presence or absence of *Dendrobium* extract in 96-well microtiter plates (flat bottom) in a final volume of 100 µL. Con A (prepared in phosphate buffered saline) was added at final concentrations of 0.5, 1 and 2 µg/mL, respectively. Aliquots of respective *Dendrobium* extract (10 µL in aqueous solution) were added at increasing final concentrations ranging from 1.6 to 100 µg/mL. Control wells were added with 10 µL of sterilized double-distilled water only. Splenocytes were then cultured for 72 h at 37°C in a humidified atmosphere of 5% CO₂ in air. At the end of the culture period, the extent of splenocyte proliferation was determined colorimetrically by MTT-based cell proliferation assay. An aliquot (10 µL) of MTT labeling reagent was added to each well under dark conditions. After 4 h of incubation, 100 µL of solubilization buffer (10% SDS in 0.01 M HCl) was added, and the mixtures were incubated in 5% CO₂ at 37°C overnight for dissolving the colored crystals. The extent of splenocyte proliferation was determined by measuring the absorbance at 570 nm using Victor V² Multi-label Counter (Perkin Elmer, Turku, Finland). Stimulation index (SI) was calculated using the equation: SI = mean absorbance of cells stimulated with Con A/ mean absorbance of cells not stimulated with Con A. The extent of Con A-stimulated proliferation of isolated splenocytes was estimated by computing the area under the curve (AUC₁) of the graph plotting stimulatory indices against Con A concentrations. Data of AUC₁ were expressed as the percentage of non-*Dendrobium* extract-treated control, and AUC₂ was computed from a graph plotting percentages of control against tested concentrations of *Dendrobium* extract. The immunopotentiating activity was expressed as the difference in AUC₂ values between the *Dendrobium* extract-untreated control and *Dendrobium*-treated group.

2.4. Measurements of Interleukin-2 (IL-2), IL-6 and Interferon-γ (INF-γ) Levels

Isolated mouse splenocytes were cultured with *Dendrobium* extract (25, 50, 100 µg/mL, final concentration) in the presence or absence of Con A (2 µg/mL) for 24 h. IL-2, IL-6 and INF-γ levels in the culture medium was then measured using ELISA kits (InvitrogenTM Mouse IL-2 ELISA kit, Invitrogen, Frederick, MD, USA; InvitrogenTM Mouse IL-6 ELISA kit, Invitrogen, Camarillo, CA, USA; ELISA^{PRO} kit for mouse IFN-γ, MABTECH,

Inc., Cincinnati, OH, USA).

3. Results and Discussion

As shown in **Figure 1**, Con A-stimulated the proliferation of mouse splenocytes, as indicated by a dose-dependent increase in stimulatory index. While the aqueous extract of *D. officinalis* (DO) alone did not produce any detectable effect on mouse splenocyte proliferation (data not shown), it caused a dose-dependent enhancement in Con A-stimulated proliferation of mouse splenocytes *in vitro*. When the extent of Con A-stimulated proliferation was estimated by computing the AUC of the Con A dose-response curve (AUC_1) and then by the AUC of the DO dose-response curve (AUC_2), the extent of Con A-stimulated proliferation of splenocytes induced by DO was found to be increased by 23%, when compared with the DO-untreated and Con A-stimulated control. Among the tested aqueous extracts of *Dendrobium* species, *D. nobile* (DN) and *D. loddigesii* (DL) produced a slight enhancing effect on Con A-stimulated proliferation (1.2 and 3.6%, respectively), whereas *D. fimbriatum* (DF) caused a mild suppressive effect (−6.4%) (**Figure 2**). Among the four tested *Dendrobium* Caulis samples (DC_{1-4}), D_3 and D_4 showed a slight enhancing effect on Con A-

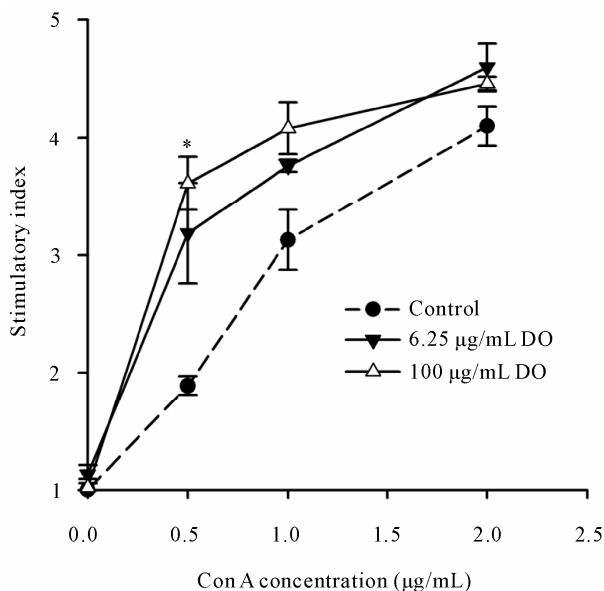


Figure 1. Effect of *D. officinalis* extract on Con A-stimulated proliferation of mouse splenocytes. Isolated mouse splenocytes were incubated with increasing concentrations of Con A (0 - 2 µg/mL) in the absence or presence of an aqueous extract of *D. officinalis* (DO) (1.6 - 100 µg/mL), as described in Materials and methods. Data of two representative concentrations (6.25 and 100 µg/mL) of DO are shown. Values given are mean \pm S.D., with data obtained from triplicate assay samples. *Significantly different from *Dendrobium* extract-untreated control.

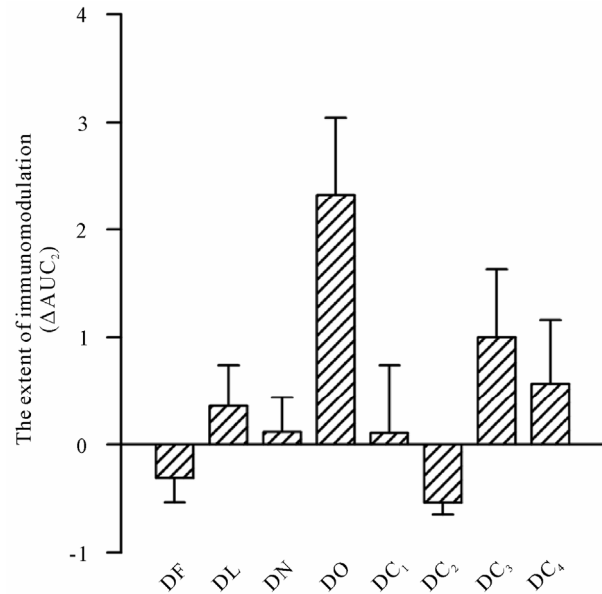


Figure 2. Immunopotentiating activity of *Dendrobium* extracts in Con A-stimulated mouse splenocytes. Isolated mouse splenocytes were incubated with Con A and *Dendrobium* extracts (*D. fimbriatum*, DF; *D. loddigesii*, DL; *D. nobile*, DN; DO; *Dendrobium* Caulis, DC_{1-4}), as described in Figure 1. The extent of immunopotentiality was estimated and data were expressed as ΔAUC_2 , according to the computation procedure described in Materials and methods. The value of AUC_2 for *Dendrobium* extract-untreated and Con A-stimulated control was 10. Values given are mean \pm S.D., with data obtained from triplicate assay samples.

stimulated proliferation of mouse splenocytes (5.4% and 10%), but D_1 showed undetectable and D_2 produced suppressive effect (−5.4%).

Splenocyte proliferation is a complex event that involves interaction of cytokines such as IL-1 and IL-2 and expression of their receptors [29]. The ability of *Dendrobium* extract to enhance Con A-stimulated splenocyte proliferation may therefore be mediated by the modulation of cytokine expression and/or function. As shown in **Figure 3(a)**, Con A caused an increase in IL-2 production in cultured mouse splenocytes (**Figure 3(b)**). DO enhanced the Con A-stimulated IL-2 production in mouse splenocytes, with the extent of stimulation being 18% at 100 µg/mL. In addition, Con A also stimulated IL-6 and INF- γ production in mouse splenocytes (**Figure 3(b)**, (c)), and the Con A-stimulated IL-6 and INF- γ production were enhanced by DO, with the degree of stimulation being 55% and 24%, respectively, at a concentration of 100 µg/mL. IL-6 and INF- γ , which are cytokines secreted by T helper cells, can activate neutrophils [30-32] and macrophages [33-35], respectively. The enhancement of Con A-stimulated splenocyte proliferation by DO was associated with the stimulation of cytokine secretion, thereby producing a generalized immunopoten-

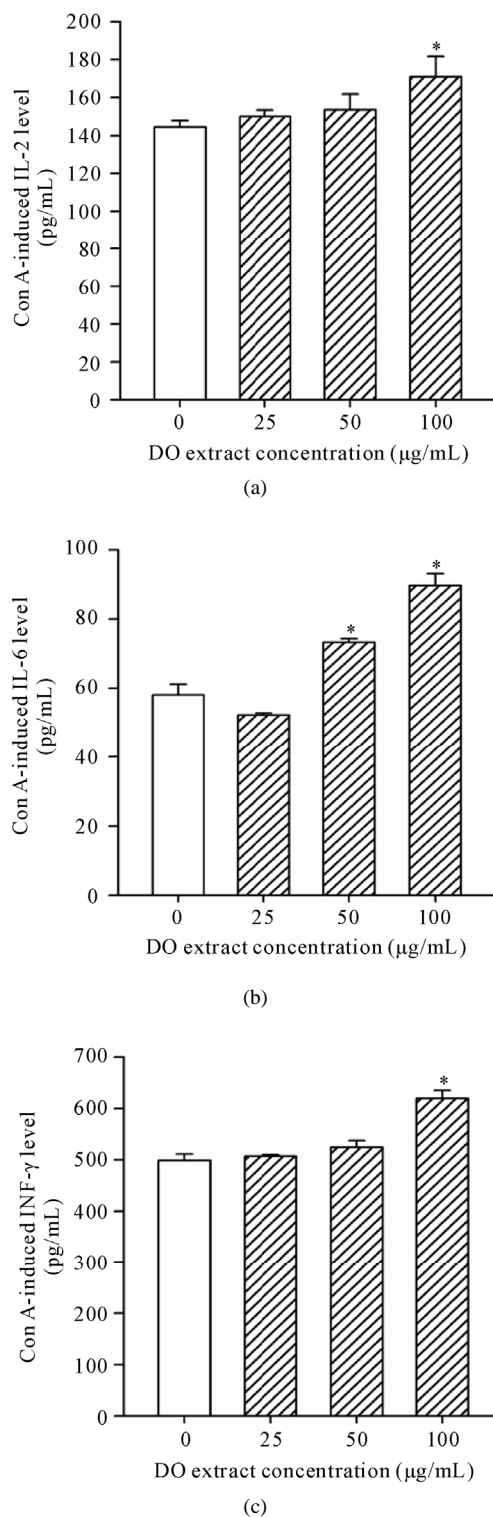


Figure 3. Effects of *D. officinalis* extract on cytokine levels in Con A-stimulated mouse splenocytes. Mouse splenocytes were treated with Con A (2 µg/mL) and DO (25 - 100 µg/mL). (a) IL-2, (b) IL-6 and (c) INF-γ levels were measured in the cultured medium. Values given are mean ± S.D., with data obtained from triplicate assay samples. *Significantly different from the DO-untreated and Con A-stimulated control.

tiating effect.

An earlier study in our laboratory has shown that “yin-nourishing” Chinese tonic herbs can enhance the Con A-stimulated mitogenic response of mouse splenocytes both *in vitro* and *ex vivo* [28]. In the present study, the finding that *D. officinalis* produced a marked immunopotentiating action further supports the use of splenocyte mitogenic assay for the assessment of “yin-nourishing” action. Interestingly, among the tested *Dendrobium* species and *Dendrobii Caulis* samples, *D. officinalis* is the most potent in immunopotentiation. The finding also provides a pharmacological basis for the adoption of *D. officinalis* as a premium *Dendrobii Caulis* for “yin-nourishing” in the latest edition of Chinese Pharmacopoeia [12]. The observation that DO obtained from water extraction at room temperature rather than 60°C did not produce stimulatory effect on mouse splenocyte proliferation suggests the involvement of polysaccharides in immunopotentiating action (unpublished data).

4. Conclusions

The results indicated that the aqueous extract of *D. officinalis* produced immunopotentiating action in mouse splenocytes, which was more prominent than those of other tested *Dendrobium* species and *Dendrobii Caulis* samples. The *in vitro* immunopotentiating assay may be used for assessing the pharmacological activity of *Dendrobium* species. The finding that *D. officinalis* produced a more potent immunopotentiating action is consistent with its “yin-nourishing” action in Chinese medicine, which is more effective than other *Dendrobium* species in clinical use.

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