

EFFECTS OF SIX ANTI-INFLAMMATORY CHINESE HERBS ON LPS/IFN- γ -INDUCED NITRIC OXIDE PRODUCTION IN RAW264.7 MACROPHAGES

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The present study was designed to evaluate whether the six Chinese herbs (*Davallia divaricata*, Dd; *Nothapodytes foetida*, Nf; *Orobanchae caerulescens*, Oc; *Spiranthes sinensis*, Ss; *Taxillus liquidambaricolus*, Tl; *Viscum alniformosanae*, Va) commonly used as anti-inflammatory drugs in traditional Chinese medicine, influence the induction of nitric oxide (NO), a mediator involved in inflammation. RAW264.7 cell was used as target cell and activated by lipopolysaccharide (LPS) plus interferon- γ (IFN- γ). Our results indicated that two out of six ethanolic crude extracts of Chinese herbs (Nf and Tl) significantly suppressed LPS/IFN- γ -induced NO production in a dose-dependent manner. It is unlikely that cytotoxicity was involved, because no cell deaths were observed. The result was correlated with their putative pharmacological activities. The remaining ethanolic extracts of the other Chinese herbs only had little or no effect on NO production.

Key Words: Nitric oxide, Macrophage, Anti-inflammation, *Nothapodytes foetida*, *Taxillus liquidambaricolus*.

INTRODUCTION

Bacteria infection or immunological stimuli including lipopolysaccharide (LPS), interferon- γ (IFN- γ), or interleukin-1, cause the expression on an inducible isoform of nitric oxide synthase (iNOS) which, once expressed, produces large amounts of nitric oxide (NO).¹ In pathological conditions, macrophages can greatly increase their production of NO. The high amounts of NO are potentially cytotoxic, capable on injuring the surrounding cells and tissues indiscriminately. Indeed, it has been reported that excess production of NO by macrophages and other cells exposed to endotoxin may contribute to local (e.g., polyarthritis, osteoarthritis) or systemic inflammatory diseases.^{2,3,4} Thus, it has been argued that inhibition of NO over-production may have the therapeutic benefit in patients with inflammatory diseases.

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Chinese herbs have been generally used in traditional Chinese medicine for a long times. There has been a relative scarcity of definitive evidence to prove their pharmacological activity. In the present study, six Chinese herbs that have anti-inflammatory activities were selected for NO inhibitory assay. Their putative activity include relief cough, treatment of rheumatism, reduction of swelling, enhancement of blood circulation, and detoxification. The species of six Chinese herbs were *Davallia divaricata* (Dd), *Nothapodytes foetida* (Nf), *Orobanchae caerulescens* (Oc), *Spiranthes sinensis* (Ss), *Taxillus liquidambaricolus* (Tl), and *Viscum alniformosanae* (Va). In the present study, a macrophage-like cell line RAW264.7 was used as target cells. The six Chinese herbs were extracted by ethanol. The effects of these crude extracts on LPS/IFN- γ induced NO production were determined.

MATERIALS AND METHODS

Plant material

All Chinese herbs were obtained in a market in Taipei and identified by Mr. J.C. Ou (Associate investigator in pharmacognosy, National Research Institute of Chinese Medicine). A voucher specimen is maintained in the herbarium of our institute.

Extraction and isolation

Six hundred gm of dried Chinese herbs were extracted with ethanol (5L) for three times. The extract was concentrated in vacuum to yield dark brown mass. All crude extracts were dissolved by dimethyl-sulfoxide (DMSO) and stored at 4 °C until for use.

Cell culture

The murine macrophage cell line RAW 264.7 (American Type Culture Collection ATCC, TIB 71, Rockville, MD) was cultured in 75 cm² plastic flasks (Corning-Costar) with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μ g/ml streptomycin as previously described.⁵ Cells were passed every 4 days. For experiments, macrophages were detached by vigorous pipetting and, after centrifugation, plated in fresh medium. These cells were activated with LPS (1 μ g/ml) plus IFN- γ (50 U/ml), and cultured for 24 hr at 37 °C in an atmosphere of 5% CO₂ plus air.

Nitrite measurement

Nitrite production, an indicator of NO synthesis, was measured in the supernatant of RAW264.7 macrophages as described previously.⁵ Briefly, the cells were cultured in 96-well plates with 200 μ l of culture medium until cells reached confluence (approximately 200,000 cells per well). In order to induce iNOS, fresh culture medium containing 1 μ g/ml LPS plus 50 U/ml IFN- γ was added. Nitrite accumulation in the medium was measured at 24 hr after the application of LPS/IFN- γ . To assay drug's effect on nitrite production, crude extract (final concentration, 2 to 100 μ g/ml) was added together with LPS/IFN- γ . Viability was assessed by the MTT test.

Nitrite was measured by adding 100 μ l of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid) to 100 μ l samples of medium. The optical density at 550 nm (OD₅₅₀) was measured

with a microplate reader. concentrations were calculated by comparison with OD₅₅₀ of standard solutions of sodium nitrite prepared in culture medium.

Cell respiration

Cell respiration, an indicator of cell viability, was assessed by mitochondria-dependent reduction of MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan.⁶ Cells in 96-well plates were incubated (37 °C) with MTT (5 mg ml⁻¹ for 4 h). Culture medium was removed by aspiration and cells were solubilized in acid-SDS (100 µl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of OD₅₇₀ against OD₆₃₀.

Statistical Analysis

All values in the figures, table and text are expressed as Mean±SE. Significant differences between groups were assessed with Student's *t*-test. A *p*-value less than 0.05 was considered to be statistically significant.

RESULTS

Effects on nitrite production

Unstimulated macrophages, after 24 hr of culture, produced the lowest levels of nitrite (3.5±2.1 µM) (Fig. 1, open bars). When these testing cells were incubated with crude extract alone, even at a concentration of 100 µM, amounts of nitrite in the medium were maintained at level similar to the unstimulated sample. A major increase of nitrite production was observed after treatment with LPS (1 µg/ml) plus IFN-γ (50 U/ml) for 24 hr; these stable products increased 7~10-fold (Fig. 1, hatched bars). With the exception of Nf and Tl, the ethanolic extracts of the other Chinese herbs had no or little effect on nitrite production in either un-stimulating or stimulating cells. However, 100 µg/ml of Va also significantly inhibited nitrite production. As shown in Fig. 1B, co-incubation of macrophages with Nf (2, 5, 10, 20, 50 and 100 µg/ml) and LPS/IFN-γ resulted in a dose-dependent reduction of nitrite accumulation. The IC₅₀ value for Nf was 18.6±1.3 µg/ml. Tl also suppressed the LPS/IFN-γ-induced NO₂⁻ production in a dose-dependent manner with an IC₅₀ of 17.8 µg/ml (Fig. 1E). Drug's effects were significantly distinguished from vehicle (data not shown). The overall efficacy of six Chinese herbs on nitrite production in macrophages is shown in Tab. 1.

Table 1. The inhibitory percentages of ethanolic extracts of *Davallia divaricata* (Dd), *Nothapodytes foetida* (Nf), *Orobanchae caerulescens* (Oc), *Spiranthes sinensis* (Ss), *Taxillus liquidambaricolus* (Tl), and *Viscum alniformosanae* (Va) on LPS/IFN-γ-induced nitrite production in RAW264.7 macrophages.

	Va	Nf	Tl	Dd	Oc	Ps
Inhibitory activity (%)	58.1±3.5	97.0±3.8	94.1±5.2	25.7±2.9	32.9±3.6	9.7±4.0

RAW264.7 macrophages were coincubation with LPS/IFN-γ and 100 µg/ml etanolic extracts for 24 hrs. Results are represented as percent inhibition and indicated as Mean±SE.

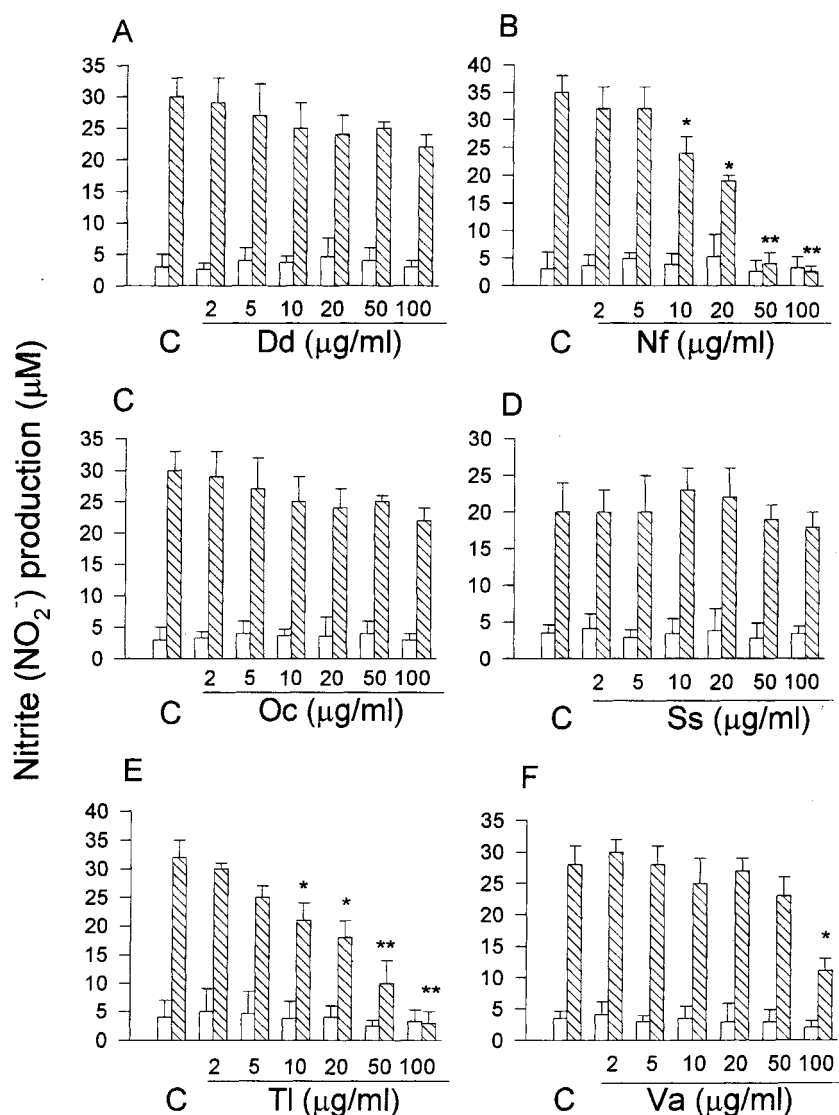


Fig. 1. Effects of *Davallia divaricata* (Dd; A), *Nothapodytes foetida* (Nf; B); *Orobanche caerulescens* (Oc; C), *Spiranthes sinensis* (Ss; D), *Taxillus liquidambaricolus* (Tl; E) and *Viscum alniformosanae* (Va; F) on LPS/IFN- γ -induced nitrite production in RAW264.7 macrophages. Cells were co-incubation with LPS/IFN- γ and six ethanolic extracts (2, 5, 10, 20, 50, and 100 $\mu\text{g/ml}$) for 24 hrs. Nitrite formation was determined by Griess methods as described in the text. Depicted is nitrite formation by RAW264.7 macrophages incubated with culture medium alone (open bars) or cell treated with LPS/IFN- γ (hatched bars). Data are expressed as Mean \pm SE. * $p < 0.05$ and ** $p < 0.01$ represents significant difference between groups in the absence (control) and presence of Chinese herbs.

Cell viability

Based on both trypan blue uptake experiments (data not shown) and the MTT test, six Chinese herbs at concentrations used were not toxic to macrophages during a 24-hr incubation (Fig. 2). That is, cell viability were not affected significantly by the six Chinese herbs treatment. However, 100 $\mu\text{g/ml}$ of Va (Fig. 2F) was associated with a small degree of cytotoxicity.

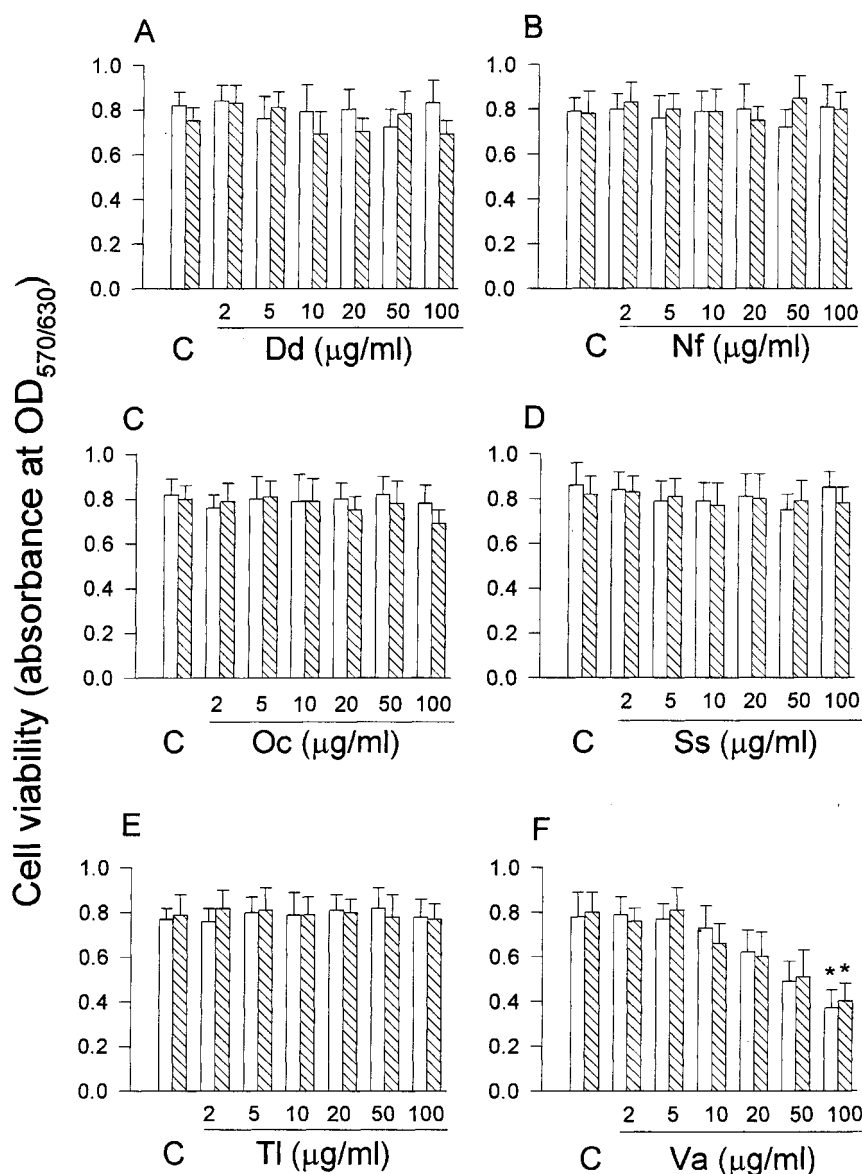


Fig. 2. Cytotoxic effects of *Davallia divaricata* (Dd; A), *Nothapodytes foetida* (Nf; B); *Orobanche caerulescens* (Oc; C), *Spiranthes sinensis* (Ss; D), *Taxillus liquidambaricolus* (Tl; E) and *Viscum alniformosanae* (Va; F) on RAW264.7 macrophages. Cells were co-incubation with LPS/IFN- γ and six ethnanolic extracts (2, 5, 10, 20, 50, and 100 $\mu\text{g/ml}$) for 24 hrs. Cell viability was determined by MTT assay as described in the text. Depicted is OD570/630 absorbance in cell treated with culture medium alone (open bars) or cell treated with LPS/IFN- γ (hatched bars). Data are expressed as Mean \pm SE. * $p < 0.05$ represents significant difference between groups in the absence (control) and presence of Chinese herbs.

DISCUSSION

NO is a short-lived bioactive molecule that participates in the physiology and pathophysiology of many system.⁷ In recent years it has become evidence that high output production of nitric oxide by iNOS is responsible for the development of a variety of diverse pathological events such as atherosclerosis, circulatory

shock, diabetes, chronic inflammation, and cancer, although the detailed molecular mechanisms are not clear. In the present study, the ethanolic crude extracts of six Chinese herbs commonly used in traditional Chinese medicine for the treatment of pain, inflammation and rheumatism were subjected to biological activity assay. Data obtained here suggesting that the ethanolic extracts isolated from *Nothapodytes foetida* (Nf) and *Taxillus liquidambaricolus* (Tl) suppressed the LPS/IFN- γ -induced nitrite production in RAW264.7 macrophages. These suggesting that Nf and Tl may act through suppressing nitrite accumulation as one of their possible antiinflammatory mechanism. This was correlated with its folk medicinal used.

It is unlikely that the ethanol extracts of Nf and Tl suppressed nitrite production through cytotoxic effects, because no cell death was noticed after the macrophages were treated with Nf and Tl. Therefore, we hypothesized that immuno-modulatory components may present in Nf and Tl. This may have important implications for the clinical treatment of diseases associated with overproduction of NO and strongly for the effectiveness of Nf and Tl in the anti-inflammatory therapy. The lose of effectiveness on the other four crude extracts may be due to their short half life. Thus, repeated application of these crude extracts to cells through out the cultured period (24 hrs) worth for further study.

The complex biochemistry of NO production affords many potential sites for regulatory action. NO production by macrophages is regulated by various factors (LPS, IFN- γ , transforming growth factor- β), and the induction of iNOS requires gene transcription and new protein synthesis, since actinomycin-D and cycloheximide inhibited the LPS- or IFN- γ -induced NO generation.⁸⁻¹¹ Thus, NO production by iNOS may be regulated at many sites, including transcription, post-transcription, translation, and post-translational modification.¹² Although our data clearly revealed that Nf and Tl dose-dependently inhibited nitrite accumulation (an indicator of NO synthesis), however, the precise mechanism by which this effect is mediated still remains to be clarified. The present finding provides a mechanism by which the anti-inflammatory properties of Nf and Tl could be mediated.

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在巨噬細胞探討六種抗發炎中草藥 對 LPS / IFN- γ 所誘發一氧化氮 生成之影響

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本實驗探討六種民間常用抗發炎中草藥的作用機轉是否與干擾發炎物質一氧化氮 (Nitric oxide, NO) 的生合成有關。六種中草藥分別是骨碎補 (*Davallia divaricata*, Dd)、青脆枝 (*Nothapodytes foetida*, Nf)、列當 (*Orobanchae caerulea*, Oc)、清明草 (*Spiranthes sinensis*, Ss)、大葉桑寄生 (*Taxillus liquidambaricolus*, Tl)、及槲寄生 (*Viscum alniformosanae*, Va)。以 LPS/IFN- γ 誘發培養的巨噬細胞產生過量一氧化氮, 藉此模擬體外發炎反應, 結果顯示其中的青脆枝及大葉桑寄生這二種中藥之乙醇抽提物可以明顯抑制由 LPS/IFN- γ 所誘發的一氧化氮生成, 且無明顯細胞毒性, 與其民間治療用途相符合, 並暗示其抗發炎機轉可能與干擾 NO 生合成途徑有關, 其餘四種中草藥並無顯著活性。

關鍵詞：一氧化氮, 巨噬細胞, 抗發炎, 青脆枝, 大葉桑寄生。

