行政院國家科學委員會補助專題研究計畫成果報告

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計畫編號：NSC90－2320－B－041－012－
執行期間：90　年　8 月　1 日至　91 年　9 月　30 日

計畫主持人：吳明娟
共同主持人：

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執行單位：嘉南藥理學院食品衛生系

中　華　民　國　91　年　12　月　10　日

行政院國家科學委員會專題研究計畫成果報告

中式飲料抗發炎之保健功能探討

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一、中文摘要

本年度計劃我們針對台灣本土特有的中式飲品包括山葡萄、蓮心、蓮葉及香茹的各種溶劑萃取液進行抑制老鼠巨噬細胞活化的抗發炎測試。我們發現香茹抑制 LPS 引發 RAW264.7 細胞的 NO 的產生效果最明顯。香茹的乙醇萃取液抑制 NO 的效果與劑量成正比。以西方吸漬法及 RT-PCR 分析發現，香茹可抑制 iNOS 基因的表達，因此香茹的抗發炎作用可能源於抑制 iNOS 基因的轉錄。

關鍵詞：香茹、山葡萄、蓮葉

Abstract

In the present study, we investigate the effects of various Chinese herbs, including *Ampelopsis brevipedunculata* (Maxim.) Trautv. stem (AB), leaf and plumule of lotus (*Nelumbo nucifera* Gertn.) as well as Hsiang Ju (*Glossogyne tenuifolia* Cassini) on nitric oxide (NO) production in endotoxin- stimulated macrophages. The ethanol extract of Hsiang Ju exhibited the best inhibition effect on LPS activated RAW 264.7 cells among the tested samples. To elucidate the detailed inhibitory mechanism of the ethanol extract of Hsiang Ju, inducible nitric oxide synthase (iNOS) expression was investigated. Western blot analysis revealed that Hsiang Ju (0.1 and 0.25 mg/ml) inhibited the expression of iNOS protein in a dose-dependent manner. RT-PCR assay further demonstrated that the iNOS mRNA level was decreased by Hsiang Ju. A mRNA stability analysis suggested that the reduced iNOS mRNA levels in Hsiang Ju-treated cells did not result from the accelerating degradation of the transcript. Taken together, the present data shows that the potent inhibition of LPS-stimulated NO production in macrophages may constitute the anti-inflammatory effects of Hsiang Ju. The primary mechanism responsible for this effect is the inhibition of iNOS expression that is probably mediated at transcription level by the ethanol extract of Hsiang Ju.

Keywords: **Keywords**: *Glossogyne tenuifolia*, Nitric oxide (NO), inducible nitric oxide synthase (iNOS)

Introduction

Nitric oxide (NO) is synthesized from L-arginine by constitutive and inducible nitric oxide synthase (cNOS and iNOS) in numerous mammalian cells and tissues (Nathan and Xie, 1994). Constitutively expressed NO by neuronal NOS (nNOS) and endothelial NOS (eNOS) is a key regulator of homeostasis. However, NO synthesized by iNOS is induced by bacterial lipoposaccharide (LPS) or immunological stimuli (IFN-γ) (Kilbourn and Griffith, 1992). The sustained NO production by iNOS has been implicated as a mediator of inflammation. It has been reported that excess production of NO by macrophages and other cells exposed to endotoxin may contribute to septic shock (Rees *et al.*, 1990), cerebral injury (Dawason et al., 1993), myocardical ischemia (Matheis et al., 1992), local or systemic inflammatory disorders, diabetes, arteriosclerosis, and other diseases (Stefanovic-Racic et al., 1993; Connor et al., 1995; Wu and Thiemermann, 1996). Thus, the inhibition of NO synthesis stands as an important therapeutic goal.

Stem of *Ampelopsis brevipedunculata* (Maxim.) Trautv. (AB)、Lotus (*Nelumbo nucifera* Gertn.) plumule and leaf (lian zi

xin、lian yeh)、Hsiang Ju (*Glossogyne tenuifolia* Cassini) are plants used as antipyretic, detoxication and anti-inflammatory remedies in folk medicine. However, informations regarding the mechanisms of their actions remain unclear.

In this study, we investigate the possible anti-inflammatory mechanisms of these herbal extracts on LPS-stimulated murine macrophage cell line, RAW 264.7. The present study suggests that inhibition of NO release and suppression of iNOS gene expression is the key factor contributing to the anti-inflammatory effect of Hsiang Ju, and the regulatory step of iNOS gene expression is primary at the mRNA level.

Results

1. Effects of different herbal extracts on the NO production

The efficacy of various concentrations (0.5, 0.1, 0.05, 0.01 mg/ml) of the ethanol extract of Hsiang Ju, water and methanol extracts of lotus plumule, methanol extract of lotus leaf and methanol extract of AB on NO production in LPS-stimulated macrophages is shown in Figure 1. Among them, the methanol extracts of Hsiang Ju and lotus plumule exhibit the most significant inhibition effects. The MTT assay revealed that the NO inhibitory effect exerted by the methanol extract of lotus plumule was due to its cytotoxicity. However, no cytotoxicity could be detected for the methanol extract of Hsiang Ju at concentration lower than 0.25 mg/ml. We further tested the effect of various solvent extracts of Hsiang Ju on the NO inhibition, and found that the ethanol extract was the most potent.

2. Effect of ethanol extract of Hsiang Ju on NO production

The efficacy of ethanol extract of Hsiang Ju on NO production in non-stimulated macrophages and LPS-stimulated macrophages is shown in Figure 2. Unstimulated macrophage (macrophage incubated with vehicle) produced the low basal level (2.1 ± 0.3 μM). Macrophages cultured with Hsiang Ju extract for 20 hrs did not affect the nitrite production too much, although a significantly elevated NO production (5.4 ± 0.38 μM) was observed at 0.25 mg/ml of Hsiang Ju extract.

When the cells were stimulated with LPS (10 ng/ml) for 20 hrs, this induced a 21.5-fold increase in nitrite production from the basal level to 45.31 ± 2.22 μM. In these experiments, the ethanol extract of Hsiang Ju (0.05, 0.1 and 0.25 mg/ml) evoked a concentration-dependent inhibition of nitrite release to 26.71 ± 7.52, 16.54 ± 4.57 and 6.25 ± 0.25 μM. Significant inhibition by the extract was observed at 0.05 mg/ml and a greater than 84% inhibition was noted at the concentration equal to 0.25 mg/ml. Based on the MTT test, cell viability after 20 hrs of 0.25 mg/ml of Hsiang Ju treatment was greater than 95% when compared with the control groups (data not shown). This indicated that the level of Hsiang Ju was not toxic to macrophages. The results implied that the inhibition of nitrite production by ethanol extract of Hsiang Ju was not due to cell death.

3. Effect of ethanol extract of Hsiang Ju on iNOS expression

To determine whether the NO inhibitory effect of ethanol extract of Hsiang Ju was due to inhibition of iNOS expression, Western blot analysis was carried out on the whole cell lysates using a monoclonal antibody for mouse macrophage iNOS. The dose responses for the inhibition of nitrate production and iNOS protein expression by the ethanol extract of Hsiang Ju are shown in Figure 3. Figure 3A shows the nitrate concentration in the supernatant of corresponding treatment, as indicated in Figure 3B. Figure 3C shows the quantification of iNOS protein expression analyzed with gel analysis software, normalized by calculating the ratio of iNOS to α-tubulin. Unstimulated macrophages did not express detectable level of iNOS protein. RAW 264.7 macrophages did not express detectable levels of iNOS protein after 12 hrs of incubation with medium alone. However, LPS caused a dramatic increase in iNOS protein expression. In the presence of 0.1 and 0.25 mg/ml of ethanol extract of

Hsiang Ju reduced the levels of iNOS protein by 87.2% and 100 % (Figure 3C), and NO generation by 63.1% and 92.8 % (Figure 3A), respectively, in LPS-stimulated cells. However, the expression of housekeeping gene, α-tubulin, is minimally affected. This suggested that the inhibition of iNOS protein expression by the ethanol extract of Hsiang Ju was not due to nonspecific or cytotoxic effects.

4. Effect of ethanol extract of Hsiang Ju on iNOS mRNA level

To determine whether the reduction in iNOS protein expression by the ethanol extract of Hsiang Ju would occur at the level of transcription, we used RT-PCR to examine the level of iNOS mRNA in activated macrophages. After 12 hrs of stimulation, LPS caused a 5.9-fold increase in iNOS mRNA expression (Figure 4). Ethanol extract of Hsiang Ju, in conjunction with the stimuli, blocked this induction. Figure 4B shows the quantification of iNOS mRNA expression by gel analysis software, normalized by calculating the ratio of iNOS to β-actin. A significant suppression of iNOS mRNA expression by ethanol extract of Hsiang Ju was observed. At 0.1 and 0.25 mg/ml, the ethanol extract of Hsiang Ju inhibited the levels of iNOS mRNA by 86.37% and 89.77%, respectively, in LPS-stimulated cells. Thus, the action of the ethanol extract of Hsiang Ju on iNOS expression was mainly at the mRNA level. The ethanol extract of Hsiang Ju may suppress iNOS transcription or render the iNOS mRNA more susceptible to degradation.

5. Effect of ethanol extract of Hsiang Ju on iNOS mRNA stability

To examine whether the ethanol extract of Hsiang Ju inhibits iNOS mRNA expression by reducing its stability, experiments were performed using the mRNA transcription disrupter, actinomycin D (added at 12 hrs after LPS induction, and indicated as time 0) (Figure 5A). In the continuous presence of actinomycin D, iNOS mRNA and β-actin mRNA expression are stable within a 12 hr period (total incubation interval for 24 hr after LPS stimulation) (Figure 5B). Figure 5C shows the quantification of iNOS mRNA expression by gel analysis software, normalized by calculating the ratio of iNOS over β-actin. In the presence of 0.25 mg/ml of ethanol extract of Hsiang Ju, iNOS mRNA levels were comparable with those of the control groups in the four tested intervals (0, 4, 8 and 12 hr). This data indicates that the inhibition of iNOS mRNA expression by ethanol extract of Hsiang Ju is not due to the decrease of post-transcriptional stability of iNOS.

Discussion and conclusions

Inducible nitrite oxide synthase (iNOS) has shown to be present in human inflammatory diseases and to promote the formation of deleterious peroxynitrite (Ischiropoulous et al., 1992). In the present study, we have shown for the first time the novel activity and mechanisms of ethanol extract of Hsiang Ju (*G. tenuifolia* Cassini) on the inhibition of NO production in LPS-stimulated macrophages. The inhibition of NO release was not attributable to cytotoxicity as assessed by the MTT assay and the expression of the housekeeping α-tubulin gene.

It has been known that the reduction of NO generation may result from one or more mechanisms: down-regulation of iNOS gene expression, inhibition of iNOS enzyme activity, and/or scavenging of $NO^{\cdot}$ molecules. Data from Western blot demonstrated that Hsiang Ju suppressed iNOS expression in LPS-stimulated macrophages. This result is consistent with NO reduction and indicates that the ethanol extract of Hsiang Ju inhibits NO generation in LPS-activated cells principally through repression of iNOS expression.

The expression of iNOS gene can be regulated at different levels, including transcriptional, post-transcriptional, translational, and post-translational steps (Nathan & Xie, 1994; Rao, 2000). Data obtained from RT-PCR showed that Hsiang Ju inhibited LPS-mediated iNOS mRNA expression. The levels of iNOS mRNA were consistent with the levels of iNOS protein.

This result suggests that the reduction in iNOS protein may primarily result from the decrease of iNOS mRNA.

The mRNA expression can be regulated both by up-stream transcriptional initiation and by down-stream mRNA degradation. The present data obtained from addition of actinomycin D indicated that the decrease in mRNA stability is not a factor contributing to the inhibitory effect of iNOS mRNA expression exerted by Hsiang Ju.

In conclusion, the ethanol extract of Hsiang Ju inhibited the production of NO in LPS-stimulated macrophages by suppressing the iNOS expression. The reduction of iNOS expression could occur primarily through mRNA synthesis. It is also possible that the inhibition of iNOS expression and NO production under stimulation may explain, in part, the beneficial effects of Hsiang Ju as an anti-inflammatory agent.

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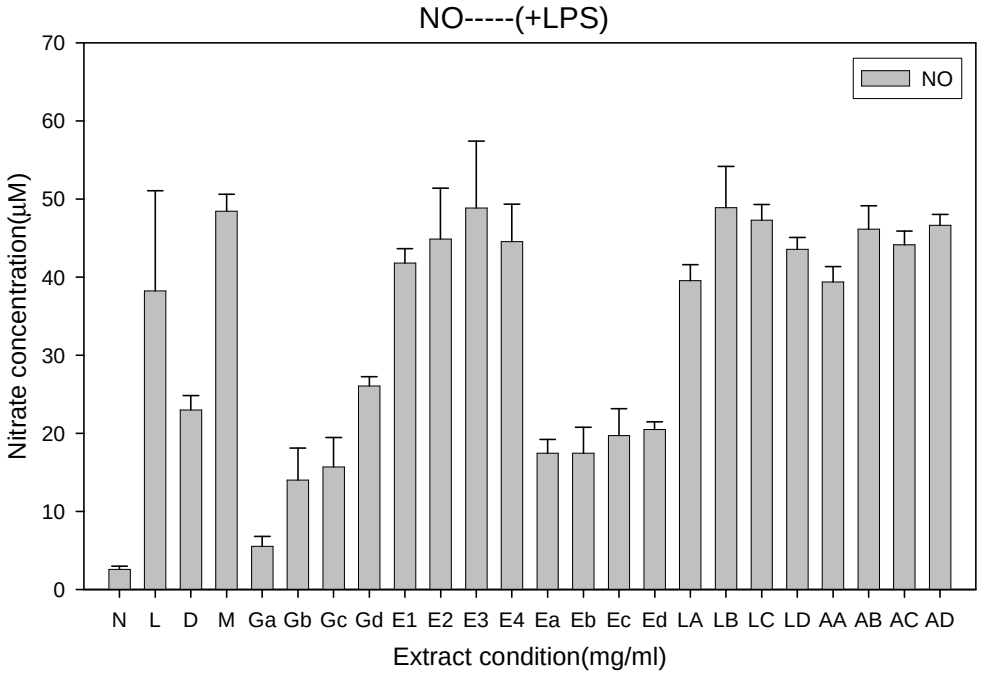

Figure 1. Effect of the various herbal extracts on NO productions in activated macrophages. N: normal; L: LPS treated cells, D: DMSO as vehicle; M: methanol as vehicle; Ga-Gd: methanol extract of Hsian Ju; E1-E4: water extract of lotus plumule; LA-LD: methanol extract of lotus plumule; AA-AD: methanol extract of AB. All the tested concentrations are 0.5, 0.1, 0.05, 0.01 mg/ml, referred to A-D or 1-4.

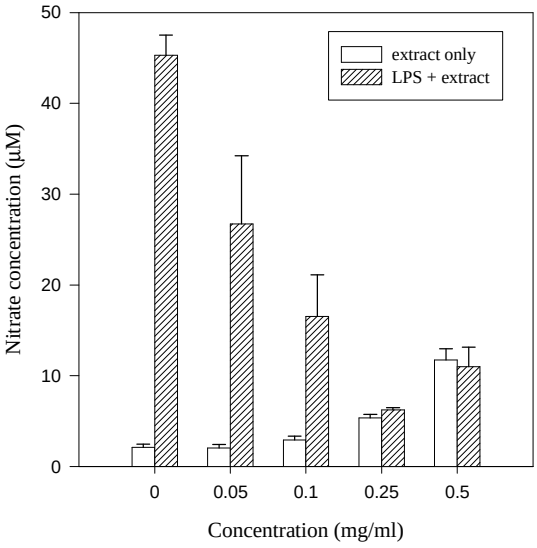

Figure 2: Effect of ethanol extract of Hsiang Ju on LPS-induced nitrite formation by RAW 264.7 macrophages.

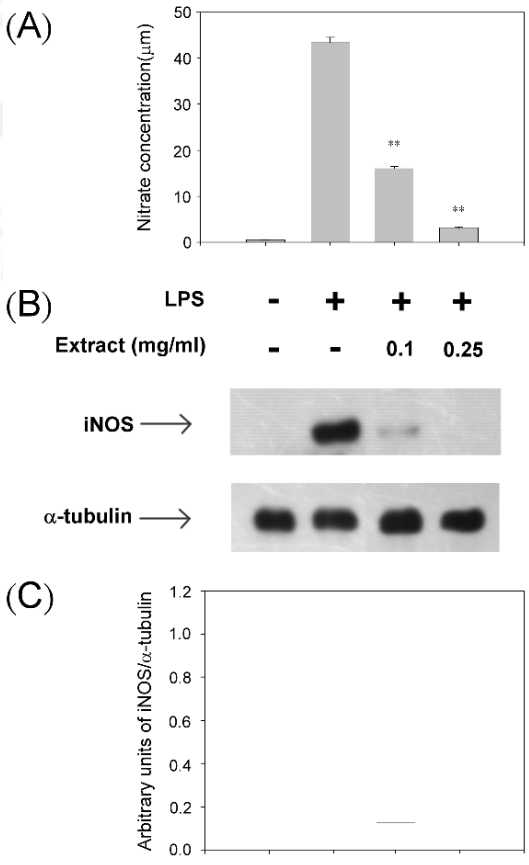

Figure 3. Inhibition of iNOS protein

expression in activated macrophages by ethanol extract of Hsiang Ju. The culture medium was collected for nitrite assay (A) and the extraction of total protein for iNOS protein and α-tubulin analysis (B). Band intensities were quantified by gel analysis software and indicated as relative fold of iNOS/α-tubulin (C).

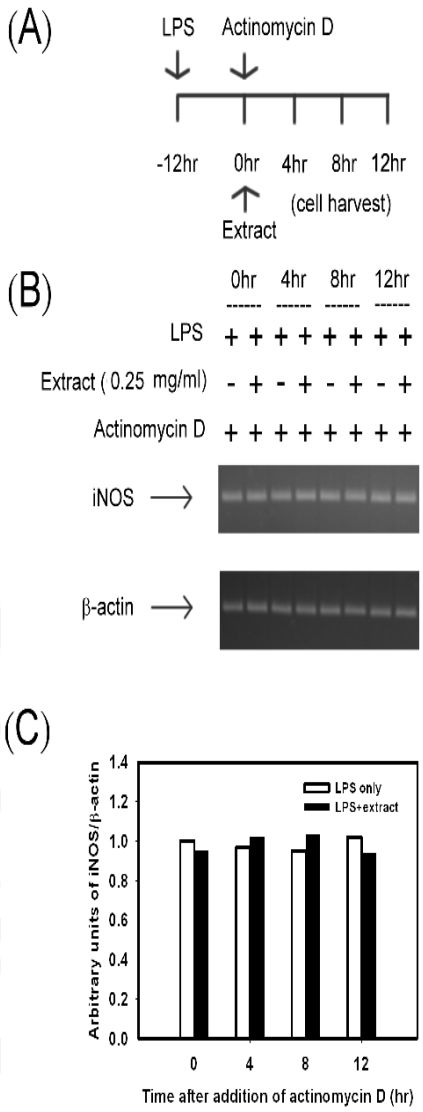

Figure 4. RT-PCR analysis of iNOS mRNA expression. (A) RAW 264.7 macrophages were incubated in the presence of LPS (10 ng/ml) with or without indicated concentrations of ethanol extract of Hsiang Ju for 12 hrs. (B) Band intensities were quantified by gel analysis software and indicated as a relative fold of iNOS/β-actin.

Figure 5. Effect of the ethanol extract of Hsiang Ju on iNOS mRNA stability. After macrophages were stimulated with LPS (10 ng/ml) for 12 hrs, actinomycin D (5 μg/ml) was added to the medium to interrupt further mRNA synthesis. Ethanol extract of Hsiang Ju (0.25 mg/ml) was added immediately following actinomycin D for further 4, 8, and 12 hr incubation. The iNOS mRNA stability was analyzed in the continuous presence of actinomycin D (A). RT-PCR was performed for iNOS and β-tubulin (B). Band intensities were quantified by gel analysis soft and indicated as a relative fold of iNOS/β-actin (C).