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## The inhibitory effects of andrographolide and extracts of some herbal medicines on the production of proinflammatory cytokines by LPS-stimulated human blood cells

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**Thamaree S, Rugrungham K, Ruangrungsi N, Thaworn N, Kemsri W. The inhibitory effects of andrographolide and extracts of some herbal medicines on the production of proinflammatory cytokines by LPS-stimulated human blood cells. Chula Med J 2001 Aug; 45(8): 661 - 70**

**Objective :** *To investigate the effects of andrographolide, an active principle isolated from *Andrographis paniculata*, and extracts of tested herbal medicines including, *Andrographis paniculata*, *Clinacanthus nutans*, *Gynura pseudochina* and *Gynura integrifolia*, on the production of proinflammatory cytokines by lipopolysaccharide (LPS) - stimulated human blood cells.*

**Design :** *Experimental study*

**Materials :** *All plants used were obtained from the botanical garden in the Faculty of Pharmaceutical Sciences, Chulalongkorn University. The chemicals and ELISA kits were purchased from Sigma Chemical Co. and Genzyme Corp., respectively.*

**Methods :** *Heparinized human blood obtained from normal healthy volunteers of ages 18 – 20 years, was stimulated with LPS in the presence or absence of tested compounds. After incubation times of 6 or 24 hours, supernatant levels of tumor necrosis factor -  $\alpha$  (TNF- $\alpha$ ), interleukin -1-  $\beta$  (IL-1 $\beta$ ) and interleukin-6 (IL-6) were quantified by ELISA.*

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**Results :** *Andrographolide as well as the extract of Andrographis paniculata, dose-dependently inhibited the production of TNF- $\alpha$  by LPS-stimulated human blood cells but did not alter the production of IL-1- $\beta$  or IL-6. The extracts of Clinacanthus nutans and Gynura pseudochaina dose dependently inhibited the production of IL-1- $\beta$  but did not alter the production of TNF- $\alpha$  and IL-6. The extract of Gynura integrifolia slightly inhibited the production of IL-1- $\beta$ .*

**Conclusion :** *In conclusion, our study revealed that the traditional herbal medicines empirically used as antiinflammatory herbs inhibited production of one or more of the proinflammatory cytokines including TNF- $\alpha$ , IL-1- $\beta$  and IL-6. The antiinflammatory effect of a single herb might be involved the inhibition of inflammatory mediators of other pathways, eg. the arachidonic acid pathway. The extract containing various constituents was found to be more effective than the pure compound. Combined use of multiple herbal medicines was more likely to be effective for inhibition of the proinflammatory cytokine production. The mechanisms by which such herbal medicines suppressed proinflammatory cytokine production remain to be further studied. Toxicological studies of effective herbal medicines are suggested.*

**Key words :** *Andrographolide, Proinflammatory cytokines, Herbal medicine, Anti-inflammation.*

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โสภิต ธรรมอารี, เกียรติ รักษ์รุ่งธรรม, นิจศิริ เรืองรังษี, นงนุช ถาวร, วันดี เข็มศรี. ฤทธิ์ยับยั้งการสร้าง proinflammatory cytokines ของสาร andrographolide และสารสกัดพืชสมุนไพรบางชนิดต่อเซลล์เม็ดเลือดมนุษย์ที่ถูกกระตุ้นด้วย LPS. จุฬาลงกรณ์เวชสาร 2544 ส.ค.; 45(8): 661 - 70

**วัตถุประสงค์** : เพื่อศึกษาฤทธิ์ของ andrographolide ที่ได้จากใบต้น *Andrographis paniculata* สารสกัดด้วย ethanol จากพืช *A. paniculata*, *Clinacanthus nutans*, *Gynura pseudochina* และ *G.integrifolia* ต่อการสร้าง proinflammatory cytokines โดยเซลล์เม็ดเลือดมนุษย์ที่ถูกกระตุ้นด้วย lipopolysaccharide (LPS)

**สถานที่ที่การวิจัย** : ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

**รูปแบบการวิจัย** : การทดลอง

**วัสดุ** : พืชทุกชนิดที่ใช้รวบรวมได้จากสวนสมุนไพรในคณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย สารเคมีและชุด ELISA ซื้อมาจาก Sigma Chemical Co. และ Genzyme Corp. ตามลำดับ

**วิธีการ** : นำเลือดจากอาสาสมัครปกติที่มีสุขภาพดีซึ่งมีสาร heparin ป้องกันการแข็งตัวของเลือดมากระตุ้นด้วย LPS ในสภาวะที่มีหรือไม่มีสารทดสอบหลังเวลา 6 หรือ 24 ชั่วโมง แยกส่วนน้ำขึ้นบนมาวิเคราะห์หาปริมาณของ tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1-6 (IL-1- $\beta$ ) และ interleukin-6 (IL-6) โดย ELISA

**ผลการศึกษา** : Andrographolide ตลอดจนสารสกัดของ *Andrographis paniculata* ทำให้เม็ดเลือดที่ถูกกระตุ้นด้วย LPS ลดการสร้าง TNF- $\alpha$  โดยขึ้นกับขนาดสารทดสอบแต่ไม่รบกวนการสร้าง IL-1- $\beta$  และ IL-6 สารสกัด *Clinacanthus nutans* และ *Gynura pseudochina* ยับยั้งการสร้าง IL-1- $\beta$  โดยขึ้นกับขนาดสารทดสอบ แต่ไม่รบกวนการสร้าง TNF- $\alpha$  และ IL-6 สารสกัด *Gynura integrifolia* ยับยั้งการสร้าง IL-1- $\beta$  ได้เพียงเล็กน้อย

**วิจารณ์และสรุป** : การศึกษานี้แสดงว่าพืชสมุนไพรที่มีประสมการณ์การใช้เป็นยาต้านการอักเสบสามารถยับยั้งการสร้าง proinflammatory cytokines ชนิดใดชนิดหนึ่ง ได้แก่ TNF- $\alpha$ , IL-1- $\beta$  และ IL-6 ฤทธิ์ต้านการอักเสบของสมุนไพรชนิดใดชนิดหนึ่งอาจเกี่ยวข้องกับการยับยั้งสารสื่อของการอักเสบจากวิธีอื่น ๆ เช่น arachidonic สารสกัดที่มีส่วนประกอบของสารหลายชนิดจะมีประสิทธิภาพดีกว่าสารบริสุทธิ์เดี่ยว การใช้ยาสมุนไพรร่วมกันน่าจะมีประสิทธิภาพยับยั้งการสร้าง proinflammatory cytokines ได้ดีกว่าสมุนไพรเดี่ยว กลไกที่พืชสมุนไพรลดการสร้าง proinflammatory cytokines ยังต้องศึกษาต่อไปเสนอแนะให้มีการศึกษาพิษวิทยาของพืชสมุนไพรที่มีประสิทธิภาพเหล่านี้

A number of herbal medicines, including *Andrographis paniculata* (Acanthaceae); *Clinacanthus nutans* (Acanthaceae); *Gynura pseudochina* (Compositae); and *Gynura integrifolia* (Compositae) have been widely used as antiinflammatory herbs in Chinese and Thai traditional medicine.<sup>(1)</sup> However, the mechanism of antiinflammatory activity is not clearly understood. It is well established that the proinflammatory cytokines including tumor necrosis factor (TNF- $\alpha$ ); interleukin-1- $\beta$  (IL-1- $\beta$ ); and interleukin-6 (IL-6) which are produced mainly by blood monocytes and tissue macrophages appear to be integrally involved in the processes of inflammation.<sup>(2,3)</sup> The antiinflammatory activity of these herbal medicines may be attributed to interference with the production of proinflammatory cytokines. Moreover the over production of TNF- $\alpha$ , IL-1- $\beta$  and IL-6 is involved in many inflammatory state disorders.<sup>(4,5)</sup> Control of the production and/or release of cytokines would represent a valuable target for pharmacological intervention. Removal of monocytes from their natural milieu may alter their subsequent immune response patterns. It is, therefore, the purpose of this study to investigate the effect of andrographolide, a purified diterpene lactone from the plant *Andrographis paniculata* and alcohol extracts of *A paniculata*; *Clinacanthus nutans*; *Gynura pseudochina*; and *G. integrifolia* on the production of proinflammatory cytokines by lipopolysaccharide (LPS)-stimulated human blood cells.

## Materials and Methods

### Chemicals

Purified E.Coli 026:B6 lipopolysaccharide (LPS), RPMI-1640 medium, L-glutamine, penicillin - streptomycin were purchased from Sigma Chemical Co.

### Plant Materials

All plants used in this study were obtained from the botanical garden in the Faculty of Pharmaceutical Sciences, Chulalongkorn University. The materials were authenticated by the Department of Botany of the same faculty and their voucher specimens are kept in the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences. Andrographolide was isolated and purified from the aerial parts of *Andrographis paniculata*, Wall ex Nees, as described.<sup>(6)</sup> The ethanol extracts of dried leaves of *Clinacanthus nutans* (Burm.f.) Lindau; of dried rhizome and leaves of *Gynura pseudochina* DC. var. *hispida* Thv. and *Gynura integrifolia* Gagnep were prepared at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. The solvent was removed under reduced pressure at 50°C.

### Human whole blood stimulation

Peripheral blood from healthy consenting male donors, 18 - 20 years of age, was drawn into heparinized syringes (12.5 U heparin/ml) and three-fold diluted in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin and 300 ml fractions were distributed into 24-well multidisc plates (costar corporation, USA). Blood samples were preincubated (60 min at 37°C) in a humidified 6 % CO<sub>2</sub> - atmosphere with 100  $\mu$ l of drug solvent (final concentration 0.02 % DMSO in RPMI 1640) or with 100  $\mu$ l of appropriate concentrations of test compounds before being stimulated by the addition of 100  $\mu$ l of LPS at a final concentration of 40 ng/ml. After indicated culture times, cell-free supernatant fluids were collected by centrifugation

(2,000 rpm, 15 min at 4°C) and stored at -70°C until tested for the presence of cytokines.

### Cytokine measurements

TNF- $\alpha$  levels were assayed by sandwich ELISA's developed in-house. IL-1- $\beta$  and IL-6 were assayed by using commercially available ELISA kits (Genzyme Corp., USA). Plates were read at 450 nm in a Titertek microplate reader. Cytokines were calculated from standard curves using recombinant human TNF- $\alpha$ , IL-6 or IL-1- $\beta$ . Validation experiments indicated that, for each cytokine tested, standard curves were unaffected by the presence of test compounds, discounting the possibility that the compound interfered with ELISA determinations.

### Data analysis

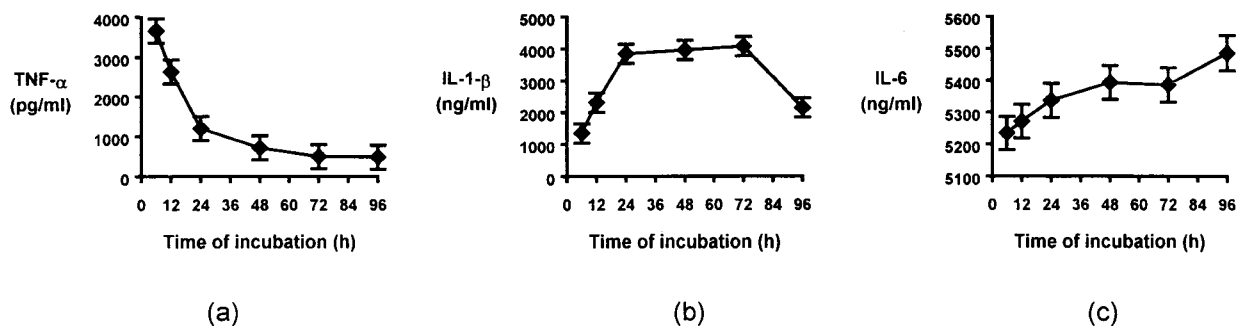
Data were expressed as mean  $\pm$  SD. Comparison between two groups were compared by Student's unpaired t-test. A p-value of < 0.05 was considered significant. The percentage changes of

cytokine production were calculated and compared between groups.

### Results

#### Cytokine production by LPS-stimulated human blood

To determine optimal incubation times, blood from three volunteers was cultured in the absence or presence of LPS. Six, 12, 24, 48, 72 and 96 h after culture start, supernatant levels of TNF- $\alpha$ , IL-1- $\beta$  and IL-6 determined by ELISA were not found in supernatants of unstimulated blood cultures. Addition of LPS induced substantial production of all measured cytokines (Figure 1). The maximal production level of TNF- $\alpha$  was reached as early as 6 h after culture start. Whereas maximal production level of IL-1- $\beta$  was reached between 24 to 72 h and of IL-6 was reached between 24-96 h after culture start. On the basis of these data, inhibition of proinflammatory cytokine production was assayed in supernatants collected at 6 h for TNF- $\alpha$  or at 24 h for IL-1- $\beta$  and IL-6.



**Figure 1.** Kinetics of TNF- $\alpha$  (a), IL-1- $\beta$  (b) and IL-6 (c) release from LPS-stimulated human blood cells. The points represent mean  $\pm$  SD of duplicate determinations from three separate experiment.

### Effects of andrographolide and ethanol extracts of tested herbal medicines on cytokine production by LPS-stimulated human blood

As shown in Table 1 and 2, andrographolide and the extract of *Andrographis paniculata* at the same concentration of 20 µg/ml or 54.35 µM of andrographolide suppressed upto 96 % of LPS-stimulated TNF-α production from human blood but not the production of IL-1β and IL-6. The extracts of *Clinacanthus nutans* as well as of *Gynura pseudochina* at the concentration of 20 µg/ml suppressed up to

83 % and 77 % of LPS-stimulated IL-1β production, from human blood, respectively (Table 3 and 4). The extract of *Gynura integrifolia* at the concentration of 0.16 and 20 mg/ml suppressed the production of IL-1β up to 61 % and 46 % from LPS-stimulated human blood, respectively, but the suppression was not statistically significant (Table 5). The inhibitory effects of test compounds, at the same concentration of 20 µg/ml, on the production of proinflammatory cytokines by LPS-stimulated human blood cells are shown in Table 6.

**Table 1.** Effects of andrographolide on cytokine production by LPS-stimulated human blood cells.

The values are mean ± SD of duplicate determinations from three subjects.

Concentration		TNF-α	IL-1β	IL-6
(µg/ml)	µM	(pg/ml)	(ng/ml)	(ng/ml)
		at 6 h	at 24 h	at 24 h
20	54.35	57.23 ± 14.35*	5,353.0 ± 2,852.69	5,565.48 ± 116.92
4	10.87	1,520.72 ± 332.25	4,959.0 ± 71.01	5,485.77 ± 112.17
0.8	2.17	1,460.32 ± 470.86	4,157.0 ± 1,460.12	5,415.92 ± 75.94
0.16	0.43	1,717.67 ± 532.62	3,842.33 ± 2,005.14	5,421.67 ± 159.51
0	0	1,536.19 ± 343.33	3,842.33 ± 2,005.14	5,337.0 ± 251.02

**Table 2.** Effects of an ethanol extract obtained from *Andrographis paniculate* on cytokine production by LPS-stimulated human blood cells. The values are mean ± SD of duplicate determinations from three subjects.

Concentration	TNF-α	IL-1β	IL-6
(µg/ml)	(pg/ml)	(ng/ml)	(ng/ml)
	at 6 h	at 24 h	at 24 h
20	49.63 ± 14.74*	3,243.93 ± 530.01	2,695.82 ± 204.25**
4	1,974.64 ± 360.82	4,112.67 ± 291.096	5,240.14 ± 231.97
0.8	2,311.19 ± 1177.72	3,698.24 ± 479.232	5,354.61 ± 125.81
0.16	2,019.61 ± 918.65	4,037.33 ± 344.03	5,402.21 ± 325.83
0	1,536.19 ± 343.33	3,842.33 ± 2,005.14	5,337.0 ± 251.02

\* p < 0.02 when compared with the control group (concentration 0 mg/ml)

\*\* p < 0.01 when compared with the control group (concentration 0 mg/ml)

**Table 3.** Effects of an ethanol extract obtained from *Clinacanthus nutans* on cytokine production by LPS-stimulated human blood cells. The values are mean  $\pm$  SD of duplicate determinations from three subjects.

Concentration ( $\mu\text{g/ml}$ )	TNF- $\alpha$ (pg/ml) at 6 h	IL-1 $\beta$ (ng/ml) at 24 h	IL-6 (ng/ml) at 24 h
20	2,054.23 $\pm$ 858.88	664.00 $\pm$ 514.23*	5,339.498 $\pm$ 271.45
4	2,425.3 $\pm$ 1,010.43	1,225.33 $\pm$ 1,042.42	5,386.34 $\pm$ 300.63
0.8	2,245.98 $\pm$ 871.31	1,024.67 $\pm$ 1,451.22	5,323.06 $\pm$ 351.21
0.16	2,795.27 $\pm$ 1,385.78	929.33 $\pm$ 1,179.59	5,385.52 $\pm$ 300.13
0	1,536.19 $\pm$ 343.33	3,842.33 $\pm$ 2,005.14	5,337.0 $\pm$ 251.02

\* p < 0.05 when compared with the control group (concentration 0  $\mu\text{g/ml}$ )

**Table 4.** Effects of an ethanol extract obtained from *Gynura pseudochina* on cytokine production by LPS-stimulated human blood cells. The values are mean  $\pm$  SD of duplicate determinations from three subjects.

Concentration ( $\mu\text{g/ml}$ )	TNF- $\alpha$ (pg/ml) at 6 h	IL-1 $\beta$ (ng/ml) at 24 h	IL-6 (ng/ml) at 24 h
20	1,523.64 $\pm$ 511.28	869.33 $\pm$ 625.15*	5,398.67 $\pm$ 261.21
4	2,101.91 $\pm$ 624.68	2,083.00 $\pm$ 2,527.61	5,240.89 $\pm$ 297.104
0.8	1,755.54 $\pm$ 573.90	1,891.33 $\pm$ 2,705.29	5,348.54 $\pm$ 106.04
0.16	1,871.45 $\pm$ 962.17	1,802.33 $\pm$ 2,775.92	5,382.23 $\pm$ 229.21
0	1,536.19 $\pm$ 343.33	3,842.33 $\pm$ 2,005.14	5,337.0 $\pm$ 251.02

\* p < 0.05 when compared with the control group (concentration 0  $\mu\text{g/ml}$ )

**Table 5.** Effects of an ethanol extract obtained from *Gynura integrifolia* on cytokine production by LPS - stimulated human blood cells. The values are mean  $\pm$  SD of duplicate determinations from three subjects.

Concentration ( $\mu\text{g/ml}$ )	TNF- $\alpha$ (pg/ml) at 6 h	IL-1 $\beta$ (ng/ml) at 24 h	IL-6 (ng/ml) at 24 h
20	1,763.50 $\pm$ 847.70	2,072.67 $\pm$ 1,869.82	5,522.75 $\pm$ 335.95
4	1,514 $\pm$ 447.61	3,325.67 $\pm$ 1,464.79	5,369.08 $\pm$ 362.93
0.8	1,200 $\pm$ 192.98	3,773.33 $\pm$ 2,124.65	5,392.91 $\pm$ 341.26
0.16	1,460.31 $\pm$ 205.15	1,485.0 $\pm$ 1,471.53	5,470.98 $\pm$ 79.095
0	1,536.19 $\pm$ 343.33	3,842.33 $\pm$ 2,005.14	5,337.0 $\pm$ 251.02



**Table 6.** Effects of 20 µg/ml of andrographolide and ethanol extracts obtained from medicinal plants on cytokine production by LPS-stimulated human blood cells.

	TNF- $\alpha$ (pg/ml) at 6 h	IL-1 $\beta$ (ng/ml) at 24 h	IL-6 (ng/ml) at 24 h
Control	1,536.19 $\pm$ 343.33	3,842.33 $\pm$ 2,005.14	5,337.0 $\pm$ 251.02
Andrographolide (54.35 µM)	57.23 $\pm$ 14.35*	5,353.0 $\pm$ 2,852.69	5,565.48 $\pm$ 116.92
Extract of <i>Andrographis paniculata</i>	49.63 $\pm$ 14.74*	3,243.93 $\pm$ 530.01	2,695.82 $\pm$ 204.25*
Extract of <i>Clinacanthus nutans</i>	2,054.23 $\pm$ 858.88	664.00 $\pm$ 514.23*	5,339.5 $\pm$ 271.45
Extract of <i>Gynura pseudochina</i>	1,523.64 $\pm$ 511.28	869.33 $\pm$ 625.15*	5,398.67 $\pm$ 261.21
Extract of <i>Gynura integrifolia</i>	1,763.50 $\pm$ 847.70	2,072.67 $\pm$ 1,869.82	5,522.75 $\pm$ 335.95

\* p < 0.05 when compared with the control group

## Discussion

It is well established that the active component of gram-negative bacteria, lipopolysaccharide (LPS) is one of the most potent inducers of TNF- $\alpha$ , IL-1- $\beta$  and IL-6, production by blood monocytes, lymphocytes and tissue macrophages. In the LPS-induced cytokine cascade, bacterial LPS acts on macrophages to release TNF. TNF induces macrophages to release IL-1- $\beta$ . IL-1- $\beta$  acts on macrophages and vascular endothelial cells to release IL-6 and IL-8. LPS also directly induces IL-1- $\beta$ , IL-6 and IL-8 and TNF directly induces IL-6 and IL-8, but these actions are amplified through the cascade.<sup>(7)</sup> Monocytes have been demonstrated to be the source of whole blood IL-1- $\beta$  production.<sup>(8)</sup> In addition to macrophages and vascular endothelial cells, monocytes are capable of producing IL-6.<sup>(9)</sup>

Putative LPS binding proteins in human plasma may modulate monocyte-LPS interactions resulting in TNF- $\alpha$ , IL-1- $\beta$  and IL-6 production.<sup>(11)</sup> The kinetics release of these cytokines from stimulated whole blood cells showed different times to maximal

production. TNF- $\alpha$  release peaked at 6 hours as previously reported,<sup>(10)</sup> whereas IL-1- $\beta$  and IL-6 release peaked later, from 24 to 72 hours.

Although the existence of a cytokine cascade, involving sequentially TNF- $\alpha$ , IL-1- $\beta$ , IL-6, and IL-8 in pathogenesis of inflammation has been evidenced. Nevertheless, several effects of each cytokine occur independently of the other cytokines.

Andrographolide, the active principle from *Andrographis paniculata*, as well as the ethanol extract, dose dependently inhibited the production of TNF- $\alpha$  by LPS-stimulated human blood cells but did not alter the production of IL-1- $\beta$  and IL-6. The results indicate that andrographolide might inhibit the effect of LPS in stimulating blood monocytes and/or inhibit the processes of TNF- $\alpha$  synthesis since andrographolide specifically inhibited TNF- $\alpha$  production. The extracts of *Clinacanthus nutans* as well as of *Gynura pseudochina* dose dependently inhibited the production of IL-1- $\beta$  but did not alter the production of TNF- $\alpha$  and IL-6, indicating that the extracts of these two plants might specifically inhibit the effect of LPS

and/or TNF- $\alpha$  in stimulating blood monocytes. The extracts of *Gynura integrifolia* slightly inhibited the production of IL-1- $\beta$ , similarly to the inhibitory effect of *Gynura pseudochina*. This might be due to the difference in composition of active principles in these two plants of the same genus. It is now clear that the principal function of IL-1, similar to TNF, is as a mediator of the host inflammatory response. <sup>(7)</sup> Thus andrographolide which is found, in this study, to inhibit production of TNF- $\alpha$  and the extracts of *Clinacanthus nutans* and *Gynura pseudochina* which are shown to inhibit IL-1- $\beta$  production confirm the antiinflammatory activity of these tested compounds.

In this study, we demonstrated the inhibitory effect of andrographolide and the extract of *A. paniculata* on the production of TNF- $\alpha$  by LPS-stimulated human blood cells. Interestingly the extract of *A. paniculata* inhibited IL-6 production whereas andrographolide did not. The possibility is that the extract contained many constituents which may contribute to inhibition of TNF- $\alpha$  and IL-6 production. The extracts of *Clinacanthus nutans* as well as of *Gynura pseudochina* solely inhibited IL-1- $\beta$  production. These herbal medicines may act directly on production of a particular cytokine by many ways. They may affect mRNA stability, reduce the levels of mRNA, increase disposition of the precursors or the cytokines as well.

In conclusion our study revealed that the traditional herbal medicines empirically used as antiinflammatory herbs inhibited production of one or more of the proinflammatory cytokines including TNF- $\alpha$ , IL-1- $\beta$  and IL-6. The antiinflammatory effect of a single herb might be involved the inhibition of inflammatory mediators of other pathways, eg. The

arachidonic acid pathway. The extract containing various constituents was thought to be more effective than the pure compound. Combination of multiple herbal medicines was likely to be effective for inhibition of the proinflammatory cytokine production. The mechanisms by which such herbal medicines suppressed proinflammatory cytokine production remains to be further studied. Toxicological studies of effective herbal medicines are suggested.

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