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Inhibitory Effect of Oriental Herbal Medicines on Tumor Necrosis Factor-α Production in Lipopolysaccharide-stimulated RAW264.7 cells

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Abstract – Eleven out of 118 herbal medicines which are frequently used in Korean traditional prescriptions for inflammatory diseases exhibited more than 50% of inhibition on TNF-α production in LPS-stimulated RAW264.7 cells by their total ethanol extracts with 0.1 mg/ml as a final concentration. The active 11 total extracts were prepared from *Angelica koreana, Coptis japonica, Cynanchum paniculatum, Magnolia fargesii, Magnolia officinalis, Panax ginseng, Patrinia scabiosaefolia, Pterocarpus santalius, Rhapontica uniflora, Saussurea lappa.* Of them, *Coptis japonica, Magnolia fargesii* and *Saussurea lappa* also significantly inhibited TNF-α production *in vivo*. These total extracts were sequentially fractionated with *n*-hexane, methylene chloride, ethyl acetate, *n*-butanol and water. Among the solvent-fractionated extracts with 0.05 mg/ml as a final concentration, inhibitory effects of *Angelica koreana, Magnolia fargesii, Magnolia officinalis, Pterocarpus santalinus, Rhapontica uniflora* and *Saussurea lappa* markedly showed in one or two solvent fractions suggesting that the principles may be concentrated by subfractionation as the main compounds.

Key words – TNF-α production, lipopolysaccharide-stimulated RAW264.7 cells, oriental herbal medicines.

Introduction

Tumor necrosis factor (TNF)-α a proinflammatory cytokine produced by activated macrophages (Vileek and Lee, 1991), participates in pathogenesis of acute and chronic inflammatory diseases such as septic shock, rheumatoid arthritis and allergic inflammation (Firestein 1994, Firestein *et al.*, 1994, Manogue *et al.*, 1992, Mohler *et al.*, 1994, Novgrodiski *et al.*, 1994). Thus, it enhances the production of other cytokines by autocrine stimulation (Hensel *et al.*, 1987) and induces the production of prostaglandin E_2 (PGE_2) by synovial fibroblast-type cells and of prostaglandin I_2 (PGI_2) by endothelial cells (Dayer *et al.*, 1986, Rossi *et al.*, 1985). Consequently, TNF-α has been implicated in many human diseases as a main mediator.

Because of its pivotal role in inflammatory disease, a significant effort has been focused on developing therapeutic agents that interfere with TNF-α production or action. These have included tyrphostin-related tyrosine kinase inhibitors (Novgrodiski *et al.*, 1994), pentoxifylline (Han *et al.*, 1990), thalidomide (Moreira *et al.*, 1993), various inhibitors of TNF-α processing (McGeehan *et al.*, 1994; Mohler *et al.*, 1994), a family of carbocyclic nucleosides (Bradshaw *et al.*, 1995, Firestein *et al.*, 1994, Sajjadi *et al.*, 1996), and new antiinflammatory benzylamide derivatives (Lang *et al.*, 1995).

There are some reports indicating that various natural compounds such as lignans, sesquiterpene lactones and flavonoids derived from plants may possibly possess various biological activities including antiviral, anticancer, antiallergic and immunoregulatory activities (Havsteen *et al.*, 1983, Hirano *et al.*, 1994, Schrder *et al.*, 1990, Torrance *et al.*, 1979). Therefore, it is considerable that these natural products can be developed as a new generation of drugs such as antiinflammatory drugs or anticancer drugs. In particular, recently many researchers have been reported that the compounds from natural product have the inhibitory effect of TNF-α production and have been regarded as the new types of TNF-α antagonists compared to previous developing drugs (Chae *et al.*, 1997, Cho *et al.*, 1998a, b, c, d).

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To search the active principles, in this study we first examined the inhibitory effect of total ethanol extract from medicinal plants, which are frequently used in Korean traditional prescriptions for inflammatory diseases, on TNF-α production in RAW264.7 cells stimulated by lipopolysaccharide (LPS). As the results, eleven out of 118 herbal medicines exhibited more than 50% of inhibition on TNF-α production in LPS-stimulated RAW264.7 cells.

Materials and Methods

Animals – Eight-week-old C56BL/6 male mice were purchased from B & K Universal (Fremont, CA, USA). The BALB/c mice were maintained in plastic cages under conventional conditions. Water and pelleted diets (Samyang, Daejeon, Korea) were supplied *ad libitum*.

Materials – Prednisolone and lipopoly-saccharide (LPS, *E. coil* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin, streptomycin and RPMI1640 were obtained from GIBCO (Grand Island, NY, USA). RAW264.7 cells, a murine macrophage like cell were purchased from ATCC (Rockville, MD, USA). Total ethanol fractions and their subfractions were prepared by previous method (Chae *et al.*, 1998) All other chemicals were of reagent grade.

Plant materials – Medicinal plants were purchased from a drug store (Dongyang Yackup Co.). The voucher specimens are deposited at the R & D Center in Daewoong Pharm. Co. (Sungnam, Korea).

TNF-α production *in vitro* – The inhibitory effect of testing fractions on TNF-α production was determined as previously descried (Cho *et al.*, 1998b). RAW 264.7 cells were maintained in RPMI1640 supplemented with 100 U/ml of penicillin and 100 ?g/ml of streptomycin, and 5% FBS. Cells were grown at 37and 5% CO_2 in humidified air. The isolated compounds and fractions solubilized with vehicle (89.9% propylene glycol, 10% ethanol and 0.1% dimethyl sulfoxide) were diluted with RPMI1640. In these conditions, none of the solubilization solvents altered TNF-α production in RAW264.7 cells. Before stimulation with LPS (1 μg/ml) and testing samples, RAW264.7 cells (1×10^6 cells/ml) were incubated for 18 h in 24 well plates (flat bottom; Falcon 3027, Becton Dickinson and Company, NJ, USA) with the same conditions. Stimuli and the various concentrations of testing samples were then added to the wells for 4 h. Supernatants were then collected and assayed for TNF-α content using mouse TNF-α ELISA kit (Amersham, Little Chalfont, Buckinghamshire, UK).

TNF-α production *in vivo* The methods of Novgrodiski *et al.*, (1994) was used for LPS induction of TNF-α *in vivo*. Fasted mice were orally administered with test fractions (200 mg/kg) and standard drug (prednisolone, 10 mg/kg) suspended in 0.5% sodium carboxylmethylcellulose 2 h before challenge with intraperitoneal injection of LPS (1.5 mg/mouse). After 90 min, blood was collected and serum samples were used to measure TNF-α levels by ELISA kit.

Statistical analysis – All values expressed as mean ±SEM were obtained from 3 or 6 observations. The Student's *t*-test for unpaired observation between control and experimental samples was carried out for statistical evaluation of a difference; p values of 0.05 or less were considered as statistically significant.

Results and Discussion

Herbal medicines in this study were selected by their frequent use in Korean traditional prescription for inflammation-related diseases. As a part of an elucidation of anti-inflammatory principles of herbal medicines, it is reasonable to estimate the inhibitory effect on TNF-α production. Eleven out of 118 herbal medicines exhibited more than 50% of inhibition on TNF-α production by their total ethanol

Table 1. TNF-α inhibitory activities of 118 medicinal plant extracts

Medicinal plants	Used part	Family name	% inhibition[a]
Acanthopanax senticosus	Radix	Araliaceae	8.2±4.4
Acanthopanax sessiliflorum	Cortex	Araliaceae	30.6±8.8
Achyranthes japonica	Radix	Amaranthaceae	6.7±5.5
Aconitum koreanum	Tuber	Ranunculaceae	10.9±3.2
Acorus gramines	Rhizoma	Araceae	19.9±1.0
Agastache rugosa	Herba	Labiatae	20.4±6.6

Table 1. Continued

Medicinal plants	Used part	Family name	% inhibition[a]
Aralia elata	Cortex	Araliaceae	47.9±3.4
Aralia cordata	Radix	Araliaceae	23.3±2.1
Aristolochia contorta	Fructus	Aristolochiaceae	36.7±7.2
Artemisia capillaris	Flos	Compositae	31.1±4.9
Albizzia julibrissin	Cortex	Leguminosae	0.0±6.2
Alisma orientale	Rhizoma	Alismataceae	19.7±3.7
Alpinia oxyphylla	fructus	Zingiberaceae	22.4±3.4
Amomum medium	Fructus	Zingiberaceae	20.7±5.2
Amomum xanthioides	Semen	Zingiberaceae	7.9±3.5
Anemarrhena asphodeloides	Rhizoma	Liliaceae	3.2±5.2
Angelica dahurica	Radix	Umbelliferae	29.4±8.8
Angelica gigas	Radix	Umbelliferae	36.7±1.0
Anethum graveolens	Fructus	Umbelliferae	35.0±1.6
Angelica koreana	Radix	Umbelliferae	56.1±0.7
Anthriscus sylvestris	Radix	Umbelliferae	26.3±3.9
Belamcanda chinensis	Rhizoma	Iridaceae	35.6±1.5
Bupleurum falcatum	Radix	Umbelliferae	-31.5±3.5
Caragana sinica	Radix	Leguminosae	30.1±1.0
Carthamus tinctorius	Flos	Compositae	5.4±6.8
Celosia argentea	Semen	Amaranthaceae	-3.4±4.3
Chaenomeles japonica	Fructus	Rosaceae	5.3±2.3
Chelidonium major	Herba	Papaveraceae	35.3±1.8
Chrysanthemum indicus	Flos	Compositae	5.9±4.2
Cinnamomum cassia	Cortex	Lauraceae	23.2±1.6
Citrus unshiu	Cortex	Rutaceae	3.1±4.5
Clematis mandshurica	Radix	Ranunculaceae	28.1±2.6
Crataegus pinnatifida	Fructus	Rosaceae	11.6±1.5
Cnidium officinale	Rhizoma	Umbelliferae	4.8±6.4
Coix lachryma-jobi	Semen	Gramineae	13.2±8.5
Coptis japonica	Rhizoma	Ranunculaceae	78.2±3.0
Curcuma zedoaria	rhizoma	Zingiberaceae	29.3±8.1
Cynanchum paniculatum	Radix	Asclepiadaceae	50.5±9.0
Daphne genkwa	Flos	Thymelaeaceae	40.4±6.9
Dioscorea japonica	Rhizoma	Dioscoreaceae	40.0±0.5
Dictammus albus	Radix	Rutaceae	6.8±2.0
Dolichos labalb	Semen	Leguminosae	38.8±2.9
Elsholtzia ciliata	Herba	Labiatae	17.9±1.9
Ephedra sinica	Herba	Ephedraceae	3.7±4.6
Eucommia ulmoides	Cortex	Eucommiaceae	6.6±8.0
Eugenia caryophyllata	Flos	Myrtaceae	-24.0±8.3
Farfugium japonicum	Flos	Compositae	12.3±2.0
Forsythia koreana	Fructus	Oleaceae	29.1±4.4
Fritillaria ussuriensis	Bulbus	Liliaceae	8.9± 8.4
Gardenia jasminoides	Fructus	Rubiaceae	2.4±1.9
Gentiana scabra	Radix	Gentianaceae	5.7±4.2
Glicine max	Semen	Leguminosae	14.1±1.3
Glycyrrhiza glabra	Radix	Leguminosae	40.1±4.3

Table 1. Continued

Medicinal plants	Used part	Family name	% inhibition[a]
Houttuynia cordata	Herba	Saururaceae	44.3±0.6
Kalopanax pictus	Radix	Araliaceae	30.3±5.3
Ledebouriella sesseloides	Radix	Umbelliferae	2.1±2.8
Leonurus sibiricus	Herba	Labiatae	24.0±4.0
Liriope platyphylla	Tuber	Liliaceae	27.8±2.5
Lonicera japonoca	Flos	Caprifoliaceae	5.1±3.9
Lonicera japonoca	Folium	Caprifoliaceae	12.4±0.5
Loranthus parasiticus	Herba	Loranthaceae	-7.9±3.1
Lycium chinense	Radix	Solanaceae	13.6±3.3
Magnolia fargesii	Flos	Magnoliaceae	70.5±2.0
Magnolia officinalis	Cortex	Magnoliaceae	69.5±3.1
Melia azedarach	Fructus	Meliaceae	-129.3±1.6
Morus alba (radicis)	Cortex	Moraceae	43.4±1.9
Nelumbo nucifera	Semen	Nymphaeaceae	1.0±0.4
Paeonia albiflora	Radix	Ranunculaceae	-31.9±6.1
Paeonia moutan	Radix	Paeoniaceae	13.3±2.8
Panax ginseng	Folium	Araliaceae	50.8±0.5
Patrinia scabiosaefolia	Radix	Valerianaceae	105.8±0.2
Perilla frutescens	Herba	Labiatae	18.0±1.7
Plantago asiatica	Semen	Plantaginaceae	32.0±5.5
Platycodon gradiflorim	Radix	Campanulaceae	37.8±1.1
Polygola tenuifolia	radix	Polygalaceae	-11.1±5.9
Poncirus trifoliata	Fructus	Rutaceae	20.5±5.3
Poria cocos	Hoelen	Polyporaceae	15.9±1.6
Prunella vulgare	Spica	Labiatae	39.6±3.0
Prunus ansu	Semen	Rosaceae	30.1±0.5
Prunus donarium	Cortex	Rosaceae	14.5±1.2
Prunus mume	Fructus	Rosaceae	4.0±1.8
Prunus persica	Semen	Rosaceae	1.6±0.9
Pterocarpus santalius	Lignum	Leguminosae	55.5±2.8
Pueraria thunbergiana	Radix	Leguminosae	18.4±5.3
Pueraria thunbergiana	Flos	Leguminosae	41.9±2.0
Pulsatilla chinensis	Radix	Ranunculaceae	42.7±7.2
Pyrrosia lingua	Herba	Polypodiaceae	24.9±2.5
Rhapontica uniflora	Radix	Compositae	51.6±8.6
Rheum tanguticum	Rhizoma	Polygonaceae	33.2±1.0
Rubus coreanum	Fructus	Rosaceae	10.2±4.0
Saguisorba officinalis	Radix	Rosaceae	-2.3±3.6
Salvia miltiorrhiza	Radix	Labiatae	27.1±3.7
Santalum album	Lignum	Santalaceae	65.6±3.2
Saussurea lappa	Radix	Compositae	89.0±1.1
Schizandra chinensis	Fructus	Schizandraceae	-0.8±4.6
Scrophularia buergeriana	Radix	Scrophulariaceae	-3.1±6.3
Scutellaria baicalensis	Radix	Labiatae	-21.8±8.0
Siegesbeckia pubescens	Herba	Compositae	26.7±4.8
Sinomenium acutum	Rhizoma	Menispermcaeae	43.±0.1
Solanum nigrum	Herba	Solanaceae	-1.8±5.1

Table 1. Continued

Medicinal plants	Used part	Family name	% inhibition[a]
Sophora flavescence	Radix	Leguminosae	19.5±6.2
Sophora japonica	Flos	Leguminosae	44.6±2.9
Sparganium stoloniferum	Rhizoma	Sparganiaceae	0.7±2.5
Taraxacum platycarpum	Radix	Compositae	3.9±5.0
Terminalia chebula	Fructus	Caprifoliaceae	43.4±5.7
Thuja orientalis	Folium	Cucurbitaceae	23.6±1.9
Thuja orientalis	Semen	Cucurbitaceae	20.9±0.4
Torilis japonica	Fructus	Umbelliferae	22.4±1.7
Torreya mucifera	Semen	Torreyaceae	9.0±2.8
Trichosanthes kirillowi	Radix	Cucurbitaceae	40.9±4.5
Typha orientalis	Pollen	Typhaceae	33.5±4.6
Xanthium strumarium	Furctus	Compositae	8.6±3.6
Zanthoxylum piperitum	Fructus	Rutaceae	37.3±5.7
Zingiber officinale	Rhizoma	Zingiberaceae	38.6±2.1
Zizyphus jujuba	Semen	Rhanmaceae	30.4±1.1

[a]: Data are indicated as mean±SEM (n=3). Each ethanol fraction, prepared by previous method (Chae *et al.*, 1998), with 100 μg/ml as a final concentration was added to LPS-activated RAW264.7 cells.

extracts with 0.1 mg/ml as a final concentration (Table 1). These potent herbal medicines were *Angelica koreana, Coptis japonica, Cynanchum paniculatum, Magnolia fargesii, Magnolia officinalis, Panax ginseng, Patrinia scabiosaefolia, Pterocarpus santalius, Rhapontica uniflora, Saussurea lappa*. Of them, moreover, three kinds of herbals showing inhibitory activity of more than 70% such as *Coptis japonica, Magnolia fargesii* and *Saussurea lappa* significantly inhibited TNF-α production *in vivo* with inhibitory effects of 70.2, 77.3 and 75.9%, respectively (Fig. 1). It suggested that these herbal medicines may have the same inhibitory effect even *in vivo*. Although their activities were lower than that of prednisolone (93.9%) as a positive standard drug, the results indicated the possibility that the inhibitory effect as a single principle may be higher than or comparable to that of prednisolone.

Interestingly, among the tested herbal medicines there were the several plants significantly enhancing TNF-α production in LPS-stimulated RAW264.7 cells. These herbal medicines were *Melia azedarach, Eugenia caryophyllata, Paeonia albiflora, Bupleurum falcatum*. These effects will require further studies in terms of searching the immunostimulating principles from these natural products because TNF-α is a well-known tumor necrosis protein released from macrophages (Manogue *et al.*, 1992).

The 11 methanol extracts were independently subjected to sequential fractionation with *n*-hexane (Hx), methylene chloride (MC), ethyl acetate (EtOAc), *n*-butanol (BuOH) and water (Table 2). Among the solvent-fractionated extracts with 0.05 mg/ml as a final concentration, inhibitory effects of *Angelica koreana, Magnolia fargesii, Magnolia officinalis, Pterocarpus*

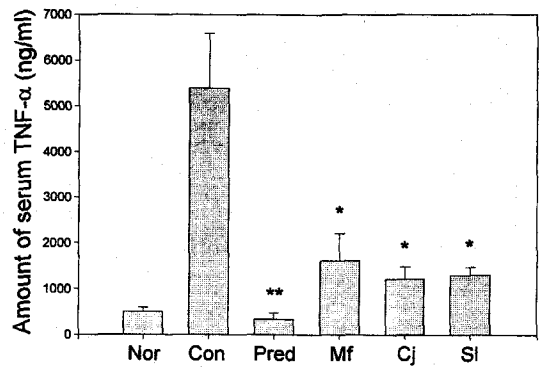

Fig. 1. Inhibitory effect of herbal medicines on TNF-α production in LPS-treated mouse. Fasted mice were administered with the test fractions and standard drug (Nor. normal; Con. control; Pred. prednisolone; Mf. *Magnolia fargesii*; Cj. *Coptis japonica*; Sl. *Saussurea lappa*) 2 h before challenge with intraperitoneal injection of LPS (1.5 mg/mouse). After 90 min, blood was collected and serum samples were used to measure TNF-α levels by ELISA kit. Values are expressed as the mean± SEM of 6 animals. **p<0.01, *p<0.05 represent significant difference compared to control.

Table 2. Inhbitory effect of solvent-fractioned extracts on TNF-α production in LPS-stimulated RAW264.7 cells

Medicinal plants	% inhibition[a]				
	Hx fr.	MC fr.	EtOAc fr.	BuOH fr.	H_2O fr.
Angelica koreana		84.9±1.5	59.6±3.7	11.4±2.4	-1.9±2.1
Coptis japonica	63.6±1.4	69.7±2.1	46.6±1.4	53.6±2.0	-4.4±3.3
Cynanchum paniculatum	65.8±3.1	62.2±0.8	50.7±5.3	49.6±1.8	-36.5±9.2
Magnolia fargesii	42.1±2.0	60.7±1.8	24.6±7.2	24.0±5.8	1.0±8.4
Magnolia officinalis	100.0±1.0	70.0±1.8	42.3±6.1	18.3±6.4	3.1±1.7
Panax ginseng		0.4±3.5	33.4±2.7	25.5±1.9	-7.9±4.6
Patrinia scabiosaefolia	100.2±5.7	55.2±2.1	98.5±4.1	18.5±2.1	-16.7±22.1
Pterocarpus santalius		68.5±1.7	18.5±2.5	-12.3±9.4	-25.2±19.0
Rhapontica uniflora		39.1±3.8	-13.1±6.8	51.6±4.4	1.0±7.1
Santalum album	28.115.4	14.5±8.4	12.8±12.9	23.3±5.0	11.4±3.6
Saussurea lappa	101.50.8	65.4±3.6	27.9±5.6	10.3±3.7	-11.8±11.4

[a]: Data are indicated as mean±SEM (n=3). Each subfraction, prepared by previous method (Chae *et al.*, 1998), with 50 μg/ml as a final concentration was added to LPS-activated RAW264.7 cells.

santalinus, Rhapontica uniflora and *Saussurea lappa* markedly showed in one or two solvent fractions suggesting that the principles were concentrated by subfractionation as the main compounds. On the other hand, *Cynanchum paniculatum, Panax ginseng, Patrinia scabiosaefolia* and *Santalum album* showed different pattern. Furthermore, because *Patrinia scabiosaefolia* showed the strong cytotoxicity, the effect appeared to inhibit TNF-α production through non-specific reaction.

We isolated the principles from one of active fraction of these plants. In *Angelica koreana*, the main compounds from MC fraction were imperatorin, isoimperatorin and osthol (Cho *et al.*, 1998a). In *Saussrea lappa*, it was reported that sesquiterpene lactones such as cynaropicrin, reynosin and santamarine showed the potent inhibitory effect on TNF-α production and CINC-1 induction from hexane fraction (Cho *et al.*, 1998b, Jung *et al.*, 1998). In addition, we found some lignan compounds as the principles of TNF-α inhibitor from MC fraction of *Magnolia fargesii* (Chae *et al.*, 1998) and from total ethanol fraction of *Coptis japonica* (Cho *et al.*, 1998d). Ginsenoside Rb_1 and Rb_2 from *Panax ginseng* also exhibited inhibitory effect on TNF-α production (Cho *et al.*, 1998c). However, because most of these compounds from the active fraction exhibited low inhibitory activity, it was regarded that the isolation of compounds from the other fractions will be further required to demonstrate the potent inhibitory activity of the medicinal plants on TNF-α production.

In addition to these compounds, possible principles from the plants were expected as magnolol and honokiol, and dehydrodieugenol from *Magnolia officinalis* (Baek *et al.*, 1992; Wang *et al.*, 1995), costunolide and dehydrocostus lactone form *Saussurea lappa* (Taniguchi *et al.*, 1995), denudatin B, and magnone A and B from *Magnolia fargesii* (Jung *et al.*, 1988, Yu, *et al.*, 1990), triterpenoid saponin, ursolic acid and oleanolic acid glycosides from *Patrinia scabiosaefolia* (Kang *et al.*, 1997, Nakanishi *et al.*, 1993), 3-hydroxy-4-methoxy-acetophenone from *Cynanchum paniculatum* (Sun *et al.*, 1994) and protoberberine alkaloids from *Coptis japonica* (Lee and Kim, 1996).

In conclusion, we have shown that eleven medicinal plants potently inhibited TNF-α production in LPS-stimulated RAW264.7 cells suggesting the possibility that the inhibitory effects of the plants may represent an important aspect of antiinflammatory effect of the oriental herbal medicines. Furthermore, because there have been a few reports about inhibitory effect of the medicinal plants on cytokine production, it is regarded that this result will give an important evidence to understand the anti-inflammatory effects of them. Furthermore, in the future it is required detailed study at the active compound level.

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