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Anti-inflammatory Activity of *Chrysanthemum indicum L.* Extract in Lipopolysaccharide-treated Rats

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Abstract – This study for developing a new anti-inflammatory medicine was sought by investigating the anti-inflammatory properties of *C. indicum L.* extract. Rats were treated with either saline (control) or *C. indicum L.* extract and then injected with LPS. We found that the plasma concentration of IL-1β, IL-6, TNF-α and IL-10 peaked at 5h after LPS injection, and the plasma concentration of IL-6 and TNF-α showed a tendency to decrease, and IL-10 concentration showed a tendency to increase with increasing levels of *C. indicum L.* extract. In the liver concentration of cytokines at 5 h post LPS injection, IL-1α, and IL-6 decreased with increasing concentration of *C. indicum L.* extract, however TNF-α and IL-10 did not differ significantly the treatment groups.
Key words – *Chrysanthemum indicum L.,* Cytokines, Anti-inflammatory activity, Lipopolysaccharides

Introduction

Sepsis is a common clinical problem and cause of death, with extremely high mortality rates in patients undergoing major surgery (Chao *et al*., 2000). Septic shock causes the release of a series of proinflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF-α), IL-8 or IL-6, and anti-inflammatory cytokines, such as the IL-1 receptor antagonists (RAs) and IL-10 (Gabay and Kushner., 1999). The successful treatment of sepsis in humans continues to be a substantial clinical challenge (Hudson *et al*., 1995), and glucocorticosteroids, which inhibit many of the functions of activated macrophages including secretion of cytokines such as IL-β, and TNF-α from cells, remain the most effective therapy for inflammatory disorders. However, despite the rapid and proven efficacy of topical glucocorticosteroids, their usefulness is limited by their side effects (Belvisi *et al*., 2001). The development of anti-inflammatory medicines with fewer adverse effects and better curative effects is needed.

Lipopolysaccharides (LPS) are structural components of the outer membranes of Gram-negative bacteria and are associated with tissue injury and fatal outcome in septic shock. Pro-inflammatory cytokines are produced in response to LPS (Aono *et al*., 1997), and effective cytokine modulating agents usually inhibit intraperitoneally administered LPS-stimulated cytokine synthesis in animals.

Chrysanthemum indicum L. is a perennial herb, and its major components are apigenin, luteolin A, cumambrin B, arteglasin A, acacetin, lactone, angeloyljadin, essential oil, sesquiterpene, and alkyl glucoside compounds (Ryu *et al*., 1994). One of these components, luteolin, exhibit anti-inflammatory (Simoes *et al*., 1988) and anti-cancer properties (Ryu *et al*., 1994). Especially, recent studies reported a high anti-inflammatory effect of *Chrysanthemum indicum L*. (Cheng *et. al*., 2005; Lee *et al*., 2009) and this plant showed the potentialities of anti-inflammatory drugs. However, more inflammatory biological values of *in vivo* and *in vitro* of functional components from this plant remain to be investigated.

In this study a basis for developing a new anti-inflammatory medicine was sought by investigating the anti-inflammatory properties of *C. indicum L.* extract. Rats were treated with either saline (control) or *C. indicum L*. extract and then injected with LPS. The anti-inflammatory effects of this extract were compared among the different treatment groups.

Experimental

Animals and treatment – Male Sprague-Dawley rats (n = 32) weighing 217.52 ± 7.18 g (mean ± SD) were used in this study. Rats were housed in temperature and humidity controlled rooms and were allowed free access to a basic diet and water for 1 week before the experiment. They were then assigned randomly to a control group (normal, saline 5 ml/kg: $n = 8$), or one of three *C. indicum L.* extract groups: 100 mg/kg ($n = 8$), 200 mg/kg ($n = 8$), or 300 mg/kg ($n = 8$).

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Experimental diet and water – All of animals were provided with a normal diet (Table 1) and water *ad libitum* for the 5 week experimental period.

***C. indicum L.* extract** – *C. indicum L.* (500 g, dry weight, root) was divided and extracted three times at 5h each in a cooling water reflux cistern, and decompression concentrated, producing 105g of ethanol extract. This *C. indicum L.* extract was administered orally using a Jones tube at 5 pm every day for 5 weeks. The control group was given normal saline in the same way and at the same time.

LPS injection – After the 5 week experimental period, all of the rats, regardless of experimental group, were given an intra-abdominal injection of 5 mg/kg LPS.

Sampling and analysis – Blood samples were taken from all of the animals at the end of the 5 week experimental period, just before LPS injection (0 h) and 2 and 5 h afterwards. These samples were taken with the rats under ether anesthesia and using the cardiac puncture method, and were immediately centrifuged at 3000 rpm for 10 min. Sera were collected, frozen, and kept at −80 °C until analysis. Five hours after LPS injection, all of the animals underwent a mid-abdominal incision, and their livers were harvested. Liver cytokine samples were prepared as follows. A 1 g aliquot of liver particles was homogenized in 5 ml of ice cold phosphate-buffered saline (PBS; pH 7.4) containing a protease inhibitor cocktail (Tablete Complete Roche, Germany). The samples were centrifuged at 15,000 rpm for 15 min at 4 °C. The resulting supernatant was filtered through a 0.45 μm filter (Millex-HA, Millipore, France) and then centrifuged again at 15,000 rpm for 15 min at 4 °C. Liver extracts were removed and kept at −80 °C until cytokine analysis was performed. Plasma and hepatic cytokine (IL-1β, TNF-α, IL-6, and IL-10) concentrations were determined by enzyme-linked immunosorbent assay, using commercial kits (Biosource International, USA). The minimum detectable concentration of TNF-α was 0.7 pg/ml, and that of the remaining cytokines was 3-8 pg/ml. Hepatic levels of cytokines were calculated per 1 g of wet tissue in 5 ml of PBS. Plasma cytokine concentrations are expressed as picograms per milliliter, and hepatic cytokine levels as picograms per milligram of tissue.

Table 1. Composition of experimental diet.

Ingredients	Composition(%)
Casein	20.0
α−Corn starch	35.0
Sucrose	11.0
Lard	4.0
Corn oil	1.0
Mineral mix[1)]	3.5
Vitamin mix[2)]	1.0
Cellurose powder	23.5
DL-methione	0.3

[1)]Mineral mix. (g/kg diet): $CaCO_3$, 29.29; $CaHPO_42H_2O$, 0.43; KH_2PO_4, 34.30; NaCl, 25.06; $MgSO_47H_2O$, 9.98; Feric citrate hexahydrate, 0.623; $CuSO_45H_2O$, 0.516; $MnSO_4H_2O$, 0.121; $ZnCl_2$, 0.02; KI, 0.005; $(NH_4)_6 MO_7O_{24}4H_2O$, 0.0025.

[2)]Vitamin mix (mg/kg diet): Thiamine-HCl, 12; Riboflavin, 40; Pyrodoxin-HCl, 8; Vitamin-B_{12}, 0.005; Ascorbic acid, 300; D-biotin, 0.2; Menadione, 52; Folic acid, 2; D-calcium pantothenate, 50; P-aminobenzoic acid, 50; Nicotinic acid, 60; Cholin choloride, 2000 (IU/kg diet); Rethinyl acetae, 5000 (IU/kg diet); Cholecalciferol, 250 (IU/kg diet).

Statistical analysis – Results were analyzed using a one-way ANOVA in an SPSS package, and each group's significance met $p < 0.05$ for Duncan's multiple range test.

Results and Discussion

Gram-negative bacterial endotoxins or LPS, are associated with tissue injury and fatal outcome in septic shock (Eduard *et al*., 2004). It has been demonstrated both experimentally and clinically that sepsis causes the production of a series of proinflammatory cytokines, such as IL-1β, TNF-α, IL-8, and IL-6, and anti-inflammatory cytokines, such as IL-1 RA and IL-10 (Gabay and Kushner, 1999). The time points chosen in the present study to investigate the anti-inflammatory activity of the ethanol extract of *C. indicum L.* in LPS-exposed rats were performed on the basis of experimental results from previous studies (Mathiak *et al*., 2000). Levels of liver cytokines (IL-1β, IL-6, TNF-α, and IL-10) were measured at 5 h after LPS injection. The chosen concentration of LPS (5 mg/kg) was based upon the finding that this concentration causes endotoxic shock and increases the cytokine concentrations in the liver and blood (Aono *et al*., 1997; Barton *et al*., 2001; Sang *et al*., 1999). IL-1β is a proinflammatory cytokine that has been implicated as a mediator of LPS toxicity *in vivo* and *in vitro*. In the study presented here, the concentration of plasma IL-1β (Table 2) peaked at 5 h after LPS injection, which is similar to Mathiak *et al*., (2000) finding that IL-1β concentration peaked at between 4 and 6 h after LPS injection. The plasma concentration of IL-6 (Table 3) peaked at 5 h after LPS injection. This finding is similar to that reported by Mathiak *et al*., (2000) Who demonstrated a peak in IL-6 concentration at 4-6 h after LPS injection. In addition, we found that the plasma IL-6 levels in the groups treated with *C. indicum L.* extract were lower than those of the controls, and confirming its anti-inflammatory properties. Plasma TNF-α concentration (Table 4) increased up 2h after LPS injection and then plateaued until the end of the 5 h measurement period. TNF-α levels at 5h post injection in the groups treated with *C. indicum L.* at 200 and 300mg/kg, were lower than that in the control group. TNF-α is a peptide

Table 2. Effect of *Chrysanthemum indicum L.* ext. on plasma IL-1β concentration in lipopolysaccharide-exposed rats.

Treatment		IL-1β (pg/ml),	Time (h)*
	0h	2h	5h
Control(saline, 100mg/kg)	17.15 ± 4.77^{NS}	79.45 ± 9.51^{NS}	184.11 ± 37.59^{NS}
Chrysanthemum indicum L. ext. (100 mg/kg)	15.44 ± 3.61^{NS}	92.17 ± 11.35^{NS}	202.85 ± 23.17^{NS}
Chrysanthemum indicum L. ext. (200 mg/kg)	17.06 ± 4.15^{NS}	84.46 ± 10.33^{NS}	193.29 ± 35.64^{NS}
Chrysanthemum indicum L. ext. (300 mg/kg)	14.83 ± 3.78^{NS}	72.15 ± 10.75^{NS}	211.45 ± 30.11^{NS}

*: 0h, 2h and 5h after LPS injection.
NS: Not significantly different ($P > 0.05$).

Table 3. Effect of *Chrysanthemum indicum L.* ext. on plasma IL-6 concentration in lipopolysaccharide-exposed rats.

Treatment		IL-6 (pg/ml),	Time (h)*
	0h	2h	5h
Control(saline, 100mg/kg)	25.72 ± 5.48^{NS}	231.74 ± 26.58^{NS}	872.74 ± 49.52^{b}
Chrysanthemum indicum L. ext. (100 mg/kg)	22.14 ± 6.35^{NS}	224.15 ± 30.43^{NS}	821.19 ± 65.47^{b}
Chrysanthemum indicum L. ext. (200 mg/kg)	30.78 ± 4.77^{NS}	160.97 ± 21.61^{NS}	735.36 ± 54.28^{a}
Chrysanthemum indicum L. ext. (300 mg/kg)	28.35 ± 5.06^{NS}	177.59 ± 25.43^{NS}	612.54 ± 57.36^{a}

*: 0h, 2h and 5h after LPS injection.
a,b: Means in the same column with different superscripts are significantly different ($p < 0.05$). NS: Not significantly different ($P>0.05$).

Table 4. Effect of *Chrysanthemum indicum L.* ext. on plasma TNF-α concentration in lipopolysaccharide-exposed rats.

Treatment		TNF-α (pg/ml),	Time (h)*
	0h	2h	5h
Control (saline, 100 mg/kg)	10.11 ± 2.56^{NS}	511.92 ± 49.15^{b}	614.68 ± 71.35^{b}
Chrysanthemum indicum L. ext. (10 0mg/kg)	8.75 ± 2.04^{NS}	409.15 ± 54.28^{a}	635.21 ± 58.43^{b}
Chrysanthemum indicum L. ext. (20 mg/kg)	11.62 ± 1.95^{NS}	357.83 ± 60.59^{a}	451.95 ± 33.78^{a}
Chrysanthemum indicum L. ext. (300 mg/kg)	9.44 ± 1.81^{NS}	380.49 ± 54.75^{a}	355.13 ± 41.85^{a}

*: 0h, 2h and 5h after LPS injection.
a,b: Means in the same column with different superscripts are significantly different ($p < 0.05$). NS: Not significantly different ($P > 0.05$).

Table 5. Effect of *Chrysanthemum indicum L.* ext. on plasma IL-10 concentration in lipopolysaccharide-exposed rats.

Treatment		IL-10 (pg/ml)	Time (h)*
	0h	2h	5h
Control(saline, 100mg/kg)	11.95 ± 4.36^{NS}	87.66 ± 9.34^{b}	92.71 ± 15.05^{a}
Chrysanthemum indicum L. .ext.(100mg/kg)	10.21 ± 3.14^{NS}	70.48 ± 6.53^{a}	84.58 ± 17.88^{a}
Chrysanthemum indicum L. ext.(200mg/kg)	9.84 ± 3.58^{NS}	79.76 ± 7.44^{ab}	141.75 ± 12.72^{b}
Chrysanthemum indicum L. ext.(300mg/kg)	11.18 ± 3.95^{NS}	92.39 ± 7.61^{b}	125.64 ± 13.49^{b}

*: 0h, 2h and 5h after LPS injection.
a,b: Means in the same column with different superscripts are significantly different ($p < 0.05$). NS: Not significantly different ($P > 0.05$).

mediator released by monocytes and macrophages in response to various stimuli including bacterial LPS (Chamulitrat *et al*., 1995). It is thought to be the principal mediator of the deleterious effects of endotoxin (Harbrecht *et al*., 1994). Over production of this proinflammatory cytokine is associated with a wide range of pathologic conditions and has therefore led to recent efforts to establish ways of down-regulating its production or inhibiting its effects in vivo (Marriot *et al*., 1998). In the study presented here, the finding that plasma TNF-α concentrations decreased with increasing levels of *C. indicum L*. extract suggests that the *C. indicum L*. extract was concerned with its anti-inflammatory properties. The plasma concentration of IL-10 (Table 5) had increased by 2 h after LPS injection and reached a peak at 5 h. Levels of this cytokine were higher in the 200 and 300 mg/kg of the *C. indicum L*. extract treated groups than in the control group at 5 h after LPS injection. IL-10 is a potent pleiotropic anti-inflammatory cytokine that is produced by lymphocytes and

macrophages (Thompson *et al*., 1998). It controls the synthesis of inflammatory cytokines such as IL-6 and TNF-α and is known to decrease the T-cell activation in vitro and in vivo (Sang *et al*., 1999; Moreira *et al*., 1993). In the study presented here, the concentration of *C. indicum L*. provided to the animals appeared to exert a dose-response effect on plasma levels of IL-10, the latter being higher at 5 h post-LPS injection with higher concentrations of the extract. It could be that the IL-10 effected the concentrations of other cytokines.

The liver concentration of IL-1β (Fig. 1) decreased at 5 h after LPS injection, with the decrease being greater for a higher concentration of *C.indicum L*. extract. Activated Kuffer cells in the liver are capable of releasing IL-1β (Simpson *et al*., 1997). Sang *et al*. (1999) reported that intraperitoneal injection of LPS in rats results in a significant elevation in the liver levels of IL-1β within 30 min postinjection. Our results are in agreement with these reports.

Sang *et al*. (1999) reported that the intraperitoneal injection of LPS results in a significant elevation of liver IL-6 mRNA levels within 30 min post injection, and that this response is still observed 3 h post injection. Our finding of large increment in liver IL-6 concentration at 5 h post LPS injection is consistent with those results. However, the liver concentration of IL-6 decreased with increasing concentration of *C. indicum L*. extract, suggesting that this herb was concerned with antiinflammatory function. TNF-α is no longer detected 3-4 h after exposure to LPS (Klapproth *et al*., 1980). In the study present here, liver TNF-α concentrations at 5 h after LPS injection were low, and were not significantly different among the treatment groups. This result might be attributable to the post LPS injection sampling times. The liver is a major source of IL-10. The

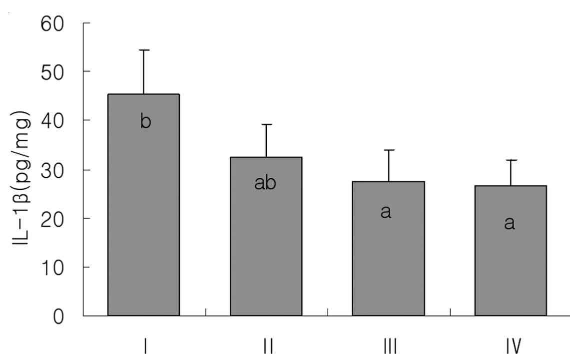

Fig. 1. Effect of *Chrysanthemum indicum L.* ext. on liver IL-1β, concentration in lipopolysaccharide-exposed rats. [a,b]: Means with different superscripts are significantly different ($p < 0.05$). I : Control (saline, 100 mg/kg), II : *Chrysanthemum indicum L.* ext. (100 mg/kg), III : *Chrysanthemum indicum L.* ext. (200 mg/kg), IV : *Chrysanthemum indicum L.* ext. (300 mg/kg).

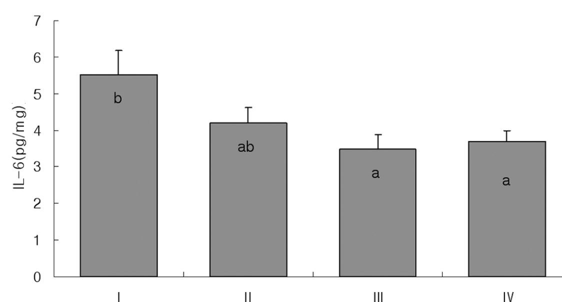

Fig. 2. Effect of *Chrysanthemum indicum L.* ext. on liver IL-6 concentration in lipopolysaccharide-exposed rats. [a,b]: Means with different superscripts are significantly different ($p < 0.05$). I : Control (saline, 100 mg/kg), II : *Chrysanthemum indicum L.* ext. (100mg/kg), III : *Chrysanthemum indicum L.* ext. (200 mg/kg), IV : *Chrysanthemum indicum L.* ext. (300 mg/kg).

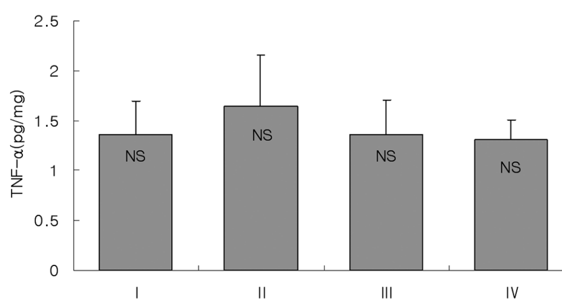

Fig. 3. Effect of *Chrysanthemum indicum L.* ext. on liver TNF-α concentration in lipopolysaccharide-exposed rats. [NS]: Not significantly different ($P > 0.05$). I : Control (saline, 100 mg/kg), II : *Chrysanthemum indicum L.* ext. (100mg/kg), III : *Chrysanthemum indicum L.* ext. (200 mg/kg), IV : *Chrysanthemum indicum L.* ext. (300 mg/kg).

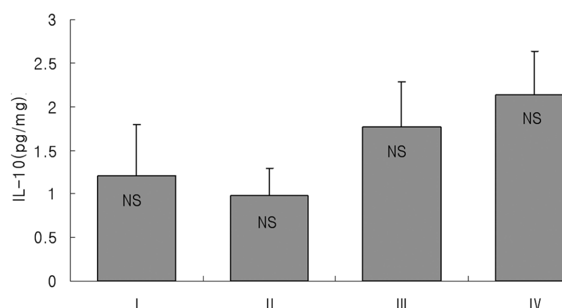

Fig. 4 Effect of *Chrysanthemum indicum L.* ext. on liver IL-10 concentration in lipopolysaccharide-exposed rats. [NS]: Not significantly different ($P > 0.05$). I : Control (saline, 100 mg/kg), II : *Chrysanthemum indicum L.* ext. (100mg/kg), III : *Chrysanthemum indicum L.* ext. (200 mg/kg), IV: *Chrysanthemum indicum L.* ext. (300 mg/kg).

potential cellular sources of IL-10 in the liver include macrophages, Kupffer cells, T and B lymphocytes, and hepatocytes (Louis *et al*., 1997). In rodents, IL-10 down-regulates proinflammatory cytokine synthesis, including the

LPS-induced synthesis of the major proinflammatory cytokine TNF-α in Kupffer cells, and IL-6 (Simpson *et al*., 1997; Thompson *et al*., 1998). In our study, liver IL-10 concentrations at 5 h post LPS injection did not differ significantly the treatment groups. These results can be thought because 5 h after LPS shocked the liver, as a main source of IL-10, its major part of this cytokine to the rest of economy through blood torrent.

Acknowledgements

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