เชียงใหม่สัตวแพทยสาร 2553; 8(2): 93-103

Original article

Effects of medicinal plant extracts on interleukin-10 and tumor necrosis factor alpha gene expressions in porcine peripheral blood mononuclear cells

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Abstract The present study evaluated the immunomodulatory activity of four medicinal plant extracts, i.e. *Centella asiatica* L. Urban, *Clinacanthus nutans* (Burm.f.) Lindau, *Andrographis paniculata* (Burm.f.) Wall. ex Nees, and *Allium sativum* L in modulating interleukin-10 (IL-10) and tumor necrosis factor alpha (TNF-α) gene expressions in peripheral blood mononuclear cells of the pigs. The results showed that the extract of *A. sativum* significantly enhanced IL-10 mRNA expression and reduced TNF-α gene expression. The extracts of *C. nutans* and *A. paniculata,* on the other hand, significantly reduced IL-10 mRNA expression and slightly enhanced TNF-α gene expression. The extract of *C. asiatica* enhanced both IL-10 and TNF-α mRNA expressions, but withhigh variation. The results of this study suggested that the extract of *A. sativum* may have a potential to reduce inflammation, and the extracts of *C. nutans* and *A. paniculata* may have a potential to enhance immune response of the pigs.
Chiang Mai Veterinary Journal 2010; 8(2): 93-103

Keywords: medicinal plants, interleukin-10, tumor necrosis factor alpha, peripheral blood mononuclear cell, pig

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Introduction

Exploitation of medicinal plants is currently an area of interest for swine production industry, particularly for organic pig production. One objective for the utilization of medicinal plants is to enhance immune response of the

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Article received date : June 21, 2010.

pigs.[1, 2] There are several medicinal plants that have been reported to possess immunomodulatory activities, but yet have never been evaluated in swine. For examples, these plants are *Centella asiatica* L. Urban (Family *Umbelliferae*), *Clinacanthus nutans* (Burm.f.) Lindau (Family *Acanthaceae*), *Andrographis paniculata* (Burm.f.) Wall. ex Nees (Family *Acanthaceae*), and *Allium sativum* L (Family *Alliaceae*).

In humans and mice, the immunomodulatory activities of these medicinal plants have been reported.[3-14] *C. asiatica* has been reported on the up-regulation of anti-inflammatory cytokine production, i.e. interleukin-10 (IL-10), and downregulation of pro-inflammatory cytokine production, i.e. IL-6, IL-12, tumor necrosis factor alpha (TNF-α), and interferon gamma (IFNγ).[3-5] *C. nutans* has been reported on the suppression of inflammatory mediators, i.e. reactive oxygen species and pro-inflammatory enzymes, i.e. myeloperoxidase, cyclooxygenase, and lipoxygenase.[6] *A. paniculata* has been reported on the promotion of lymphocyte proliferation and antibody production, and inhibition of IL-12, TNF-α, and IFNγ production.[7-11] *A. sativum* has been reported on the promotion of IL-10 production, and inhibition of IL-1, IL-6, IL-8, IL-12, and TNF-α production.[12-14]

The present study assessed the immunomodulatory activities of these four medicinal plants on the porcine peripheral blood mononuclear cells (PBMC) for the expression of IL-10 and TNF-α. We aimed that this study would suggest potential use of these medicinal plants for immunomodulatory purposes in pigs.

Materials and methods

1. Plant materials and plant extracts

The stems and leaves of *C. asiatica, C. nutans,* and *A. paniculata;* and the bulbs of *A. sativum* were collected in Sansai, Chiang Mai, Thailand during April-September, 2009. The plant materials were dried (50°C) prior to pulverization. The pulverized *C. asiatica* and *C. nutans* (100g each) were macerated in methanol for three 24-hour period and the solvents were collected and combined.[4, 6] The pulverized *A. paniculata* and *A. sativum* (100g each) were macerated in 95% ethanol using the same protocol as *C. asiatica* and *C. nutans*.[7, 15] All collected extracts were centrifuged (1000 xg, 10 min, 4°C), and the supernatants were filtered through filter paper no.1 (Whatman, UK). The filtrates were evaporated (below 40°C) and freeze-dried to remove the solvents. The residues of each extract (0.5 g) were

dissolved in 10 ml of RPMI^{++} (RPMI-1640 with L-glutamine, 10% heat-inactivated fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (250 ng/ml) (all from Gibco, Grand Island, NY)), and filtered through 0.22 µm membrane (Sartorius, Germany). The crude extracts were then placed into Detoxi-Gel Endotoxin Removal columns (Thermo Fisher Scientific Inc, Rockford, IL) to remove lipopolysaccharide (LPS). The LPS contamination of each extract after LPS removal was less than 0.0625 EU/ml as determined by a limulus amebocyte lysate test kit (Wako, Japan). The extracts were kept at -20°C until use.

2. Determination of the effects of plant extracts on cell viability

PBMC were isolated as previously described.[16] Briefly, blood was collected from four 24-week-old pigs seronegative for porcine reproductive and respiratory syndrome virus from a commercial pig producer. The blood was placed into 5 mM EDTA (J.T.Baker, Phillipsburg, NJ), diluted 1:1 with sterile phosphate buffered saline (PBS), and isolated for PBMC using lymphocyte separation medium (Histopaque®-1077, Sigma, St. Louis, MO). Contaminating red blood cells were lysed with cold lysis buffer (0.156 M ammonium chloride, 10 mM sodium bicarbonate, and 1 mM EDTA). Isolated PBMC were resuspended in RPMI^{++} and adjusted to 5×10^6 cells/ml. One hundred microliters of which were placed into each well of a 96-well flat-bottom plate (Nunc, Denmark) and each well received equal volume of plant extracts at varying final concentrations (50, 25, 12.5, 6.25, 3.13, and 1.56 mg/ml). Cells were incubated with the plant extracts for 16 hours in a humidified incubator (37°C, 5% CO_2), and were determined for viability by trypan blue staining (Gibco). PBMC that received only RPMI^{++} but not plant extract were served as negative controls of the assay.

3. Determination of immunomodulatory effects of plant extracts

One hundred microliters of PBMC were placed into each well of a 96-well flat-bottom plate in duplicates. The wells then received 50 µl of concanavalin A (conA; 5 µg/ml final concentration; Sigma), 50 µl of LPS (5 µg/ml final concentration; Fluka, Germany), and 100 µl of plant extracts at optimal 1.56 mg/ml final concentration. Cells were incubated in a humidified incubator for 16 hours. PBMC that received conA and LPS but not plant extracts were served as positive controls, and those that received only RPMI^{++} were

served as negative controls. PBMC that received methanol or ethanol diluted in RPMI^{++} at the same concentration (v/v) as plant extracts were served as vehicle controls.

4. Real-time PCR

At the end of incubation period, PBMC were harvested, washed with PBS, and extracted for total RNA, using the NucleoSpin® RNA II kit (Macherey-Nagel, Bethlehem, PA). Contaminating DNA was eliminated by Dnase I provided with the kit. cDNA was synthesized using RevertAid™ First Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD). Real-time PCR was performed on the MJ Research PTC-200 thermal cycler in a total reaction volume of 25 μl, consisting of 2 μl cDNA template, 0.3 μM each of forward and reverse primers for porcine IL-10, TNF-α, and ribosomal protein L32[17], and 12.5 μl PCR buffer (Maxima™ SYBR Green qPCR master mix, Fermentas). The PCR condition was 'initial denaturation' (95°C, 10 min); and 40 cycles of 95°C (15s), 60°C (30s), and 72°C (30s). The threshold cycles (C_T) of all genes were used for calculation of cytokine gene expression by $\Delta\Delta C_T$ method. The melting curve analysis was performed after the completion of PCR cycles. PCR products were determined for size correction by agarose gel electrophoresis (2.5% agarose (Research Organics, Cleveland, OH) in TBE buffer (National diagnostics, Atlanta, GA) with 0.5 μg/ml ethidium bromide (Bio Basic Inc., Canada)) and visualized under ultraviolet light with Quantity One software (version 4.5.0, Bio-Rad, Hercules, CA).

5. Statistical analysis

All statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL). Mean differences of cytokine gene expressions were tested by one-way analysis of variance, followed by Dunnett's test using mean of positive control as a control group. $P<0.05$ was set as statistically significant level.

Results

1. Effect of plant extracts on PBMC viability

Plant extract preparations were determined for their cytotoxicity to PBMC, and their least cytotoxic concentrations were obtained. As shown in Table 1, all plant extracts at 1.56 mg/ml (except that of *C. nutans*) provided highest PBMC viability, compared to other concentrations tested. The extract of *C. nutans* at 1.56 mg/ml, yet, provided second highest PBMC viability, compared to its other concentrations. Thus, this

concentration was chosen for subsequent investigations.

2. IL-10 mRNA expression

Compared to positive control, PBMC incubated with extracts of *C. asiatica* and *A. sativum* demonstrated increased IL-10 mRNA expression, while those incubated with extracts of *C. nutans* and *A.paniculata* demonstrated significantly reduced cytokine mRNA expression (Fig. 1). The methanol and ethanol controls showed significantly and slightly reduced IL-10 mRNA expression, respectively.

3. TNF-α mRNA expression

Compared to positive control, PBMC incubated with extracts of *C. asiatica* and *A. paniculata* demonstrated increased TNF-α mRNA expression, while those incubated with extract of *A. sativum* demonstrated slightly reduced cytokine mRNA expression (Fig. 1). PBMC incubated with extract of *C. nutans* demonstrated unmodulated TNF-α mRNA expression. The methanol and ethanol controls showed significantly and moderately increased TNF-α mRNA expression, respectively.

Discussion

The present study evaluated the immunomodulatory activities of four medicinal plants, i.e. *C. asiatica, C. nutans, A. paniculata,* and *A. sativum* on IL-10 and TNF-α mRNA expressions in porcine PBMC. The immunomodulatory activities of these medicinal plants have never been assessed in swine.

Among these four plants, extract of *A. sativum* showed significantly highest activity in enhancing IL-10 mRNA expression and reducing TNF-α mRNA expression. These findings suggest that *A. sativum* may likely have an anti-inflammatory capacity and may be utilized for anti-inflammation purposes in pigs. These results were compatible with previous reports in humans and mice that extract of *A. sativum* could promote IL-10 production and inhibit TNF-α, IL-1, IL-6, IL-8 and IL-12 production.[12, 13, 18, 19]

In constrast to *A. sativum,* extract of *A. paniculata* significantly reduced IL-10 mRNA expression, and slightly increased TNF-α mRNA expression. These findings suggested that this medicinal plant may have a pro-inflammatory capacity and may be exploited to enhance immune response of the pigs. These results were, however, different from previous reports in mice and rats that extract of *A. paniculata* possessed anti-inflammatory activity, i.e. suppression of TNF-α, IL-12, and

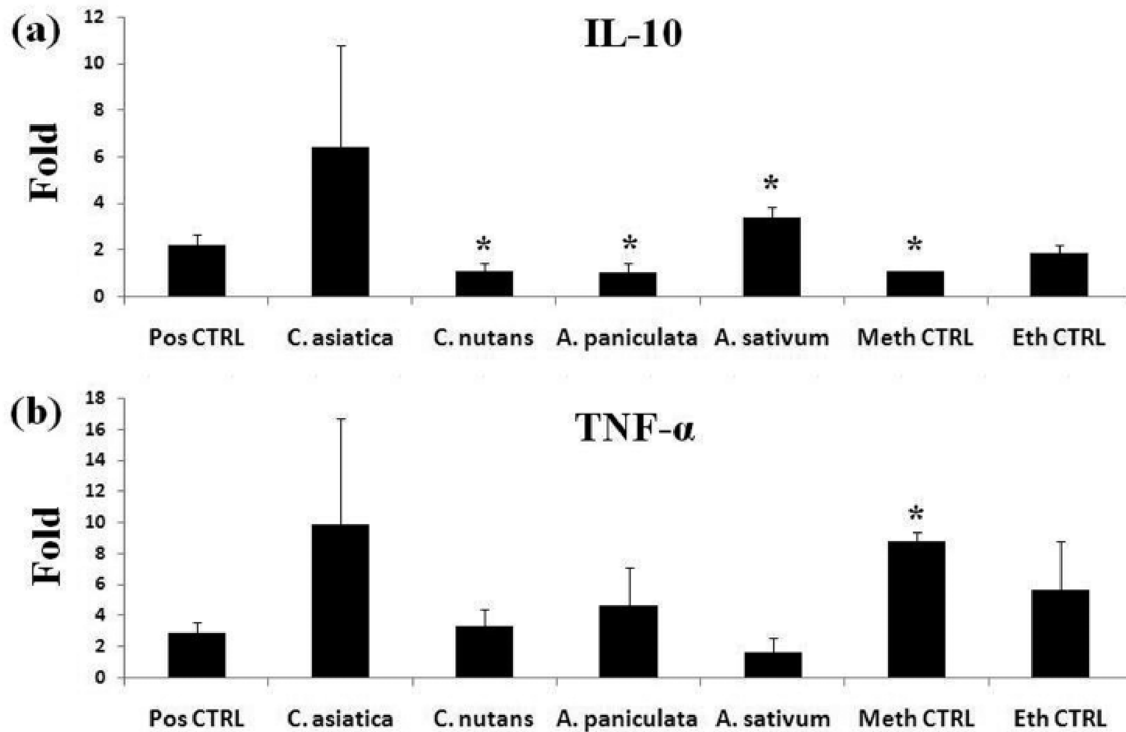

Figure 1. Effect of *C. asiatica*, *C. nutans*, *A. paniculata*, and *A. sativum* extracts on IL-10 (a) and TNF-α (b) mRNA expressions of PBMC after incubating with plant extracts in the presence of ConA and LPS for 16 hours prior to real-time PCR analysis. PBMC treated with culture media alone and stimulated with ConA and LPS served as the positive control (Pos CTRL). PBMC treated with culture media-diluted solvent and stimulated with ConA and LPS served as the vehicle control (Meth CTRL and Eth CTRL). Error bars indicated the SEM. Asterisk (*) represented significant mean difference of cytokine mRNA expression between treatment and positive control groups (p<0.05).

Table 1. Mean±standard error of mean (SEM) of %viable PBMC after cultivation with varying concentrations of plant extracts for 16 hours. CTRL represents PBMC that were cultivated with culture media alone (without plant extracts).

	Concentration (mg/ml)						
	50	25	12.5	6.25	3.13	1.56	0 (CTRL)
C. asiatica	44.16±10.80	43.09±9.74	42.58±8.92	47.45±9.49	44.21±9.34	50.39±9.91	55.43±10.84
C. nutans	60.26±15.05	43.17±15.47	40.47±12.89	51.08±10.63	49.94±12.01	52.52±7.61	-
A. paniculata	28.95±4.79	39.62±4.07	34.90±4.31	43.87±4.41	30.65±4.67	44.14±3.74	-
A. sativum	53.84±19.45	56.40±13.03	54.59±9.64	43.87±12.96	44.10±11.76	57.40±8.54	-

IFNγ production.[8-11, 20] Different observations made in the present study and previous reports are likely attributed to the use of crude plant extract versus plants' purified bioactive constituents for immunomodulatory evaluation. In this study, the crude plant extract was used and, of which, several bioactive constituents may be present. These constituents may have varying immunomodulatory activities, and they may stimulate cells in different manner from purified bioactive constituents. The observation of this study, however, suggested at least that the predominant effect of the extract of *A. paniculata* in pigs may be towards pro-inflammation.

The extract of *C. nutans* significantly reduced IL-10 mRNA expression and had no effect on TNF-α mRNA expression. However, these findings must be interpreted with caution, since the methanol vehicle control for this plant extract also showed modulatory effects on both cytokine mRNA expression. Based on the assumption that there might be a trace amount of methanol left in the plant extract after the preparation, it could be speculated that the IL-10 reducing effect observed in *C. nutans* treatment was the effect from the leftover methanol and probably not from the *C. nutans* itself. Like the observation of IL-10 mRNA expression, it could be suspected that the *C. nutans* extract actually had a suppressing effect on TNF-α mRNA expression as compared to the enhanced cytokine expression observed in the methanol control. These ambiguous results can be made clear by using purified bioactive constituents of the plants in order to rule out the effect of solvent and other contaminants. Reported bioactive constituents of this plant are, for examples, flavonoids and stigmasterol.[21]

Unlike other plant extracts, the extract of *C. asiatica* induced both IL-10 and TNF-α mRNA expressions. These results were also detected with high variation. These findings were partially compatible with previous reports in humans and mice that extracts of *C. asiatica* could enhance IL-10 production but reduce TNF-α and IL-6 production.[3, 5] High variation in cytokine gene expressions might be attributed to the effect of methanol, if any, in the plant extracts, since the methanol control could modulate IL-10 and TNF-α mRNA expressions (Fig. 1). Like *A. paniculata* and *C. nutans,* for more definitive result, future studies on immunomodulatory activity of *C. asiatica* should consider the use of

purified bioactive constituents of this plant, eg. asiatic acid and asiaticoside, in order to rule out the effect of contaminant.[3, 5]

In conclusion, this study reports the potential of *A. sativum* and probably *C. asiatica* to promote anti-inflammatory cytokine gene expression, i.e. IL-10, and reduce pro-inflammatory cytokine gene expression, i.e. TNF-α. The study also reports the potential of *C. nutans, A. paniculata,* and probably *C. asiatica* to promote TNF-α gene expression and decrease IL-10 gene expression in PBMC of the pigs. The further study may conduct using the purified material to rule out other contaminants.

Acknowledgement

This work is partially supported by the Thailand Research Fund (grant# I352D03001) and the Faculty of Science, Maejo University. The authors thank Dr. Watchara Kasinrerk at Chiang Mai University for a limulus amebocyte lysate test kit. The authors also thank Ms.Reunkaew Praphrute, Prattana Nawee, and Voramas Duangkampan for excellent technical assistance.

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