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Original article

Immunomodulatory effects of *Houttuynia cordata* Thunb extract on interleukin-1 beta, interleukin-10, and interleukin-12p40 gene expressions in porcine peripheral blood mononuclear cells

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Abstract *Houttuynia cordata* Thunb has been reported for its variety of pharmacological effects in humans and experimental animals. This study aimed to evaluate the immunomodulatory effects of *H. cordata* extract on gene expressions of interleukin-1 beta (IL-1β), IL-10, and IL-12p40 in peripheral blood mononuclear cells (PBMC) of the pigs. The *H. cordata* extract at optimal dose, i.e. a highest dose that was least cytotoxic, was incubated with PBMC for either 12 or 24 h. At 6 h prior to the incubation end, the cultures received lipopolysaccharide (LPS) stimulation. Results showed that the *H. cordata* extract significantly enhanced IL-1β and slightly enhanced IL-12p40 expression of PBMC in response to LPS stimulation at both 12 and 24 h. The *H. cordata* extract, however, have no effect on IL-10 gene expression at both incubation period. These findings suggested that *H. cordata* may have a potential to enhance immune functions of the pigs by inducing pro-inflammatory cytokine expressions. **Chiang Mai Veterinary Journal 2013; 11(2): 139-147**

Keywords: *Houttuynia cordata* Thunb, interleukin-1 beta, interleukin-10, interleukin-12p40, peripheral blood mononuclear cell, pig

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Introduction

Studies on pharmacological effects of medicinal plants for use in swine medicine have been of interest for swine researchers in recent years. This is mainly in accordance with the concern of food safety, as there are efforts to reduce the residue/use of chemicals and antibiotics in swine production (Windisch et al., 2008). The ideal medicinal plants should possess one or more desired effects, which may

include growth promotion, antimicrobial effect, immunoenhancement, and anti-inflammation (Arora et al., 2011; Windisch et al., 2008).

Houttuynia cordata Thunb is a medicinal plant of a Family *Saururaceae* that is widely cultivated in China, Vietnam and northern Thailand. This medicinal plant reportedly possesses various pharmacological effects including immunomodulation, antiviral, antibacterial, anti-parasitic, and anti-cancer (Banjerdpongchai & Kongtawelert, 2011; Chen et al., 2011; Du et al., 2012; Han et al., 2009; Kim et al., 2012; Kim et al., 2008; Kim et al., 2009; Lau et al., 2008; Lee et al., 2008; Li et al., 2011; Prommaban et al., 2012; Ren, Sui, & Yin, 2011; Shin et al., 2010; Wang et al., 2002; Yadav & Temjenmongla, 2012; Yin et al., 2011). For immunomodulatory activities, *H. cordata* and its purified constituents reportedly induces phagocytic capacity of macrophages and proliferation of lymphocytes (Wang et al., 2002). The plant extracts also enhance expression of pro-inflammatory cytokines, i.e. interferon gamma (IFNγ), interleukin-1 beta (IL-1ß), IL-2, IL-6, IL-10, and tumor-necrosis factor alpha (TNFα) (Du et al., 2012; Kim et al., 2009; Lau et al., 2008; Wang et al., 2002). The medicinal plant, on the other hand, suppresses anti-inflammatory cytokine, i.e. transforming growth factor beta (TGFß) and T helper 2 cytokines, e.g. IL-4 and IL-5, both at mRNA and protein levels (Du et al., 2012; Lee et al., 2008). It also reduces allergic reaction in vitro (Han et al., 2009; Li et al., 2005; Shim, Seo, & Park, 2009), and inflammation in a carrageenan-air pouch model of the mice (Kim et al., 2012; Li et al., 2011; Shin et al., 2010). In swine-related pathogens, this medicinal plant and its bioactive constituents, e.g. quercetin 7-rhamnoside, have been reported for their antimicrobial activities against pseudorabies virus (Ren et al., 2011), and porcine epidemic diarrhea virus (Choi et al., 2009; Song, Shim, & Choi, 2011). When used as a feed additive, pulverized *H. cordata* at 1 g/kg feed reportedly improve growth performance, feed digestibility, white blood cell concentration, and meat weight at *Longissimus* muscle area of finishing pigs (Yan, Meng, & Kim, 2011). The contribution of *H. cordata* on swine immunity, however, has not been studied yet.

In order to understand the effects of *H.cordata* on swine immune responses, the present study evaluated the immunomodulatory activities of crude *H. cordata* crude extract on the gene expression of IL-1ß, IL-10, and IL-12p40 in porcine peripheral blood mononuclear cells (PBMC).

Materials and methods

Plant material and plant extract

The stems and leaves of *H. cordata* Thunb were collected in Sansai, Chiang Mai, Thailand during May-July, 2010. The plants were authenticated by the Pharmaceutical and Natural Products Department, Thailand Institute of Science and Technological Research, where their voucher specimens were deposited.

The plant materials were dried at 50˚C for 72 h prior to pulverization. For water extraction, the pulverized plants (1 kg) were boiled for 1 h in a stainless steel pot containing 3.5 L of boiling water. Periodically a volume of water was added to the pot to substitute the evaporated amount of water. The water extracts were then sieved through muslin cloth and later

filtered through filter paper no.1 (Whatman, UK). The filtrates were evaporated by spray dry to remove the water. For ethanolic extraction, the pulverized plants (1 kg) were percolated in either 50%, 70%, or 95% ethanol for five 24-h period. In each period, the percolator chamber contained 3, 2, 2, 2, and 2 L of ethanol, respectively. At the end of each period, the solvents were collected and after the 5th period all collected solvents were combined. All ethanolic 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) by rotary evaporator to remove the solvents. All crude extracts were stored at -20˚C and were protected from light until resuspension.

For resuspension, the residues of each extract were dissolved in $RPMI^{++}$ (RPMI-1640 with L-glutamine, 10% heat-inactivated fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), amphotericin B (250 ng/ml) (all from PAA, Austria), and 1% dimethyl sulfoxide (J.T.Baker, Phillipsburg, NJ). The resuspended crude extracts were vortexed, filtered through 0.22 µm membrane (Sartorius, Germany), and subsequently placed into the Detoxi-Gel Endotoxin Removal columns (Thermo Fisher Scientific Inc, Rockkford, IL) to remove lipopolysaccharide (LPS). All extracts were kept at -20˚C and were protected from light until use.

Determination of the effects of *H. cordata* extract on PBMC viability

PBMC were isolated from whole blood as previously described by Charerntantanakul et al. (2006). In brief, whole blood (20 ml) was collected from four 24-week-old pigs from a commercial pig producer. All the pigs were seronegative to porcine reproductive and respiratory syndrome virus. The blood was placed into 5 mM ethylene diamine tetraacetic acid (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). Contaminated red blood cells were lysed with cold lysis buffer (0.156 M ammonium chloride, 10 mM sodium bicarbonate, and 1 mM EDTA) (Charerntantanakul et al., 2006). Isolated PBMC were resuspended in $RPMI^{++}$ and adjusted to 5x106 cells/ml. One hundred microliters of which were placed into each well of a 96-well flat-bottom plate (Nunc, Denmark). Each well then received equal volume of *H. cordata* extract at varying final concentrations (233, 23.3, 2.33, $2.3x10^{-1}$, $2.3x10^{-2}$, $2.3x10^{-3}$, $2.3x10^{-4}$, $2.3x10^{-5}$, and $2.3x10^{-6}$ mg/ml). Cells were incubated with the *H. cordata* extract for 12 and 24 h in a humidified incubator (37˚C, 5% CO_2), and were determined for viability by trypan blue staining (Gibco, Grand Island, NY). PBMC that received only $RPMI^{++}$ but not *H. cordata* extract were served as a negative control of the assay.

Determination of immunomodulatory effects of *H. cordata* extract

One hundred µl of PBMC were placed into each well of a 96-well flat-bottom plate in duplicates. The wells then received 100 µl of *H. cordata* extract at optimal 2.3x10-2 mg/ml final concentration. Cells were incubated in a humidified incubator for 12 and 24 h. At 6 h prior to the end of incubation period, the cells received 50 µl of LPS (100 ng/ml final well

concentration; Fluka, Germany). PBMC that received LPS but not *H. cordata* extract were served as positive control, and those that received only RPMI^{++} were served as negative control.

Real-time polymerase chain reaction (PCR)

At the end of the 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. Complementary DNA (cDNA) was synthesized using RevertAidTM 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-1ß, IL-10, IL-12p40, and ribosomal protein L32(Royaee et al., 2004), and 12.5 µl PCR buffer (Maxima^{TM} SYBR Green qPCR master mix, Fermentas). The PCR condition was 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 expressions by $\Delta\Delta C_T$ method. The melting curve analysis was conducted after the completion of PCR cycles. PCR products were analyzed 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).

Statistical analysis

All statistical analyses were performed using the SPSS software version 17 (IBM, Armonk, NY). Mean differences of cytokine gene expression 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

Effect of *H. cordata* extracts on PBMC viability

The water extract and 50%, 70%, and 95%

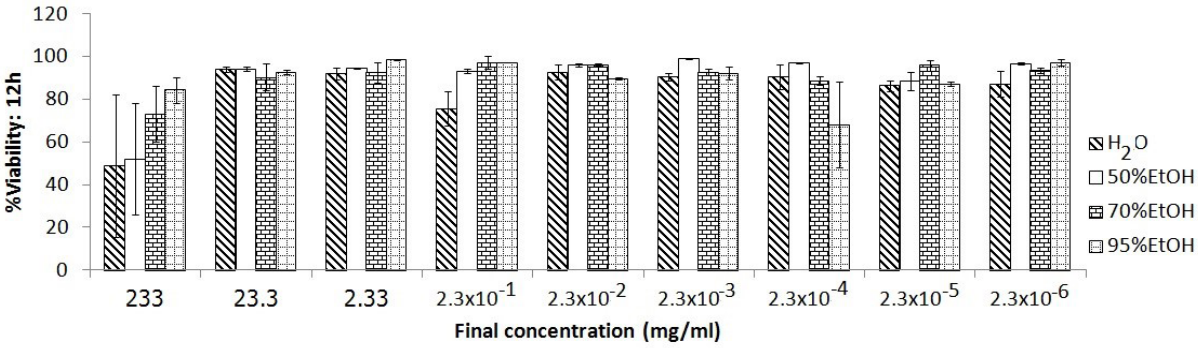

Figure 1 Effects of *H. cordata* extracts on peripheral blood mononuclear cell viability at 12 h of incubation. Error bars indicate the standard error of the mean. Data presented were obtained from two independent experiments.

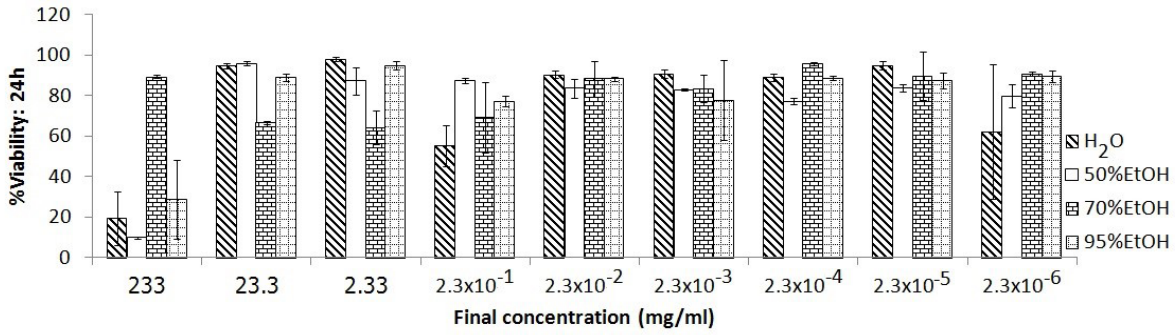

Figure 2 Effects of *H. cordata* extracts on peripheral blood mononuclear cell viability at 24 h of incubation. Error bars indicate the standard error of the mean. Data presented were obtained from two independent experiments.

ethanolic extract of *H. cordata* demonstrated cytotoxic effect at the concentration of 233 mg/ml after 12 h of incubation (Figure 1), and at the concentration between 233 and $2.3x10^{-1}$ mg/ml after 24 h of incubation (Figure. 2). No cytotoxic effect was detected at any concentration between $2.3x10^{-2}$ and $2.3x10^{-6}$ mg/ml after 12 and 24 h of incubation. The $2.3x10^{-2}$ mg/ml was the highest concentration that was least cytotoxic to PBMC in all extracts and in both time points determined. Therefore, this concentration was chosen for subsequent evaluation.

IL-1β mRNA expression

Compared to positive control, at 12 h of incubation, PBMC incubated with 95% ethanolic extract significantly increased IL-1β mRNA expression (Figure. 3). Slight increase ($p>0.1$) in cytokine gene expression was observed in PBMC incubated with *H. cordata* extracts obtained from water and 50% and 70% ethanol. At 24 h of incubation, PBMC incubated with water and 50% ethanolic extracts significantly increased IL-1β mRNA expression, whereas those incubated with 70% and 95% ethanolic extracts only slightly ($p>0.1$) increased cytokine expressions.

IL-10 mRNA expression

No detectable change in IL-10 mRNA expression was seen in PBMC incubated with all *H. cordata* extracts at 12 and 24 h of incubation (Figure. 4).

IL-12p40 mRNA expression

Compared to positive control, at 12 h of incubation, PBMC incubated with each *H.cordata* extract did not change in the expression of IL-12p40 mRNA (Figure. 5). After 24 h of incubation, however, the cells demonstrated slightly increased cytokine gene expression in all extracts.

Discussion

The present study evaluated the immunomodulatory activities of *H. cordata* crude extracts on IL-1β, IL-10, and IL-12p40 mRNA expression in porcine PBMC. The immunomodulatory effects of this medicinal plant have never been assessed in porcine model.

At 12 h of incubation, PBMC incubated with 95% ethanolic extract significantly up-regulated IL-1β mRNA expression in response to LPS

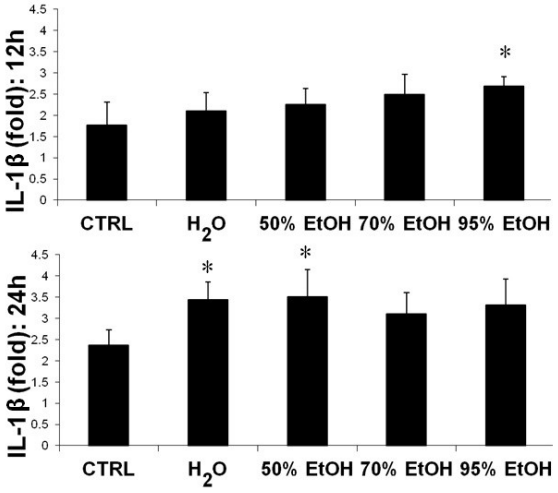

Figure 3 Effects of *H. cordata* extracts (H2O, 50% EtOH, 70% EtOH, and 95% EtOH) on interleukin-1 beta (IL-1β) mRNA expression in lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMC) after 12 and 24 h of incubation. PBMC treated with culture media alone and stimulated with LPS served as the control (CTRL). Error bars indicate the standard error of the mean. Asterisks represent significant mean difference of IL-1β mRNA expression between treatment and control group ($p<0.05$).

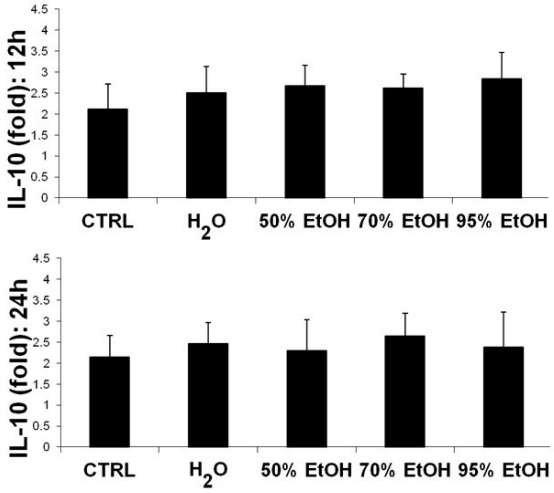

Figure 4 Effects of *H. cordata* extracts (H2O, 50% EtOH, 70% EtOH, and 95% EtOH) on interleukin-10 mRNA expression in lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMC) after 12 and 24 h of incubation. PBMC treated with culture media alone and stimulated with LPS served as the control (CTRL). Error bars indicate the standard error of the mean.

stimulation (Figure. 3). PBMC incubated with water, 50% ethanolic and 70% ethanolic extracts, however, did not change their IL-1β mRNA expression. No changes in IL-10 and IL-12p40 mRNA expression were detected in PBMC incubated with either water or ethanolic extract at this time point. These findings suggested that the 95% ethanolic extract may have a potential to induce relatively early pro-inflammatory cytokine response in porcine immune cells. Currently at least 40 compounds in *H. cordata* have been chemically characterized (Chou et al., 2009), but only a few of them have been tested for their effects on immunomodulation (Lee et al., 2008; Wang et al., 2002). Among those compounds as well as other *H. cordata* crude extracts that showed immune activity were reportedly obtained from water extraction (Han et al., 2009; Kim et al., 2009; Lee et al., 2008; Wang et al., 2002). Thus, further studies are needed to identify the constituents in the 95% ethanolic fraction that contribute to porcine IL-1β induction.

In contrast to 12-h incubation, incubation of PBMC with crude water and 50% ethanolic extracts significantly increased IL-1β expression (Figure. 3), and slightly increased IL-12p40 expression after 24 h of incubation (Figure. 5). Incubation of PBMC with 70% and 95% ethanolic crude extracts, on the other hand, slightly increased IL-1β and IL-12p40 expression. These findings suggest that *H. cordata* extracts, in a polar fraction, also contain bioactive constituents that could

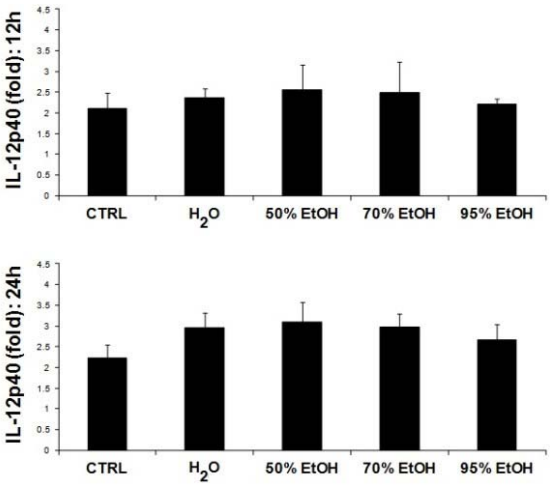

Figure 5 Effects of *H. cordata* extracts (H2O, 50% EtOH, 70% EtOH, and 95% EtOH) on interleukin-12p40 mRNA expression in lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMC) after 12 and 24 h of incubation. PBMC treated with culture media alone and stimulated with LPS served as the control (CTRL). Error bars indicate the standard error of the mean.

induce pro-inflammatory cytokine expressions of the pigs. These findings were compatible with previous reports in humans and mice that water extract of *H. cordata* could potentially promote pro-inflammatory cytokine response, e.g. IFNγ, IL-1β, IL-2, IL-6, and TNFα (Du et al., 2012; Kim et al., 2009; Lau et al., 2008; Wang et al., 2002). None of the *H. cordata* extracts affected IL-10 gene expression at 12 and 24 h of incubation (Figure. 4). These findings were different from previous report in a murine study that *H. cordata* extract could induce IL-10 expression (Lau et al., 2008). Precise reason for the different findings made in this study and a previous report was not known, but a different dose and time of exposure may be factors to consider. Further studies with various doses of *H. cordata* extract and incubation periods may be done for a more thorough investigation. The present findings at least suggest that the predominant effect of *H. cordata* in pigs may be towards pro-inflammation.

In conclusion, this study reports the potential of *H. cordata* to promote immune function of porcine PBMC by enhancing pro-inflammatory cytokine expressions, i.e. IL-1ß and IL-12p40. These findings suggested that *H. cordata* may be exploited for immunostimulation purposes in the pigs.

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ฤทธิ์ของสารสกัดพลูคาวต่อการแสดงออกของยีนอินเตอร์ลิวคิน-1 เบต้า, อินเตอร์ลิวคิน-10 และอินเตอร์ลิวคิน-12p40 ใน peripheral blood mononuclear cell ของสุกร

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[1]ห้องปฏิบัติการวิจัยเพื่อส่งเสริมภูมิต้านทานในมนุษย์และสัตว์ สาขาวิชาเทคโนโลยีชีวภาพ มหาวิทยาลัยแม่โจ้
[2]ฝ่ายเภสัชและผลิตภัณฑ์ธรรมชาติ สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย

บทคัดย่อ พลูคาวมีฤทธิ์ทางเภสัชวิทยาที่หลากหลายทั้งในมนุษย์และสัตว์ทดลอง งานวิจัยนี้ศึกษาฤทธิ์ของสารสกัดพลูคาวต่อการแสดงออกของยีนอินเตอร์ลิวคิน-1 เบต้า อินเตอร์ลิวคิน-10 และอินเตอร์ลิวคิน-12p40 ใน peripheral blood mononuclear cell (PBMC) ของสุกร สารสกัดพลูคาวที่ความเข้มข้นที่เหมาะสม ได้แก่ความเข้มข้นที่สูงที่สุดที่ไม่เป็นพิษต่อเซลล์ ถูกบ่มร่วมกับ PBMC นาน 12 หรือ 24 ชั่วโมง และที่ 6 ชั่วโมง ก่อนครบกำหนดการบ่ม เซลล์ถูกกระตุ้นด้วยสารไลโปโพลีแซคคาไรด์ ผลการศึกษาพบว่าสารสกัดพลูคาวสามารถกระตุ้นการแสดงออกของยีนอินเตอร์ลิวคิน-1 เบต้าได้อย่างมีนัยสำคัญ และกระตุ้นการแสดงออกของอินเตอร์ลิวคิน-12p40 ได้เล็กน้อยทั้งที่ 12 และ 24 ชั่วโมง สารสกัดพลูคาวไม่มีผลต่อการแสดงออกของยีนอินเตอร์ลิวคิน-10 ทั้งสองระยะเวลาที่ศึกษา ผลการศึกษานี้บ่งชี้ว่าพลูคาวน่าจะมีฤทธิ์กระตุ้นการทำงานของระบบภูมิคุ้มกันของสุกรโดยเหนี่ยวนำการสร้างไซโตคายน์ที่ส่งเสริมกระบวนการอักเสบ

คำสำคัญ พลูคาว อินเตอร์ลิวคิน-1 เบต้า อินเตอร์ลิวคิน-10 อินเตอร์ลิวคิน-12p40 peripheral blood mononuclear cell สุกร