大韓本草學會誌 제26권 제2호(2011년 6월)
Kor. J. Herbology 2011 ; 26(2) : 1-10

Chlorogenic acid 및 인동등 ethyl acetate 분획의 비장 및 흉선 세포에서의 유전자 발현 분석을 통한 면역조절효과

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Immunomodulatory effects of chlorogenic acid and ethyl acetate fraction from *Lonicera japonica* on cytokine gene expression profiles in spleen and thymus

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ABSTRACT

Objective : *Lonicera japonica* contains anti complementary polysaccharides and polyphenolic compound. Among these polyphenolic substances, chlorogenic acid is the major active component of this plant. However, the immunological mechanisms for these activities, have not been elucidated, nor the active components. To clarify immunomodulatory effects of those we examined the relationship between the activity of CD8+ T cell-mediated lysis and the frequency of cytokine profiles in spleen, thymus (especially IFN-γ , IL-4, GM-CSF etc.) expressing CD8+ T cells activated by IL-2.

Methods : To study immunomodulatory effects ethyl acetate fraction from *Lonicera japonica*, chlorogenic acid on cytokine gene expression from spleen, thymus cells, RT-PCR was performed after quantitative normalization for each gene by a densitometry using β -actin gene expression. A modified standard ^{51}Cr-release assay was used to measure cytotoxic activities of cytotoxic T cells. Spleen, thymus cells from NOD mice were stained with CD3, CD4, CD44, CD69 in staining buffer and analyzed by two color flow cytometry.

Results : We showed that ethyl acetate fraction from *Lonicera japonica* in combination with IL-2 resulted in a significant enhancement of PCR products for IFN-γ , IL-4, IL-10, GM-CSF, IL-6 and cytotoxtic CD8+ T cell proportion in spleen and thymus T cells in NOD mice. This suggests that IFN-γ , IL-6 like IL-4 may be acting as a regulatory rather than proinflammatory cytokine.

Conclusions : In conclusion, based on the results of the present study which showed that ethyl acetate fraction from *Lonicera japonica* and chlorogenic acid upregulating cytokine gene expression in spleen and thymus, we are tempted to speculate that some of the therapeutic efficacies such as anti-diabetic activity of *Lonicera japonica* are due to the immunomodulatory its ethylacetate fraction and chlorogenic acid.

Key words : Immunomodulation, Lonicera japonica thumb., chlorogenic acid, cytokines, CD8+

1. Introduction

Lonicera japonica Thunb., family Caprifoliaceae, Korean name "Indongdeung", is one of the medicinal plants widely used in Korea as an immunomodulational herb. *Lonicera japonica* Thunb. has been used to treat urinary disorders, fever and headache. It has been known as an anti-inflammatory agent in Korea from ancient times and is used widely for upper respiratory tract infections, diabetes mellitus and rheumatoid arthritis[1].

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This plant has been shown to display a wide spectrum of biological and pharmacological activities such as antibacterial, antiviral[2], antioxidant[3]. *Lonicera japonica* contains anticomplementary polysaccharides and polyphenolic compound. It is known to contain loganin, an iridoid glucoside, as the main constituent as well as other derivatives of secologanin[4]. The polyphenolic compounds inhibit the platelet aggregation, thromboxane biosynthesis and hydrogen peroxideinduced endothelial injury[5]. Recently, the 95% ethanol extract from the leaves of *Lonicera japonica* has been demonstrated to reduce HIV loading and possess immunomodulating properties[6].

Among these polyphenolic substances chlorogenic are representative. Chlorogenic acid is the major active component of this plant. Chlorogenic acid (1,3,4,5−tetrahydroxycyclohexane carboxylic acid 3−(3,4−dihydroxycinna−mate)), of which chemical structure is shown in Fig. 1, is a kind of polyphenol derivative which widely exists in higher plants like fruits, vegetables, black teas, and some traditional Chinese medicines[7]. It not only has the functions of antioxidation, of inhibiting hypertension, and of stimulating the flowering of plants, but also affects the activity of trypsin, amylase, and other enzymes. Chlorogenic acid is a phenolic acid formed by esterification of caffeic and quinic acids, is present as 50.3% of the total phenolics identified in potato peel extracts and exerts antioxidant and antimicrobial activities[8−9]. Additionally, it has been claimed to modulate the in vitro activity of G−6−PASE[10]. In previous studies, it was found that intravenous infusion of chlorogenic acid to insulin−resistant, obese Zucker (fa/fa) rats improved (P b.05) glucose tolerance by 22%[11]. It might be that chlorogenic acid can regulate glucose metabolism by modulating G−6−PASE activity in vivo.

Helper T (Th) cells have been divided into two subclasses, Th1 and Th2, according to the profile of cytokine secretion[12]. Th1 cells produce mainly IFN−γ , IL−2, TNF−a and Th2 cells IL−4, IL−5 and IL−6. Several other cytokines are secreted by both Th1 and Th2, including, IL−3, GM−CSF, IL−10, and IL−13. IFN−γ and IL−12 suppress IL−13 production, and IL−13 reciprocally suppresses IL−12 and IFN− γ production[13]. IL−10 down−regulates IFN−γ . Currently immunity to infection, diabetes mellitus, cancer, asthma and rheumatoid arthritis is thought to be controlled by distinct type 1 (Th1) and type 2 (Th2) subpopulations of T cells, as discriminated on the basis of cytokine secretion and function.

Recently, we have been screening for natural reagents from plants and fruits on the basis of the Korean traditional herb medicines and medical formulae. Several medicinal herbs have shown to promote immunity in different ways.

In the present study, we examined the immunomodulatory activity of ethyl acetate fraction from *Lonicera japonica*, chlorogenic acid on spleen and thymus cells. We found that ethyl acetate fraction from *Lonicera japonica* and chlorogenic acid activated several parameters of cytokines gene expression such as IFN−γ , IL−4, GM−CSF, IL−6, IL−2, IL−10 expression of co−stimulatory molecules, cytolytic activity and blood glucose level in NOD mice. The active component of *Lonicera japonica* appeared to be ethyl acetate fraction and chlorogenic acid. The fact that *Lonicera japonica* contains immunomodulatory polyphenolic substances may explain some of the therapeutic efficacies of *Lonicerajaponica* , which has been used in folk medicine to treat various diseases including cancer, diabetes mellitus, rhreumatoid arthritis and so on.

2. Materials and methods

2.1. Plant material and preparation of extracts

The sample of *Lonicera japonica* were purchased from Buyeo−gun agriculture technology center (Buyeo, Korea). The voucher specimens are deposited in our laboratory (Department of Herbology, College of Oriental Medicine, Sanji University Wonju 220−702, Republic of Korea). Chlorogenic acid were purchased from Sigma−Aldrich (Sigma−Aldrich,Korea). Ethylacetate (EtOAc), 1−butanol (BuOH), chloroform (CHCL3) were purchased from Merck (Darmstate, Germany). Water was prepared in a Milli−Q water purification system (Millipore, Bedford, MA,USA). All other chemicals and reagents were of analytical grade and used without further purification.

Caulis et folium of *Lonicerajaponica* (1200 g) were extracted with 1.5 L of boiled distilled water at 100℃ for 2 hrs. The extracts were concentrated to water extracts approximately 100 g and it (100 g) was successively extracted with ethyl acetate (EtOAc), 1−butanol (BuOH), chloroform (CHCL3) and water (H2O). Then, the extracts were filtered and evaporated

on a rotatory evaporator(Rotary evaporator, BUCHI B-480, Switzerland) and finally dried by a freeze drier (Freezedryer, EYELAFDU-540, Japan) to yield the extracts each fractions. The yield(w/w) of ethyl acetate (EtOAc), 1-butanol (BuOH), chloroform (CHCL3) and water (H2O) extracts were about 3.1, 7.6, 3.2 and 3.5% (Fig. 1).

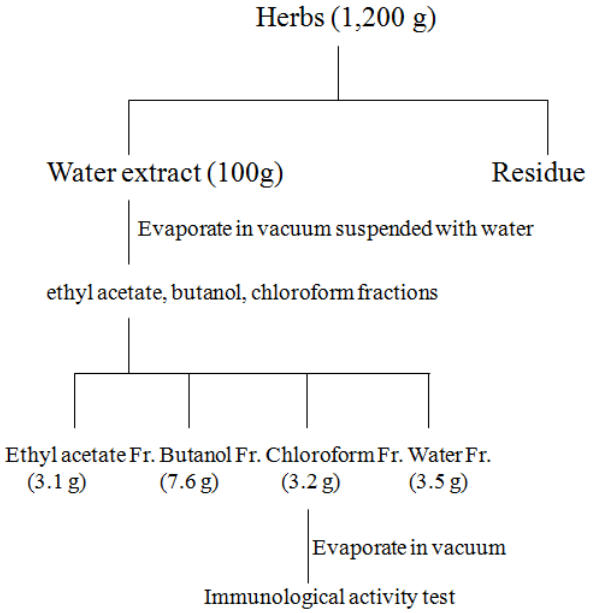

Fig. 1. Schematic diagram for partial fractions from Lonicera japonica

Extraction and Isolation. The dried plant material (1.2 kg) was extracted with distilled water and concentrated. The resulting syrup (100 g) was subsequently partitioned with CHCl3, EtoAC, butanol fractions.

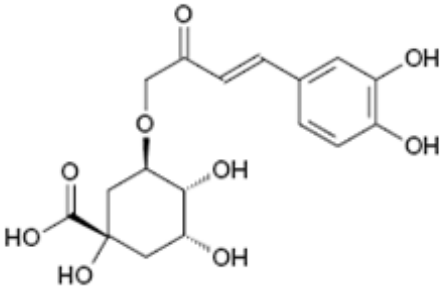

Fig. 2. The chemical structure of chlorogenic acid

2.2. Animals and blood glucose determination in NOD mice

Four to five-week-old male C57BL/6 mice were obtained at Daehan Biolink Co. LTD. (Eumsung, RepublicofKorea). NOD mice were obtained from the KRIBB (Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea)and kept in our animal facilities under specific pathogen-free conditions. Mice were treated with anti-CD3 mAbs (5 ㎍/day for 5 consecutive days i.v.) and EtOAc fraction (100 mg/kg p.o) from 8 weeks of age. In males, spontaneous diabetes begins to appear by 14 weeks of age. Mice were screened for blood glucose levels at least once a week after animals were 8 weeks old.

Blood glucose analysis was performed every week using a Lifescan One Touch Profile Meter (Issy les Mouline aux, France) with one drop of blood sampled from the tail. A 1.0g/lcalibration standard solution was assayed at least every samples. In this study, attention was focused on any sign of glycemic disturbance. The threshold for positivity was set at the mean of the pretest values plus two standard errors. Mice were considered diabetic when glucose levels reached 250mg/dl.

All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea).

2.3. RT-PCR

To study immunomodulatory effects ethyl acetate fraction from *Lonicera japonica*, chlorogenic acid on cytokine gene expression from spleen, thymus cells, RT-PCR was performed after quantitative normalization for each gene by a densitometry using β -actin gene expression. Briefly, total cellular RNA was extracted by using RNAzolB(Tel-Test, Friendswood, TX) according to the manufacturer's instruction. Aliquots(3g) of total RNA were transcribed into cDNA at 37° C for 1h in a total volume of 20㎕ with 2.5 U of moloney murine leukemia virus reverse transcriptase (Roche, Mannheim, Germany). Reverse transcribed cDNA samples were added to a PCR mixture consisting of 10x PCR buffer, 0.2 mM dNTP, 0.5 U Taq DNA polymerase (Takara, Tokyo, Japan), and 10 pmol of primers for each gene.

The primer sequences are as shown in table 1. Amplifications were performed with 25 cycles for β -actin and 30 cycles for the others. The amplification profile included denaturation at 95° C for 1 min, primer annealing at 55° C for 1 min, and extension at 72° C for 1 min, followed by an additional extension step at 72° C for 10 min. PCR products were electrophoresed and visualized by ethidium bromide staining.

Table 1. Primer sequences for RT-PCR

Gene	Primer	Sequence	Product (bp)
β-actin	Sense Anti-sense	5'-GTGGGGCGCCCCAGGCACCA-3' 5'-CTCCTTAATGTCACGCACGATTTC-3'	548
IL-2	Sense Anti-sense	5'-AACAGCGCACCCACTTCAA-3' 5'-TTGAGATGATGCTTTGACA-3'	442
IL-4	Sense Anti-sense	5'-TAGTTGTCATCCTGCTCTT-3' 5'-CTACGAGTAATCCATTTGC-3'	382
IL-6	Sense Anti-sense	5'-ATGAAGTTCCTCTCTGCAAGA-3' 5'-GGTTTGCCGAGTAGATCTCAA-3'	633
CTLA-4	Sense Anti-sense	5'-TGGTGTTGGCTAGCAGCCATG-3' 5'-TTGGATGGTGAGGTTCACTC-3'	197
CD28	Sense Anti-sense	5'-AACAAGATTTTGGTAAAGCAG-3' 5'-GAACTCAATTTTGCAGAAGTA-3'	273
IL-10	Sense Anti-sense	5'-TCCTTAATGCAGGACTTTAAGGGTTACTTG-3' 5'-GACACCTTGGTCTTGGAGCTTATTAAAATC-3'	256
IFN-γ	Sense Anti-sense	5'-AGCGGCTGACTGAACTCAGATTGTAG-3' 5'-GTCACAGTTTTCAGCTGTATAGGG-3'	247
TGF-β	Sense Anti-sense	5'-GGACCGCAACAACGCCATCTA-3' 5'-CGCACACAGCAGTTCTTCTCT-3'	525
GM-CSF	Sense Anti-sense	5'-GGATCCTCAGAGAGAAAGGCTAAG-3' 5'-GGATCCTGGGCTTCCTCATTTTTAG-3'	477
B7-1	Sense Anti-sense	5'-TGCTGTCTGTCATTGCTGGGAAACT-3' 5'-CCCAGGTGAAGTCCTCTGACACGTG-3	474
B7-2	Sense Anti-sense	5'-GGGGGATCCATGGGCTTGGCAATCCTTAT-3' 5'-TCGGGTGACCTTGCTTAGACGTGCAGG-3'	477
TNF-α	Sense Anti-sense	5'-GGCAGGTCTACTTTGGAGTCATTGC-3' 5'-ACATTCGAGGCTCCAGTGAATTCGG-3'	308

2.4. ^{51}Cr-release assay

A modified standard ^{51}Cr-release assay was used to measure cytotoxic activities of cytotoxic T cells as previously described[14]. Briefly, as a single-cell suspension of target cells, B16/F10 tumor cells were prepared and incubated for 48 hrs, and then labeled ^{51}Cr. As a effector cells, mouse T cells from healthy C57BL/6 mice we retransferred to 24-well plates with coating with anti-CD3mAb(0.5μg/well) and incubated for 48 hrs after being stimulated in medium supplemented with EtOAc fraction from *Lonicera japonica* and rIL-2(10ng/㎖) and thymus T cells were placed at the indicated effector : target cell ratios in a 96-well roundbottom microtiter tissue culture plate. ^{51}Cr-labeled target cells ($1x10^4$ cells per well) were added to the wells in triplicate and incubated for 6 h in a 37-C, 5% CO2- humidified incubator. After incubation, 100 ul of the culture supernatant was collected, and radioactivity was detected by g counting. Results are expressed as the percentage of the specific release based on the formula % specific release = (experimental release - spontaneous release)/(maximum release - spontaneous release) x 100. All data represent the standard deviation of at least three different determinants and were compared using Student's unpaired t-test.

2.5. Antibodies and flow cytometric analysis.

All antibodies(CD3, CD4, CD44, CD69) for flow cytometric analysis were purchased from Becton Dickinson (BD) PharMingen (SanDiego,CA). Spleen, thymus cells from NOD mice were stained with the indicated antibodies in staining buffer(PBS containing 1% FBS and 0.01% NaN3) for 10 min on ice, and analyzed by two color flow cytometry on a FACScan using Cell Quest software(BD Biosciences, MountainView, CA).

2.6. Statistical Analysis.

For statistical analysis of data, P-values were

analyzed using a unpaired Student's t-test software program (Startview 5.1 ; Abacus Concepts, Berkeley,CA). Results were considered statistically significant when P values were*p<0.05,**p<0.01,***p<0.001.

3. Results

3.1. mRNA expression of cytokines in splenocytes treated with ethyl acetate, butanol and chloroform fractions from *Lonicera japonica*

As shown in Fig. 3, the mRNA for β-actin was detectable in spleen cells treated with RPMI-1640 media (Lane 1), ethyl acetate fractions (Lane 2, 3), butanol fractions (Lane 4, 5), and chloroform fractions (Lane 6, 7) respectively. PCR products for IL-10, IFN-γ , TNF-a, IL-4 and TGF-b1, amplified from spleen cell RNA preparations. Especially, PCR products for IL-10, IFN-γ were increased in ethyl acetate fractions (Lane 2, 3) compared with control groups (Lane 1). The results demonstrates that components in ethyl acetate fractions significantly affected these cytokines mRNAs expression of spleen cells.

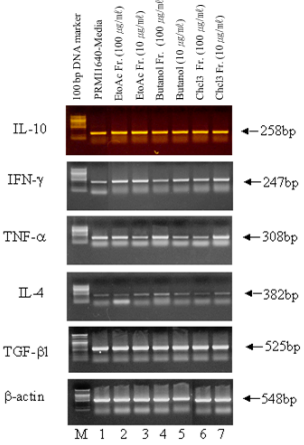

Fig. 3. mRNA expression of cytokines in splenocytes treated with EtOAc fraction from *Lonicera japonica*

The mice spleen cells were incubated in the presence of EtOAc fraction of *Lonicera japonica* for 6 hrs before the isolation of total RNA. Total RNA(3 ㎍/㎖) was used in RT reactions. cDNA was amplified (30 cycles) using primer pairs to IL-10, IFN-γ , TNF-α , IL-4, TGF-β 1 and β -actin. PCR products were visualized by ethidium bromide on 2% agarose gels. Lane M, 100bp DNA marker ; lane 1, only cells(Splenic cells) ; lane2, EtOAc fraction from *Lonicera japonica*(100㎍/㎖) ; lane 3, EtOAc fraction from *Lonicera japonica*(10㎍/㎖) ; lane 4, butanol fraction from *Lonicera japonica* (100 ㎍/㎖) ; lane 5, butanol fraction from *Lonicera japonica*(10㎍/㎖) ; lane 6, $chcl_3$ fraction from *Lonicera japonica* (100㎍/㎖) ; lane 7, $chcl_3$ fraction from *Lonicera japonica* (10 ㎍/㎖).

3.2. mRNA expression of cytokines in thymus treated with ethyl acetate, butanol and chloroform fractions from *Lonicera japonica*

As shown in Fig. 4, the mRNA for β -actin was detectable in thymus cells treated with RPMI-1640 media (Lane 1), ethyl acetate fractions (Lane 2, 3), butanol fractions (Lane 4, 5), and chloroform fractions (Lane 6, 7) respectively. PCR products for IFN-γ , IL-4, GM-CSF, IL-6, IL-10, B7-1 and B7-2 amplified from thymus cell RNA preparations. The relative expression of above genes was higher in the thymus of ethyl acetate fraction group and in both spleen, when compensated with that of the b-actin genes. Especially, PCR products for IFN-γ , IL-4, GM-CSF, IL-6 were increased in ethyl acetate fractions (Lane 2, 3) compared with control groups (Lane 1). The results demonstrates that components in ethyl acetate fractions significantly affected these cytokines mRNAs expression of thymus cells.

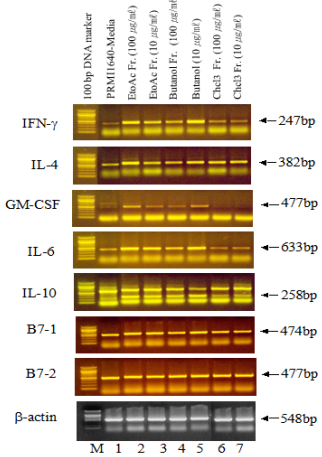

Fig. 4. mRNA expression of cytokines in thymus treated with EtOAc fraction from *Lonicera japonica*

The mice thymus cells were incubated in the presence of EtOAc fraction from *Lonicerajaponica* for 6 hrs before the isolation of total RNA. Total RNA(3 ㎍/㎖) was used in RT reactions. cDNA was amplified (30 cycles) using primer pairs to IFN-γ , IL-4, GM-CSF, IL-6, IL-10, B7-1, B7-2, and β -actin. PCR products were visualized by ethidium bromide on 2% agarose gels. Lane M, 100bp DNA marker ; lane 1, only cells (Spleen cells) ; lane2, EtOAc fraction

from *Lonicera japonica* (100 ㎍/㎖) ; lane 3, EtOAc fraction from *Lonicera japonica* (10 ㎍/㎖) ; lane 4, butanol fraction from *Lonicera japonica* (100 ㎍/㎖) ; lane 5, butanol fraction from *Lonicera japonica* (10 ㎍/㎖) ; lane 6, chloroform ($CHCl_3$) fraction from *Lonicera japonica* (100 ㎍/㎖) ; lane 7, chloroform ($CHCl_3$) fraction from *Lonicera japonica* (10 ㎍/㎖)

3.3. mRNA expression of cytokines in thymus co-stimulated with EtOAc fraction from *Lonicera japonica* and anti-CD3mAb

As shown in Fig. 5, the mRNA for β-actin was detectable in thymus cells treated with RPMI-1640 media (Lane 1), CD3 (Lane 2) only, CD3 in the presence of PMA (100 ng/㎖) and ionomycin (1 ㎍/ml), or treated with EtoAc fractions (100, 50, 10, 1 ㎍/㎖), chlorogenic acid (1, 0.1, 0.01, 0.001 ng/㎖) respectively. PCR products for IFN-γ, IL-4, GM-CSF and IL-2 amplified from thymus cell RNA preparations. Especially, PCR products for IFN-γ, IL-4 were increased in ethyl acetate fractions (100, 50, 10 ㎍/㎖), chlorogenic acid (1 ng/㎖) compared with control groups (Lane 2). The results demonstrates that components in ethyl acetate fractions, chlorogenic acid significantly affected these cytokines mRNAs expression of thymus cells.

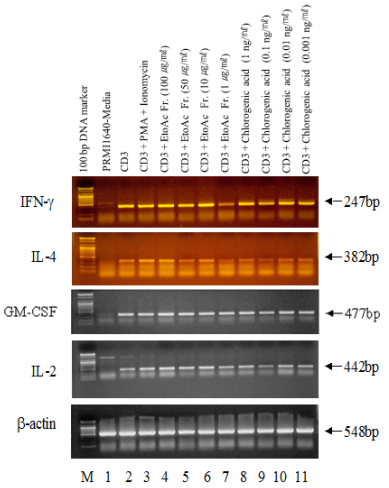

Fig. 5. mRNA expression of cytokines in thymus co-stimulated with EtOAc fraction from *Lonicera japonica* and anti-CD3mAb

Thymus cells were transferred to 24-well plates coated with anti-CD3mAb (0.5 ㎍/well) and stimulated in medium supplemented with PMA (100 ng/㎖), ionomycin (1 ㎍/ml), EtOAc fraction from *Lonicera japonica* and chlorogenic acid and then cultured with RPMI1640 media for 6 hours before the isolation of total RNA. Total RNA(3 ㎍/㎖) was used in RT reactions. cDNA was amplified (30 cycles) using primer pairs to IFN-γ, IL-4, GM-CSF, IL-2 and β-actin. PCR products were visualized by ethidium bromide on 2% agarose gels.

3.4 B16/F10 tumor target cells killed by rIL-2 and EtoAc fraction-activated mouse T cells

The use of cytotoxicity assays is of central importance in characterizing the function of T lymphocytes, and the ^{51}Chromium(Cr)-release assay is the most widely practiced method for analyzing the lytic activity of a cytotoxic T-cell (CTL) culture.

Cytotoxic T cell activities were measured by testing lytic activity against melanoma targets that differed in the relative amounts of MHC Class I expression. Cytotoxic T cells were highly effective in lysing melanoma targets at all effector versus target (E : T) ratios (Fig. 6). Tumor targets were lysed at all E : T ratio. The relatively weak ability to lyse cells on the part of 50 : 1 (E : T) ratio is likely explained by a low percentage of cytotoxic T cells in the preparation from thymus T cells.

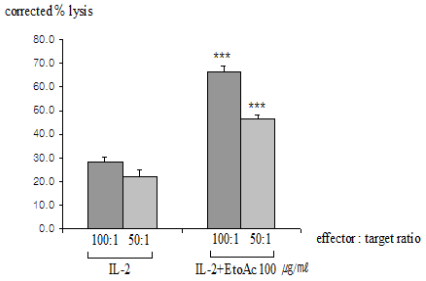

Fig. 6. Lytic activity of mouse T lymphocytes following rIL-2 incubation

As a single-cell suspension of target cells, B16/F10 tumor cells were prepared and incubated for 48 hrs, and then labeled ^{51}Cr. As a effector cells, mouse T cells from healthy C57BL/6 mice were transferred to 24-well plates with coated with anti-CD3mAb(0.5㎍/well) and incubated for 48 hrs after being stimulated in medium supplemented with EtOAc fraction from *Lonicera japonica* and rIL-2 (10 ng/㎖). ^{51}Cr labeled target cells were added to replicates wells containing effector cells and incubated 6hrs. After 6hrs incubation, the culture supernatants(100㎕) were dispensed to the wells of 96-well microtiter plates with replicates of three wells for each effector cell concontration. The ^{51}Cr release were measured in γ-scintillation counter (LKB,U.S.A) 1min/sample as described in materials and methods. Statistically significant value compared with control data by T test($^{***}p<0.001$).

3.5 Blood glucose level in NOD mice treated with EtOAc fraction from *Lonicera japonica* and anti-CD3 mAbs

Mice were considered diabetic as a positive blood glucose when glucose levels reached 250mg/dl. The blood glucose level of NOD mice were increased from 14 weeks of age (Table 2, Fig. 7). At 18 weeks of age, 90% of NOD normal mice were positive. In the EtOAc fraction from *Lonicerajaponica*-treated groups, an decrease in the blood glucose was seen between 15 and 18 weeks of age. Blood glucose levels were lower in the EtOAc fraction from *Lonicerajaponica*and anti-CD3 mAbs treated groups than the untreated mice. Although the blood glucose level of mice was also lower in two experimental groups than the untreated mice, statistical significance was not attained.

Table 2. Blood glucose in NOD mice treated with EtOAc Fraction from *Lonicera japonica* and anti-CD3 mAb compared to control NOD mice (group B) and normal mice (group A) Group A(Normal group) : It was treated with RPMI-1640 media.

Weeks (after treated EtOAc fraction and anti-CD3)	Blood glucose(mg/dℓ) MEAN±S.D.		
	Normal NOD mice (group A)	Control NOD mice **CD3** (group B)	NOD mice **CD3 + EtOAc fraction** (group C)
1	115.7 ± 14.4	119.0 ± 16.4	96.0 ± 5.1
2	100.0 ± 1.5	119.0 ± 11.3	103.7 ± 0.9
3	103.7 ± 11.3	103.3 ± 5.8	112.0 ± 7.6
4	110.0 ± 13.5	100.3 ± 0.9	122.3 ± 4.3
5	121.0 ± 11.7	115.0 ± 13.5	121.3 ± 19.6
6	131.0 ± 14.6	229.3 ± 125.3	217.3 ± 84.4
7	272.0 ± 160.0	285.0 ± 174.0	230.0 ± 92.6
8	333.0 ± 220.0	111.0 ± 9.0	142.0 ± 35.0
9	362.5 ± 207.5	97.0 ± 1.0	140.0 ± 43.0
10	344.0 ± 10.0	98.5 ± 2.5	127.0 ± 21.0
11	386.0 ± 10.0	101.5 ± 4.5	114.5 ± 25.5

Group B(Control group) : It was treated with anti-CD3 mAb (5 μg/day for 5 consecutive days i.v.)
Group C(Samples group) : It was treated with anti-CD3 mAb (5 μg/day for 5 consecutive days i.v.) and EtOAc fraction.
Experimental NOD treatment groups are compared with control NOD mice Mean of S.D. triplicate plates. P-value : Stastistically significant value as compared with data of control group by T-test.

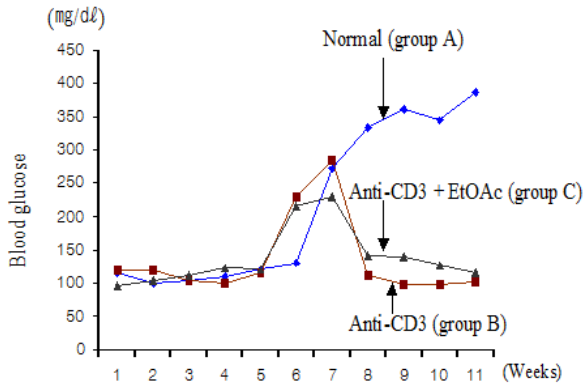

Fig. 7. Blood glucose level in NOD mice treated with EtOAc fraction from *Lonicera japonica* and anti-CD3 mAb compared to control NOD mice.

3.6 Inhibitory effect of ethyl acetate fraction from *Lonicera japonica* on spleen, thymus surface marker

Effects of ethyl acetate fraction from *Lonicera japonica* on leukocyte subsets in spleen and thymus, there were marked change in numbers of CD8+ T cells compared to control cells (Fig. 8). Ethyl acetate fraction and anti-CD3 mAb treated group resulted in further increase in CD4-CD8+ T cells (by 0.4% in spleen, 0.5% in thymus), whereas CD44+CD69+ T cells were reciprocally decreased in both anti-CD3 only, ethyl acetate fraction and anti-CD3 mAb treated groups (Fig. 8).

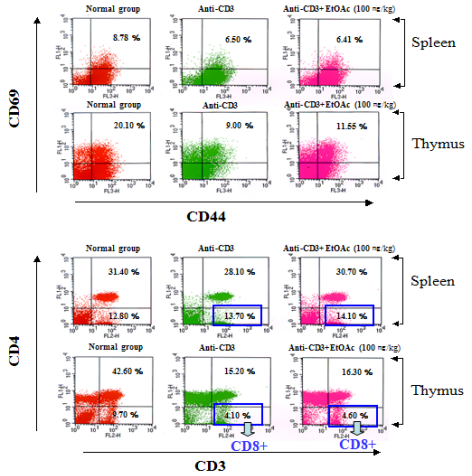

Fig. 8 EtOAc fraction from *Lonicera japonica* and anti-CD3 mAb down-regulates $CD44^{+}CD69^{+}$, $CD3^{+}CD8^{+}$ expression of spleen and thymus T cells in NOD mice.

Spleen and thymus cells in NOD mice were transferred to 24-well plates and stimulated in medium supplemented with EtOAc fraction from *Lonicera japonica* and then cultured with RPMI1640 media for 24 hours. Expression of activation markers were measured by FACS.

Discussion and conclusion

In this study, we analyzed the effect of ethyl acetate fraction and chlorogenic acid on the expression of genes expression profiles associated with immunomodulatory activity.

Particulary immune response to diabetes mellitus, cancer, asthma and rheumatoid arthritis is thought to be controlled by distinct Th1 Th2 cells, as discriminated on the basis of cytokine secretion and function. Th1 cells produce mainly IFN-γ , IL-2, TNF-a and Th2 cells IL-4, IL-5 and IL-6. Several other cytokines are secreted by both Th1 and Th2, including, IL-3, GM-CSF.

Therefore, it is of importance to examine the effect of herbal medicine and components on the expression of those genes in spleen, thymus.

Interferons (IFNs) are a family of cytokines with antiviral, antiproliferative and immunomodulatory activities[15]. The ability of most cells to secrete and respond to IFN makes the IFN system a powerful first line host defence against pathogen and an essential component of innate immunity[16].

The hepatocellular stress induced by hepatotoxins or by viruses, may lead to activation of liver resident macrophages on one side and to the release of proinflammatory cytokines such as IFN-γ on the other side[17]. The production of IFN-γ (the only type II IFN) is restricted to cells of the immune system, such as activated T cells and NK cells. IFN-γ activates cellular antiviral and microbicidial effector functions on most various levels including pathogen recognition, antigen processing and presentation, immunomodulation, or leukocyte trafficking[18].

The diversity of the possible approaches to immunotherapy of diabetes for future clinical development. The data already available in man – using cyclosporin A, hsp p277 or anti-CD3 antibody – are promising. A good future direction might be to combine various treatments. One could thus propose to start the treatment by therapy such as anti-CD3 antibody, ethyl acetate fraction and chlorogenic acid that might immediately freeze the autoimmune process (and prevent any further loss of β cells) and induce a first wave of regulation.

Although it is accepted that IDDM resulted from altered balance between T helper type 1 and 2 (Th1 and Th2) cells, the exact roles Th1 and Th2 cells play in IDDM pathogenesis.

However, it is well known that islet infiltration by both CD4+ and CD8+ T cells is required for IFN-γ and IL-10 production in islets and b-cell destruction.

We have characterized an assay that detects melanoma specific cytotoxic CD8+ T cells. Recombinant IL-2 were used to activate antigen-specific T cells to express IFN-γ . In most cases, IFN-γ production is coupled with cytotoxic activity. Thus, we examined the relationship between the activity of CD8+ T cell-mediated lysis and the frequency of IFN-γ expressing CD8+ T cells activated by IL-2.

We showed that ethyl acetate fraction from *Lonicera japonica* in combination with IL-2 resulted in a significant enhancement of cytotoxtic CD8+ T cell responses (Fig. 6). Increased proportion of CD8+ T cells were accompanied by compensatory increases in gene expression profiles (IL-2, IFN-γ , IL-4, GM-CSF, IL-6 etc.), proportions of regulatory T cells (data not shown). Blood glucose levels were lower in the EtOAc fraction from *Lonicera japonica* and anti-CD3mAbs treated groups than the untreated mice. Although the blood glucose level of mice was also lower in two experimental groups than the untreated mice, statistical significance was not attained.

An unexpected finding in the present study was that IL-6 mRNA expression in thymus parallelled that of IL-4, IFN-γ , IL-10, GM-CSF. Thus, IL-6 and IL-4 mRNA levels in thymus mononuclear leukocytes of individual C57BL/6. This suggests that IL-6, like IL-4, IFN-γ may be acting as a regulatory rather

than proinflammatory cytokine.

In addition, our in vitro data does not and actually cannot necessarily reflect the in vivo data. Thus, our findings should be interpreted with caution and require further studies examining larger number of NOD mice. The Th1/Th2 cytokine-secreting profile represents the extreme of many possible outcomes.

It can be explained that cytokines are pleiotropic ; a cytokine may be produced by more than one cell type and may exert its effect on several target cells. Thus, assignment of a specific role to Th1 and Th2 cytokines cannot be fully addressed by using these isolated conditions.

The transcriptional NF-kB is important as a mediator of cellular responses to extracellular signals. NF-kB plays a pivotal role in inflammation by virtue of its ability to induce transcription of an array of inflammatory genes, especially to the regulation of pro-inflammatory molecules such as TNF-a and IL-6[19].

NF-kB is one of the most ubiquitous transcription factors that regulate gene expressions involved in cellular proliferation, inflammatory responses, and cell adhesion. Upon stimulation with a wide variety of stimuli including LPS, NF-kB translocates from the cytoplasm to the nucleus where the NF-kB positively regulates the expression of genes involved in the immune and inflammatory responses, including iNOS, COX-2, and TNF-alpha[20].

It is well established that NF-kB plays a crucial role in the innate and acquired immune responses and chronic inflammation. The NF-kB activity is crucial for the development of TH1 responses. NF-kB is also known to be a critical transcription factor that regulates TH2 cell differentiation and TH2-dependent airway inflammation[21].

The transcription factor NFAT (nuclear factor of activated T cells) plays an essential role in IL-2 expression. Binding sites for NFATs have also been found within the promoter regions of several other cytokine genes, including IL-3, IL-4, IL-5, IL-8, IL-13, tumor necrosis factor alpha, granulocyte-macrophage colony-stimulating factor, and IFN-γ [22]. NFAT is a complex composed of a cytoplasmic subunit and an inducible nuclear component comprised of AP-1 (Fos/ Jun) family members.

The promoter-enhancer regions of several activation-associated genes, some of which have been shown to be activated or suppressed after HIV-1 infection, possess NFAT binding sites, including those encoding IL-2, IL-3, IL-4, IL-5, IL-8, GM-CSF, TNF-α .

In our results, IL-2, IFN-γ , IL-4, GM-CSF, IL-6 gene expression were positively regulated by all three major families of transcription factors, these findings suggest that there may be significant overlap in T-cells between the signal transduction circuits that activate transcription of above genes.

Previously, we reported that binding activity of transcription factors (NF-κ B, NFAT, AP-1) was increased by EtOAc fraction of *Lonicera japonica*[23].

This results suggest that IFN-γ , IL-6 like IL-4 may be acting as a regulatory rather than proinflammatory cytokine and immunomodulation of ethyl acetate fraction from *Lonicera japonica* may be due to several transcription factors including NFAT as well as through indirect effects on the transcriptional activity of NF-κ B and AP-1.

Thus, based on the results of the present study which showed that ethyl acetate fraction from *Lonicera japonica* and chlorogenic acid upregulating cytokine gene expression in spleen and thymus, we are tempted to speculate that some of the therapeutic efficacies such as anti-diabetic activity of *Lonicera japonica* are due to the immunomodulatory its ethylacetate fraction and chlorogenic acid. Moreover, its mechanisms are involved by activating NF-kB, NFAT and AP-1.

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