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Immuno-Regulatory Activities of an Isoflavone Glycoside, 4', 6-Dimethoxylsoflavone-7-*O*-β-D-Glucopyranoside and the Crude Extract Isolated from *Amorpha fruticosa* LINNE.

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ABSTRACT : The methanolic (MeOH) extract of *A. fruticosa* bark, which showed immuno-regulatory activities, was separated to purify an active compared by means of a multi-stage column chromatography. This resulted in the isolation and characterization of an isoflavone glycoside named 4', 6-Dimethoxyisofalvone-7-*O*-β-D-glucopyranoside. Immuno-regulatory activities of the crude extract of *Amorpha fruticosa* LINNE bark were compared with that of an isoflavone glycoside (4', 6-dimethoxyisoflavone-7-*O*-β-D-glucopyranoside). The crude methanolic extract of *A. fruticosa* and purified single compound showed 16% of relatively low cytotoxicity at a maximum concentration of 1.0 g/L in cultivated normal human lung cell line (HEL299). Cell growth of human T cells was increased up to 15%, 0.5 g/L of the crude extract added group. This was higher than a single compound added one. On the other hand, specific production rates of IL-6 and TNF-α from T cell were higher in the purified compound treat group (0.82×10^{-4} pg/cell and 1.08×10^{-4} pg/cell, respectively), compared to 0.5 g/L of the crude extract added group (0.65×10^{-4} pg/cell and 0.84×10^{-4} pg/cell, respectively). In addition, the growth of NK-92MI cells incubated with the crude extract was higher up to 56% over the cells grown with a single compound (0.5 g/L). In overall, the crude extract showed relatively higher immuno-regulatory activities compared with a single compound, probably due to the synergic effect given by other substances existed in the crude extract. Even though the siolated compound stimulated higher secretion of cytokines from human T cells.

Key words **:** *Amorpha fruticosa* L., 4', 6-dimethoxyisoflavone-7-*O*-β-D-glucopyranoside, Immuno-regulatory activity, cytokines, NK-92MI cell

INTRODUCTION

As a deciduous shrub, *Amorpha fruticosa* LINNE is a rosales plant belonging to the family pea originating from North America. It was introduced into Korea around the 1930's and planted throughout Korea for erosion control and to restore wasteland. Being about 3 m high, the tree branches are covered with hair, which disappears with time. It is strongly environmental and salt tolerant and grows so fast to be plantable anywhere regardless of temperature and humidity (Huh *et al.*, 1997). Researchers have been interested in *A. fruticosa* extract since 1960's and many new compounds have been isolated in the mean time. A rotenoid having the dihydrofuran ring with the isoprenoid side chain was isolated from *A. fruticosa* for the first time (Crombie *et al.*, 1964). Crude methanol extract of *A. fruticosa* seed was fractionated using dichloromethane to isolate a rotenoid, 6a, 12a-dihydro-a-toxicarol (Reisch *et al.*, 1976). Researchers reported that the seed of *A. fruticosa* is composed of fatty acids including linoleic, oleic, palmitic, and stearic acid (Reisch *et al.*, 1976; Wang *et al.*, 1974; Lee *et al.*, 1977). Amorphigenin, fomononetin, ononin, wistin and amorphaquinone were purified from the crude methanol extract of *A. fruticosa* root (Shibata and Shimizu, 1978). The compounds such as cannabinoid were isolated from *A. fruticosa* (Kemal *et al.*, 1979). Amorinin having the flavanone structure was isolated from its bark (Rozsa *et al.*, 1982). Amoradin, amoradicin, and amoradinin were also purified and their structures have been identified (Rozsa *et al.*, 1984). It was reported that *A. fruticosa* root contains the prenylated flavanone, isoamoritin (Ohyama *et al.*, 1998). Three different isoflavones and 5 different rotenoids were purified (Li *et al.*, 1993). One flavonoid glycoside, 1 ester compound, and 2 rotenoid compounds were purified from its fruit (Lee *et al.*, 2003). Despite the fact it contains so many useful substances, *A. fruticosa* is mainly

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used for erosion control and to restore wasteland. No study has been performed on its immune activity of crude extract and single compounds. This study is to evaluate immune boosting effect of the crude extract from *A. fruticosa* bark and an isoflavone glycoside purified from its bark extract, and to consider the possibility of using this compound as an immune boosting agent.

MATERIALS AND METHODS

Materials

Different parts of 10 to 15-year-old *Amorpha fruticosa* L. were harvested from roadside of the Namhae expression way in 2003. The media for cell culture, RPMI 1640 and Alpha minimum essential medium (α-MEM) were purchased from Gibco (USA). HEPES buffer was purchased from sigma (USA). Fetal bovine serum and horse serum were purchased from Gibco (USA). Gentamycin sulfate and Sulforth odamine B (SRB) needed for staining were purchased from Sigma.

Extraction and Purification

Air-dried and powdered bark of *A. fruticosa* was extracted three times with MeOH at room temperature (3 days each). The combined MeOH extracts were concentrated in vacuum at 40 ℃. The concentrated extract was partitioned with light petroleum ether (LPE), diethyl ether (Et_2O), and ethyl acetate (EtOAc).

The Et_2O-soluble (97.1 g) fraction was separated on Sephadex LH-20 column (8.0×50.0 ㎝) using MeOH-EtOH (1 : 1, v/v) solvent system to yield 36 fractions (250.0 mL each). These fractions were divided into 3 portions (ABO-1~ABO-3) on the basis of TLC profiles. ABO-2 (89.1 g) was chromatographed on Sephadex LH-20 column (8.0×45.0 ㎝) using a MeOH-EtOH (1 : 4, v/v) solvent system to yield 70 fractions (250.0 mL each). These fractions were divided into 4 portions (ABO-2-1~ABO-2-4).

A MeOH solution of ABO-2-3 was divided into MeOH insoluble fraction (ABO-2-4A) and soluble fraction (ABO-2-4B). ABO-2-4A (12.9 g) was subjected to a silicagel column (4.5×55.0 ㎝) and washed with $CHCl_3$-MeOH (10 : 1, v/v) to yield 120 fractions (100 mL each). These fractions were divided into 5 portions (ABO-2-4A-1~ABO-2-4A-5). ABO-2-4A-2 (683.3 ㎎) was purified to give a white powdered compound. : EI-MS *m/z* : 460 ($[M]^+$), 340, 312, 298 (base peak), 283, 255, 240, 227, 166, 132, 123, 89, 69, 60.; FAB-MS *m/z* : 461 ($[M+H]^+$), 460 ($[M]^+$) (Fig. 1).

General experimental procedures

NMR spectra were recorded in dimethylsulfoxide-d_6 using tetramethylsilane (TMS) as an internal standard. Chemical shifts was expressed in δ and coupling constants (*J*) in Hz. ^1H and ^{13}C NMR spectra were obtained with a Varian Unity-Inova 500 MHz, operating at 500 MHz (^1H) and 125 MHz (^{13}C). EIMS spectrum was determined on a JEOL JMS-SX102A operating at 70 eV. FABMS spectrum was determined on a JEOL 700.

Thin layer chromatography (TLC) was carried out on precoated silica gel 60 F_{254} (0.2 ㎜, Merck) plates. TLC plates were developed with solvent system A (toluene-ethyl formate-formic acid = 5 : 4 : 1, v/v/v) and B (acetone-ethyl acetate-H_2O = 10 : 10 : 1, v/v/v). TLC were visualized under UV light at 254 ㎚ and 365 ㎚.

Silica gel 60 (40~100 ㎛, Kanto Chemical Co.) and Sepha-

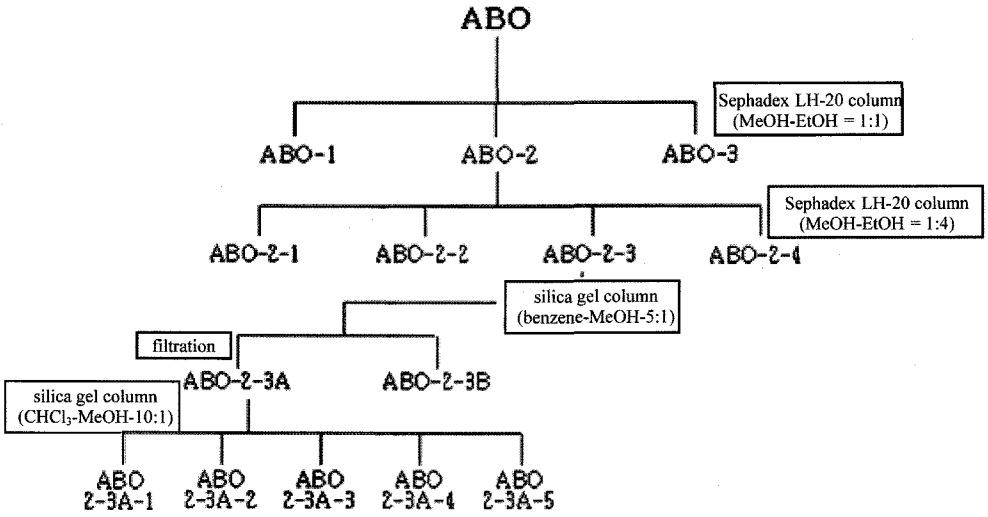

Fig. 1. Isolation scheme for AB-2; 4', 6-dimethoxyisoflavone-7-O-β-D-glucopyranoside.

dex LH-20 (Amersham Pharmacia Biotech AB) were used for the column chromatography

Cytotoxicity measurement

SRB (sulforhodamine B) assay (Doll and Peto, 1981, Kim *et al.*, 2005) to measure either cellular proliferation or cytotoxicity was performed by staining protein using the human lung cell line, HEL299 (Lung normal). After 100 μL of this cell line at a concentration of 4~5×10^4 cells/mL was placed in each 96 well plate and cultured for 24 h (37 ℃, 5% CO_2), 100 μL of each sample at 0.2, 0.4, 0.6, 0.8, and 1.0 g/L was added into each well and cultured for 48 h. After incubating, the supernatant was removed. Then, cold 100 μL of 10% (w/v) TCA (Trichloroacetic acid) was added into the well, which was left at 4 for 1 h. It was washed in distilled water 4 to 5 times. After removing TCA, the well was dried at room temperature. Then, 100 μL of 0.4% (w/v) SRB solution dissolved in 1% (v/v) acetic acid was placed into the well to perform SRB staining at room temperature for 30 min. Not binding SRB stain was removed by washing it with 1% acetic acid for 4 to 5 times and the well was dried. The staining was removed by adding 100 μL of 10mM Tris buffer. The absorbance was read at 540 ㎚ using a microplate reader (Molecular Devices, THERMO max, USA).

Measurement of humane T cell (Jurkat) growth and secretion of cytokines

Immuno-regulatory activities of the samples were examined using the human immune cells, T cell (Jurkat; ATCC, TIB-152). Cells were cultured in RPMI 1640 medium containing 10% FBS in 5% CO_2 at 37 ℃. Cell growth enhancement was determined by measuring the number of cells in the 24 well plate containing 1.0×10^4 cells/mL using a hemacytometer (Lee *et al.*, 2002, Mun *et al.*, 2004) in adding 0.5 g/L of each sample. Secretion of cytokines were quantified by measuring the amounts of IL-6 and TNF-α using IL-6 and TNF-α kits from Chemicon (USA). After adjusting the cell concentration at 1~2 ×10^4 cells/mL, 900 μL of this cell concentrate was placed into 24 well plates and cultured for 24 h (37 ℃, 5% CO_2). Then, 100 μL of 0.5 g/L cell was cultured again (37 ℃, 5% CO_2). The sample was centrifuged to obtain the supernatant, which was used to read the absorbance at 450 ㎚ using a microplate reader. The amounts of cytokines were measured using the O.D values of the standards (Han *et al.*, 1998, Park *et al.*, 2004).

Enhancement of Natural Killer (NK) cell growth

The NK-92MI cell line (ATCC, CRL-2408) was diluted to 2×10^7 cells/mL using 2 mM L-glutamine, 0.2 mM myoinositol, 20 mM folic acid, 10 M 2-mercaptoethanol, 12.5% fetal bovine serum (FBS) and 12.5% horse serum (Myelocult) in α-MEM medium. While culturing the human T cells in T-25 Flask, the degree of proliferation was observed after placing each sample at a concentration of 0.5 g/L. It was subcultured 3 to 4 times and centrifuged to obtain the supernatant. After 900 μL of the NK-92MI cell line was aliquoted into 24 well plates at 4~5×10^4 cells/mL, 100 μL of the supernatant from T cells was placed into the well and cultured for 48 h. Then, the degree of NK-92MI cell activation was observed for 6 days using a cell counter (Yueran *et al.*, 2003; Limdbolum, 2002).

Statistical analysis

SAS (Statistical Analysis system) PC package(SAS Institute; Cary, NC, USA) was used for statistical analysis. All measurements were expressed in mean ± SD (Norman and Smith, 1981).

Results and Discussion

Extraction and Isolation

The air-dried and powdered bark of *A. fruticosa* was extracted three times with MeOH at room temperature for 3 days each.

The structure of a compund was deduced from analysis of the ^1H and ^{13}C NMR data (Table 1) aided with 2D NMR measurements (^1H-^1H COSY, NOESY, TOCSY, HMQC, and HMBC).

The ^1H NMR spectrum of a compound showed signals characteristic of the isoflavone moieties. A 1H signal at δ 8.23 (1H, *s*, H-2) was assigned to H-2. Two 1H signals at δ 7.35 (1H, *s*, H-8) and 7.64 (1H, *s*, H-5) represented the aromatic H-8 and H-5. Two 2H signals at δ 7.00 (2H, *dd* J=2.0, 7.0 Hz, H-3'/H-5') and 7.49 (2H, *dd*, J=2.0, 7.0 Hz, H-2'/H-6') were assigned to *ortho* and *meta* coupled aromatic H-3'/H-5' and H-2'/H-6'. Two 3H signals were found at δ 3.84 (3H, *s*, OMe-4') and δ 3.96 (1H, *s*, OMe-6).

The ^{13}C NMR spectrum of the compound showed resonances for all twenty-three carbons in adding two methoxyl, twelve primary, one secondary, and eight quaternary carbons. The signal at δ 177.69 (*s*, C-4) was assigned to the carbonyl carbon.

Direct one-bond $^1H/^{13}C$ connectivities of the compound were established by HMQC spectrum which showed that H-2 was connected with δ 154.99 (C-2, *d*). Similarly, H-8 and H-5 showed connectivity with δ 105.38 (C-8, *d*) and 106.23 (C-5, *d*), respecterively.

The long-range heteronuclear interactions of the compound were established by HMBC spectrum which showed that OMe-4' and OMe-6 were connected with δ 161.19 (C-4', *s*)

Table 1. NMR data for compound 1 in dimethylsulfoxide-d_6.

position	δ_H (ppm)	δ_C (ppm)
2	8.23 (1H, *s*)	154.99 *d*
3		125.53 *s*
4		177.69 *s*
5	7.64 (1H, *s*)	106.23 *d*
6		149.64 *s*
7		153.65 *s*
8	7.35 (1H, *s*)	105.38 *d*
9		153.65 *d*
10		119.82 *s*
1'		125.45 *s*
2'	7.49 (1H, *d*, 9.0)	131.39 *d*
3'	7.00 (1H, *d*, 9.0)	114.90 *d*
4'		161.19 *s*
5'	7.00 (1H, *d*, 9.0)	114.90 *d*
6'	7.49 (1H, *d*, 9.0)	131.39 *d*
1"	5.14 (1H, *d*, 7.5)	102.00 *d*
2"	3.55 (1H, *m*)	74.67 *d*
3"	3.55 (1H, *m*)	77.87 *d*
4"	3.43 (1H, *m*)	71.26 *d*
5"	3.55 (1H, *m*)	78.53 *d*
6"	3.72 (1H, *m*), 3.93 (1H, *m*)	64.76 *t*
OMe		
6	3.96 (1H, *s*)	56.84 *q*
4'	3.84 (3H, *s*)	55.77 *q*

Coupling constants (J in Hz) are in parentheses.

and 149.64 (C-6, *s*). An anomeric proton at δ 5.16 (1H, *d*, J = 7.5 Hz, H-1") was assigned to *β*-D-glucopyranosyl unit. The glucopyranosyl anomeric proton signal showed HMBC correlation with C-7 (δ 153.65) in the isoflavone aglycone, confirming that the *O*-glucopyranose moiety is 1"→7 attached to the aglycone.

As a result, compound **1** was characterized as 4', 6-dimethoxyisofalvone-7-*O*-*β*-D-glucopyranoside (Kajiyama *et al.*, 1993) (Fig. 2).

Cytotoxicity

Cytotoxicity of human normal lung cell line, HEL299 were increased in a dose-dependent manner by the bark crude extract and a single compound; however, their cytotoxicity was relatively as low as about 16%. At the maximum concentration of 1.0 g/L, anticancer effect was shown, respectively. It is a lower cytotoxicity (15%) of 16% and 16.6% them that was

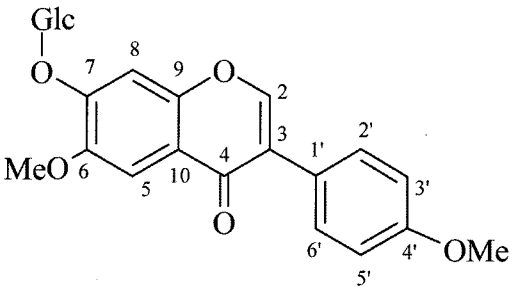

Fig. 2. Chemical structure of AB-2 (4', 6-dimethoxyisoflavone-7-*O*-β-D-glucopyranoside).

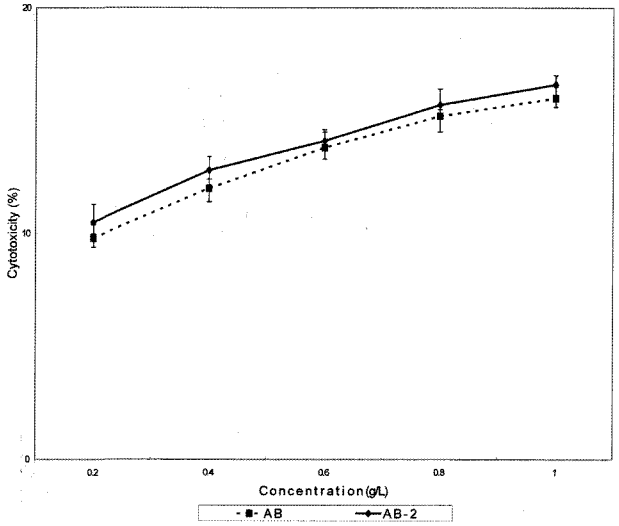

Fig. 3. Cytotoxicity of partially purified extracts (AB : *A. fruticosa* barks) and 4',6-dimethoxyisoflavone-7-*O*-β-D-glucopyranoside (AB-2) on normal cell line, HEL299.

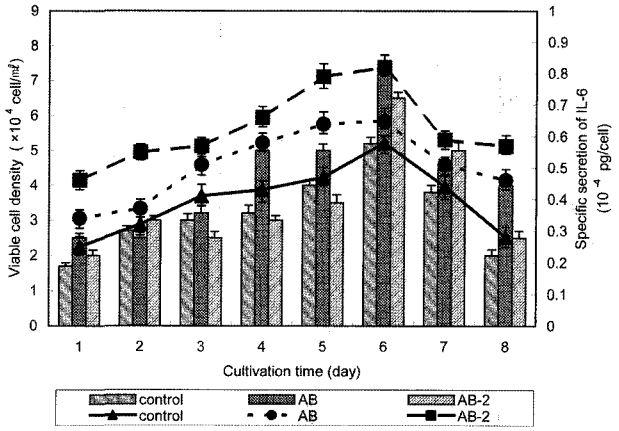

Fig. 4. The cell growth and specific secretion of IL-6 from human T cell in adding *Amorpha fruticosa* L. extracts (AB : crude extract of *A. fruticosa* barks, AB-2 : 4', 6-dimethoxyisoflavone-7-O-β-D-glucopyranoside).

shown by *Rubus schizostylus* fruit (Lee *et al.*, 2003) (Fig. 3).

Effect on human immune T cell (Jurkat) growth and secretion of cytokines

The counter of T cells (Jurkat) grown in bark crude extract treated sample was 7.5×10^4 cells/mL, which was about 30% higher than control 5.2×10^4 cells/mL. In the case of purified single compound, cell growth was 6.5×10^4 cells/mL. The amount of IL-6 released was 0.65×10^{-4} pg/cell and 0.82×10^{-4} pg/cell in the bark crude extract and purified single compound treated sample, respectively. These were higher amounts than that of control (0.58×10^{-4} pg/cell) (Fig. 4). The amount of TNF-α released was 0.84×10^{-4} pg/cell and 1.08×10^{-4} pg/cell in the bark crude extract and purified single compound treated sample, respectively (Fig. 5).

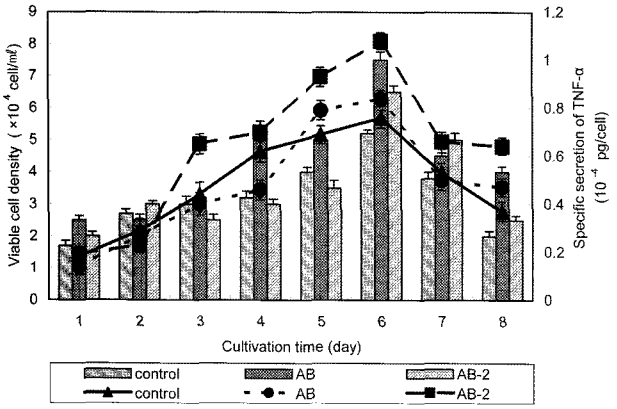

Fig. 5. The cell growth and specific secretion of TNF-α from human T cell in adding *A. fruticosa* L. extracts (AB : crude extract of *A. fruticosa* L. barks, AB-2 : 4', 6-dimethoxyisoflavone-7-O-β-D-glucopyranoside).

Effect on Natural Killer-92MI (NK-92MI) cell growth

It has been found that the cytokines released from human T cells could affect NK-92MI cell growth promotion (Yueran *et al.*, 2003; Limdbolum, 2002). It was clearly shown that NK cell growth was promoted with a high amount of cytokine released by the treatment of the bark crude extract and purifyied single compound, compared with control. Cell growth and the amount of IL-6 reached their maximum at 5 days after on set of incubation by the crude extract or a purified single compound. Both crude extract and a purified single compound showed high growth-promotion effect compared with control. The amount of cytokines released and NK cell growth were drastically increased in crude extract and purified single

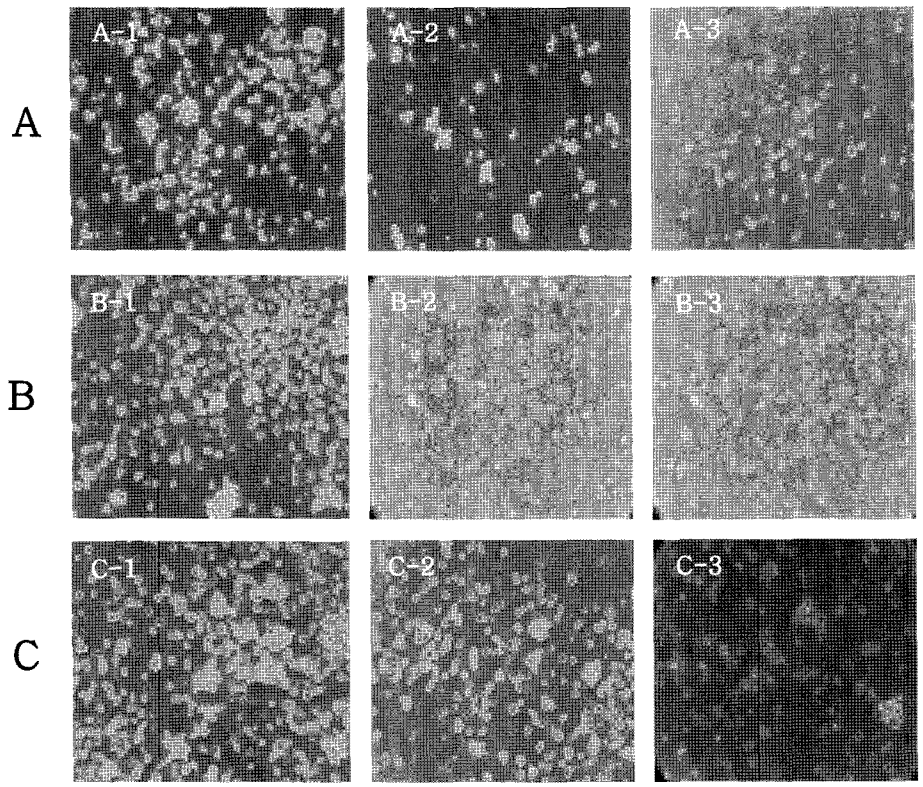

Fig. 6. Comparison of NK cell growth in adding the supernatant of human T cells after addition of the extracts (A : Control, B : crude extract of *A. fruticosa* barks, C : 4', 6-dimethoxyisoflavone-7-O-β-D-glucopyranoside).

Table 2. The induction of IL-6 and TNF-a from human T cell growth and NK cell by *Amorpha fruticosa* L.

Sample	Cultivation time (DAY)	Cytokine concentration from T cell (10^{-4} pg/cell)		Viable cell density of T cell ($\times10^4$ cells/mℓ)	Viable cell density of NK cell ($\times10^4$ cells/mℓ)
		IL-6	TNF-α		
Control	1	0.25 ± 0.0001	0.18 ± 0.0001	1.7 ± 0.001	5.5 ± 0.003
	2	0.32 ± 0.0001	0.29 ± 0.0001	2.7 ± 0.002	7.0 ± 0.005
	3	0.41 ± 0.0002	0.44 ± 0.0002	3.0 ± 0.002	7.5 ± 0.005
	4	0.43 ± 0.0002	0.62 ± 0.0003	3.2 ± 0.002	8.0 ± 0.006
	5	0.47 ± 0.0002	0.69 ± 0.0003	4.0 ± 0.003	9.5 ± 0.007
	6	0.58 ± 0.0003	0.76 ± 0.0003	5.2 ± 0.004	11.0 ± 0.007
AB*	1	0.34 ± 0.0001	0.14 ± 0.0001	2.5 ± 0.002	6.5 ± 0.004
	2	0.37 ± 0.0001	0.27 ± 0.0001	2.5 ± 0.002	9.0 ± 0.005
	3	0.51 ± 0.0002	0.4 ± 0.0002	3.2 ± 0.002	10.0 ± 0.006
	4	0.58 ± 0.0002	0.46 ± 0.0002	5.0 ± 0.003	11.0 ± 0.006
	5	0.64 ± 0.0003	0.79 ± 0.0003	5.0 ± 0.003	13.0 ± 0.007
	6	0.65 ± 0.0003	0.84 ± 0.0003	7.5 ± 0.004	14.2 ± 0.008
AB-2**	1	0.46 ± 0.0001	0.19 ± 0.0001	2.0 ± 0.002	7.0 ± 0.005
	2	0.55 ± 0.0002	0.23 ± 0.0001	3.0 ± 0.003	10.5 ± 0.007
	3	0.57 ± 0.0002	0.65 ± 0.0002	2.5 ± 0.002	11.5 ± 0.007
	4	0.66 ± 0.0002	0.7 ± 0.0002	3.0 ± 0.003	13.0 ± 0.008
	5	0.79 ± 0.0003	0.93 ± 0.0003	3.5 ± 0.003	14.0 ± 0.008
	6	0.82 ± 0.0003	1.08 ± 0.0004	6.5 ± 0.004	15.0 ± 0.008

AB* : crude extract of *Amorpha fruticosa* L. barks, AB-2** : 4', 6-dimethoxyisoflavone-7-O-β-D-glucopyranoside isolated from *A. fruticosa* barks.

compound-treated cells. The T cell growth stimulation was higher than a single compound treated group, by crude extract probably due to the crude extract showing synergic effects by other components. The crude extract from bark and a single compound stimulated T cell growth (Fig. 6). By 6 days of incubation, T cell growth, TNF-α secretion and NK cell growth were enhanced. The amount of TNF-α released by purified single compound was higher than that released by crude extract. NK-92MI cell growth was much more significant, showing the effect of cytokines, which is the products of immune cells, promoting NK cell growth (Table 2).

Discussion

Isoflavones are a diverse group of plant natural products synthesized from phenylpropanoid which play important roles in plant growth and development, and in defense against microorganisms and pests. These compounds often prossess antioxidant and estrogenic anti-cancer activity, and are also receiving considerable attension as health-promoting nutraceuticals. In this study, immuno-regulatory activities of the isoflavone in *A. fruticosa* was measured to find diffent biological activities of isoflavone which was not ger investigated.

In order to investigate immuno-regulatory activities of the crude extract of *Amorpha fruticosa* L. and a single compound isolated from, plant, their effects on Human T cell growth and the specific production of IL-6 and TNF-α were measured. It was found that human T cell growth were stimulated by adding the crude extract and this effect was higher than that of a single compound treated group. The secretion of both cytokines, IL-6 and TNF-α were also stimulated by these samples. Unlike cell growth, the secretion of cytokines was higher in an single compound treated group. Both of the crude extract and an single compound of *A. fruticosa* showed immune activation activities similar to those in *Rubus schizostylus* fruit (Lee *et al.*, 2003) and *Dendropanax morbifera* leaf (Lee *et al.*, 2002). Immune activities of both the crude extract and a single compound were similar, suggesting that a single compound was one of the promising immuno-regulatory agent containced in *Amorpha fruticosa* L.

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