Effect of new rotenoid glycoside from the fruits of *Amorpha fruticosa* LINNE on the growth of human immune cells

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Abstract A new compound, rotenoid isoflavone glycoside named, 6'-O- β -D-glucopyranosyl-12a-hydroxydalpanol was isolated from the methanolic (MeOH) fruit extract of *Amorpha fruticosa* LINNE by means of multi-stage column chromatography. Immuno-modulatory activities of this new glycoside were compared with the partitioned fractions of *Amorpha fruticosa* LINNE. Both of the fractions and purified single compound showed a 19% relatively low cytotoxicity at a maximum concentration of 1.0 g/L in a cultivated normal human lung cell line (HEL299). The purified single compound showed less cytotoxicity than the crude extracts, possibly because residual toxicants were eliminated during purification processes. Cell growth of human T cells was increased by about 15% by adding 0.5 g/L of the fractions compared to the control.

Specific production rates of interleukin-6 (IL-6) and tumor necrosis factor (TNF- α) from T cell were higher as 1.16×10^{-4} and 1.86×10^{-4} pg/cell, respectively, in the purified compound, compared to 1.38×10^{-4} and 2.22×10^{-4} pg/cell, respectively, by adding 0.5 g/L of the dichloromethane fraction. Natural killer cell-92MI (NK-92MI) growth supplemented with the supernatant of human T cell was up to 19% higher with the dichloromethane fraction compared with a new single compound at a concentration of 0.5 g/L. Overall, the dichloromethane fraction showed relatively higher immuno-modulatory activities compared with a new single compound, probably due to the synergic effect given by other substances existing in the fractions.

Keywords *Amorpha fruticosa* L. · 6'-O- β -D-glucopyranosyl-12a-hydroxydalpanol · Immuno-modulatory activity · Cytokines · NK-92MI cell

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Abbreviation

NK cell Natural killer cell

Introduction

As a deciduous shrub, *Amorpha fruticosa* LINNE was introduced into Korea around the 1930s and planted throughout Korea for erosion control and to restore wasteland. It is strong, environmentally tolerant and grows rapidly, so it can be planted

anywhere, being cold hardy and resistant to dryness (Huh et al. 1997). A rotenoid having the dihydrofuran ring with the isoprenoid side chain attached was isolated from *Amorpha fruticosa* L. for the first time (Crombie and Peace 1963; Crombie et al. 1964). Methanol crude extract of *Amorpha fruticosa* L. seed was fractionated using dichloromethane to produce a rotenoid, 6a,12a-dihydro- α -toxicarol (Reisch et al. 1976). The seed of *Amorpha fruticosa* L. 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 amorphiquinone were purified from the methanol crude extract of *Amorpha fruticosa* L. root (Shibata and Shimizu 1978). Compounds such as cannabinoid were isolated from *Amorpha* (Kemal et al. 1979). Amorinin having the flavanone structure was isolated from its bark (Rozsa et al. 1982). Amoradin, amoradin and amoradinin were also purified and their structures have been identified (Rozsa et al. 1984). It was reported that *Amorpha fruticosa* L. root contains the prenylated flavanone, isoamoritin (Ohyama et al. 1998). Three different isoflavones and five different rotenoids were purified from *Amorpha fruticosa* L. (Li et al. 1993). One flavonoid glycoside, one ester compound and two rotenoid compounds were purified from its fruit (Lee et al. 2003).

Despite the fact it contains so many useful substances, *Amorpha fruticosa* L. is mainly used for erosion control and to restore wasteland. No study has been performed on its immune activity and no comparative study has been done on the immuno-activity of fractions from *Amorpha fruticosa* L. fruits. This study investigates immunity-boosting effect of a newly purified rotenoid glycoside purified from its fruits extract by observing the growth of human immune cell lines as well as the secretion of cytokines from them. It is known that there have been difficulties in growing human natural killer (NK) cells in vitro and they could not be cultivated alone without adding several kinds of cytokines (Toomey et al. 2003). It could be interesting if the plant extracts could induce the secretion level of cytokines from B or T cells, and it could result in improving the growth of NK cells. The enhancement of the growth of human

NK cells was also examined to clearly understand immuno-modulatory effect of the compound originating from the plant, supplementing the supernatant from the culture medium of human B and T cells. It could provide useful information for utilizing the plants for the culture of mammalian cells and to evaluate the possibility of using this compound as an immunity-boosting agent.

Materials and methods

Materials

Different parts of 10–15-year-old *Amorpha fruticosa* L. were harvested from roadside of the Namhae express way in 2003. The media needed for cell culture, i.e., RPMI 1640 and alpha minimum essential medium (α -MEM) were purchased from GIBCO (USA). Hepes buffer was purchased from SIGMA (USA). Fetal bovine serum (FBS) and horse serum were purchased from GIBCO (USA). Gentamycin sulfate was purchased from Sigma. Sulforhodamine B (SRB), needed for staining, was purchased from Sigma.

Extraction and purification

The air-dried and powdered fruits of *A. fruticosa* was extracted 3 times with MeOH at room temperature for 3 days each. The combined MeOH extracts were concentrated under vacuum at 40 °C. The concentrated MeOH extract was dissolved in water and successively partitioned with *n*-hexane, dichloromethane, (CH_2Cl_2) and ethyl acetate (EtOAc).

The CH_2Cl_2 -soluble (30.0 g) was first separated on the Sephadex LH-20 column using MeOH/EtOH (1:1, v/v) as an eluent to yield 85 fractions (50.0 mL each). On the basis of TLC profiles, Fractions 39–48 were combined and dried to give a crude mass of 6.0 g. This was further chromatographed over the silica gel column using benzene/MeOH (5:1, v/v) as an eluent to yield 120 fractions (15.0 mL each), which were divided into three fractions, Fractions 11–19 (1.7 g), 35–64 (364.0 mg), and 100–120 (1.9 g). These fractions were separately purified to give four pure

compounds. Fractions 35–64 (364.0 mg) was chromatographed over the silica gel column using $\text{CHCl}_3/\text{MeOH}$ (15:1, v/v) as an eluent to collect 200 fractions (7.0 g each) by a fraction collector. On the basis of TLC profiles, fractions 110–140 were pooled together and further purified by preparative TLC in the solvent system of $\text{CHCl}_3/\text{MeOH}$ (3:1, v/v) to yield a pure new compound (40.3 mg). The CH_2Cl_2 soluble fractions were chromatographed on the silica gel and Sephadex LH-20 column, which resulted in the isolation of one new rotenoid glycoside. The chemical structures of compounds were elucidated by using their spectral (^1H -NMR, ^{13}C -NMR, DEPT, COSY, NOESY, HMBC, HMQC, FAB-MS, EIMS, HR-EIMS, IR, and UV) and chemical data. Among these fractions, three samples were selected for the comparison of immuno-modulatory effects such as the crude MeOH extracts (crude), dichloromethane fraction from the partition of the soluble CH_2Cl_2 fraction of *A. fruticosa* (DMF) and a new rotenoid glycoside (ARF).

Measurement of cytotoxicity on normal human cell line

A SRB (sulforhodamine B) assay (Doll and Peto 1981) 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 $4\text{--}5 \times 10^4$ cells/mL was placed in each 96 well plate and cultured for 24 h (37 °C, 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 culturing, the supernatant was removed. Then, a cold 100 μL of 10% (w/v) trichloroacetic acid (TCA) was added into the well, which was left at 4 for 1 h. It was washed in distilled water 4–5 times. After removing the 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. Non-binding SRB stain was removed by washing with 1% acetic acid for 4–5 times and the well was dried. The staining was removed by adding 100 μL of 10 mM Tris buffer. The absorbance was read at 540 nm using a

microplate reader (Molecular Devices, THERMO max, USA).

Measurement of human T cell growth and secretion of cytokines

Immuno-modulatory activities of the samples were examined using the normal human immune cells, T cells (H9; ECACC, 85050301). Cells were cultured in RPMI 1640 medium containing 10% FBS in 5% CO_2 at 37 °C. 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) by adding 0.5 g/L of each sample. Secretion of cytokines was quantified by measuring the amounts of Interleukin-6 (IL-6) and Tumor Necrosis Factor- α (TNF- α) using IL-6 and TNF- α kits from Chemicon(USA). After adjusting the cell concentration at $1\text{--}2 \times 10^4$ cells/mL, 900 μL of this cell concentrate was placed into 24 well plates and cultured for 24 h (37 °C, 5% CO_2). Then, 100 μL of 0.5 g/L cell was cultured again (37 °C, 5% CO_2). The sample was centrifuged to obtain the supernatant, which was used to read the absorbance at 450 nm using a microplate reader. The amounts of cytokines were measured using the O.D. values of the standards (Han et al. 1998).

Enhancement of human 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, 0.1 mM 2-mercaptoethanol, 12.5% 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 3–4 times and centrifuged to obtain the supernatant. After 900 μL of the NK-92MI cell line was aliquoted into 24 well plates at $4\text{--}5 \times 10^4$ cells/mL, 10–100 μL of the supernatant from the T cells was then placed into the well and cultured for 48 h. The growth of NK-92MI cell was then 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 $\pm$ SD (Norman and Smith 1981).

Results

The chemical structure of a new compound isolated from *Amorpha fruticosa* L.

The new compound was isolated as a yellow solid. The UV λ_{max} (log ϵ) of the new compound appeared at 296 nm (4.23). The IR spectrum showed a characteristic absorption for the conjugated carbonyl (1610 cm^{-1}) and hydroxyl group (3421 cm^{-1}). The EI-MS spectrum of the new compound showed a molecular ion peak at m/z 590 ($[M]^+$) along with other major daughter ion peaks at m/z 428, 408, 208, (base ion) and 165, suggesting $\text{C}_{29}\text{H}_{34}\text{O}_{13}$ for the molecular formula of new compound, which was finally confirmed by HR-EI-MS. The presence of a glucose moiety in the new compound was also determined by ion chromatography (Dionex AS50 series with a Dionex Carbpac PA 10 column) and co-TLC with an authentic sample of D-glucose (Aldrich) after the acid hydrolysis of the new compound. The purified aglycon part after acid hydrolysis of the new compound had similar spectral data as was previously reported for 12a-hydroxydalpanol. On the basis of all the spectral and chemical data, the structure of the new compound was established as 6'-O- β -D-glucopyranosyl-12a-hydroxydalpanol (Fig. 1). The ^1H - and ^{13}C -NMR data of the new compound are shown in Table 1.

Cytotoxicity of the extracts and the enhancement of human immune cell growth

Cytotoxicities of human normal lung cell lines, HEL299 were all increased in a dose-dependent manner for the fruits fractions and a purified rotenoid glycoside; however, their cytotoxicity was relatively low at about 19%. At the maximum concentration of 1.0 g/L, relatively low cell growth inhibition was observed at 19.0%,

Fig. 1 Chemical structure of a new compound (ARF: rotenoid glycoside isolated from *Amorpha fruticosa* L. fruits : 6'-O- β -D-glucopyranosyl-12a-hydroxydalpanol)

Table 1 ^1H -(500 MHz) and ^{13}C -NMR (125 MHz) data of new compound

Position	1 (MeOH- d_4)	
	δ_{H} (ppm)	δ_{C} (ppm)
1	6.71 (1H, s)	111.41 d
1a		108.69 s
2		143.76 s
3		151.65 s
4	6.52 (1H, s)	101.13 d
4a		149.29 s
6	4.48 (1H, m), 4.51 (1H, m)	64.88 t
6a	4.57 (1H, m)	76.48 d
7a		157.52 s
8		113.33 s
9		167.96 s
10	6.47 (1H, d, 8.5)	104.67 d
11	7.76 (1H, d, 8.5)	129.36 d
11a		113.97 s
12		191.76 s
12a		68.02 s
4'	3.13 (1H, m), 3.22 (1H, m)	27.16 t
5'	4.49 (1H, m)	90.76 d
6'		78.03 s
7'	1.38 (3H, s)	22.42 q
8'	1.25 (3H, s)	20.31 q
Gluc		
1''	4.50 (1H, d, 7.5)	97.63 d
2''	3.15 (1H, m)	73.97 d
3''	3.32 (1H, m)	77.01 d
4''	3.28 (1H, m)	70.56 d
5''	3.26 (1H, m)	76.58 d
6''	3.66 (1H, m), 3.86 (1H, m)	61.67 t
2	3.68 (3H, s)	55.99 q
3	3.78 (3H, s)	55.20 q

Fig. 2 Cytotoxicity of the fractions from *Amorpha fruticosa* L. fruits on normal cell line, HEL299 (Crude: the crude MeOH extract of *Amorpha fruticosa* L. fruits, DMF: dichloromethane fraction of *Amorpha fruticosa* L. fruits, ARF: newly isolated compound, rotenoid glycoside of *Amorpha fruticosa* L. fruits)

15.3%, and 14.3% for the crude, DMF and ARF, respectively. Its cytotoxicity was similar to 15% shown by the addition of the fruit extracts of *Rubus schizostylus* (Lee et al. 2003, Fig. 2). It was also found that the cytotoxicity of the crude extract on human cells was higher than those of purified samples, DMF and ARF. Even least cytotoxicity was measured by adding the finally purified single compound ARF, because the potent toxicants could be removed during purification processes. This interesting result has least been reported for natural chemical compounds, and it could provide promising a clinical application for a new compound. As shown in Fig. 3, human T cell (H9) growth was compared with

Fig. 3 Comparison of human T cell growth by adding 0.5 g/L of the fractions of *Amorpha fruticosa* L. fruits (Crude: the crude MeOH extract of *Amorpha fruticosa* L. fruits, DMF: dichloromethane fraction of *Amorpha fruticosa* L. fruits, ARF: newly isolated compound, rotenoid glycoside of *Amorpha fruticosa* L. fruits)

0.5 g/L supplementation of three different samples, and estimated as 6.2×10^4 cells/mL by adding DMF (the fruits dichloromethane fraction), which was about 26% higher than 4.6×10^4 cells/mL shown by the control, no addition of the samples. In the case of ARF (purified single compound), cell growth was 5.2×10^4 cells/mL, which was also higher than that shown by the control, but slightly lower than that by adding DMF. It could be due to the loss of active compound and/or less synergic effects than DMF because even potent toxicants were reduced during the separation process.

The secretion of cytokines, IL-6, and TNF- α from the growth of human T cells

It is also very important to know the level of secreted cytokines associated with the cell growth, not only improvements in cell growth. Figures 4 and 5 illustrate the secretion of both cytokines according to the human T cells shown in Fig. 3. The concentration of secreted cytokines from the cell growth should be expressed as a specific concentration secreted from each cell, not as total volume in a culture flask to clearly understand the immuno-modulatory effects of the samples. The amount of IL-6 released from the cell growth was measured as 1.38×10^{-4} and 1.16×10^{-4} pg/cell by adding 0.5 g/L of DMF (dichloromethane fraction) and ARF (purified single compound),

Fig. 4 Specific secretion of IL-6 from human T cells by adding 0.5 g/L of the fractions of *Amorpha fruticosa* L. fruits (Crude: the crude MeOH extract of *Amorpha fruticosa* L. fruits, DMF: dichloromethane fraction of *Amorpha fruticosa* L. fruits, ARF: newly isolated compound, rotenoid glycoside of *Amorpha fruticosa* L. fruits)

Fig. 5 Specific secretion of TNF- α from human T cells by adding 0.5 g/L of the fractions of *Amorpha fruticosa* L. fruits (Crude: the crude MeOH extract of *Amorpha fruticosa* L. fruits, DMF: dichloromethane fraction of *Amorpha fruticosa* L. fruits, ARF: newly isolated compound, rotenoid glycoside of *Amorpha fruticosa* L. fruits)

respectively. These amounts were definitely higher than 0.98×10^{-4} pg/cell for the case of the control, without adding any samples (Fig. 4). A similar trend was observed for TNF- α secretion. The amount of TNF- α released was estimated as 2.22×10^{-4} and 1.86×10^{-4} pg/cell in the case of adding DMF and ARF, respectively, which were also higher than the case of the control (Fig. 5). This result was well correlated to the growth of human T cell, implying that the samples can positively play an important role in immunomodulatory functions by improving both cell growth and cytokine secretion.

Enhancement of NK cells associated with human T cell growth

To better understand the complex immuno-modulatory effect, it is also necessary to know the role of the growth of T cell associated with NK cell growth. Figure 6 clearly showed that NK cell growth was promoted significantly, with a large amount of cytokine released by supplementing DMF or ARF, compared with the crude or the control (no addition). NK cell concentration was increased with the addition of amounts of the T cell supernatant grown with 0.5 g/L of DMF or ARF; however, the crude did not seem to greatly improve the NK cell growth. Interestingly enough, in Fig. 6, the NK cells showed a different growth pattern by adding the supernatant grown with DMF or ARF. The cell growth was not

Fig. 6 Comparison of human NK cell growth supplemented with the supernatant of T cell growth by adding the fractions of *Amorpha fruticosa* L. fruits according to the addition concentration (Crude: the crude MeOH extract of *Amorpha fruticosa* L. fruits, DMF: dichloromethane fraction of *Amorpha fruticosa* L. fruits, ARF: newly isolated compound, rotenoid glycoside of *Amorpha fruticosa* L. fruits)

continuously increased with the addition of amounts of the supernatant from T cell growth with DMF while NK cell was gradually improved with the increased addition of the supernatant. This might imply the purified glycoside could play a role in improving immune activity differently. This result can support the hypothesis that the cytokines released from human T cells affect NK-92MI cell growth promotion. It was also found that NK cells could not grow well without the growth factors secreted from human T cells. In Fig. 7, morphology of each NK cell was also compared after 6 days cultivation supplemented with the supernatant from the culture of human T cells grown with DMF and ARF. The best cell growth was observed by adding the partitioned fraction, DMF, followed by the case of adding a rotenoid glycoside, ARF. This result could also provide the information that cell growth and the amount of IL-6 were at maximum by 6 days of cultivation in both fractions and purified single compound. Both fractions and a purified rotenoid glycoside showed high growth promotion compared with the control. When the fractions and purified single compound were compared, the amount of cytokines released was greatly increased but the enhancement of NK cell growth was similar by adding DMF (dichloromethane fraction) and ARF (purified rotenoid glycoside),

Fig. 7 Morphology of NK cells supplemented with the supernatant from human T cells by adding 0.5 g/L of rotenoid glycoside and partitioned fraction of *Amorpha fruticosa* L., respectively. (A-control, no addition; B-DMF, dichloromethane fraction; C-ARF, rotenoid glycoside)

probably due to the dichloromethane fraction showing the synergic effects of other components. In the case of T cells, the dichloromethane fraction and a purified rotenoid glycoside showed high cell growth compared with control. By 6 days of cultivation, all the T cell growth, TNF- α secretion and NK cell growth were enhanced. The amount of TNF- α released was higher in the dichloromethane fraction and purified single compound compared with the control, and

accordingly, NK-92MI cell growth was much more significant, showing the effect of cytokines, which are the products of immune cells, promoting NK cell growth (Table 2).

To investigate the availability of a newly isolated compound from *Amorpha fruticosa* L. fruit, the immuno-modulatory activities of the fractions and a purified rotenoid glycoside were compared. Interestingly enough, human T cell growth was better by adding the dichloromethane

Table 2 The specific secretion of IL-6 and TNF- α from human T cell growth and NK cell growth by adding the fractions (0.5 g/L) from fruits of *Amorpha fruticosa* L

Sample	Cultivation time (day)	Cytokine concentration from T cell (10^{-4} pg/cell)		Viable cell density of T cell ($\times 10^4$ cells/mL)	Viable cell density of NK cell ($\times 10^4$ cells/mL)
		IL-6	TNF- α		
Control	3	0.61 ± 0.0002	0.91 ± 0.0002	2.8 ± 0.002	6.9 ± 0.005
	4	0.68 ± 0.0002	1.03 ± 0.0003	3.2 ± 0.002	7.6 ± 0.006
	5	0.81 ± 0.0002	1.21 ± 0.0003	3.8 ± 0.003	9.2 ± 0.007
	6	0.98 ± 0.0003	1.54 ± 0.0003	4.6 ± 0.004	10.8 ± 0.007
DMF ^a	3	0.74 ± 0.0002	1.12 ± 0.0002	3.3 ± 0.003	7.6 ± 0.005
	4	0.93 ± 0.0003	1.48 ± 0.0002	4.2 ± 0.003	9 ± 0.006
	5	1.06 ± 0.0003	1.61 ± 0.0003	4.7 ± 0.004	10.2 ± 0.007
	6	1.31 ± 0.0004	2.02 ± 0.0004	5.8 ± 0.004	12.9 ± 0.008
ARF ^b	3	0.65 ± 0.0002	1.01 ± 0.0002	3 ± 0.002	7.5 ± 0.007
	4	0.8 ± 0.0002	1.21 ± 0.0002	3.6 ± 0.003	8.3 ± 0.008
	5	0.93 ± 0.0003	1.42 ± 0.0003	4.3 ± 0.003	9.9 ± 0.008
	6	1.16 ± 0.0003	1.86 ± 0.0004	5.3 ± 0.004	11.6 ± 0.008

^a DMF, dichloromethane fraction of *Amorpha fruticosa* L. fruits

^b ARF, newly isolated compound, rotenoid glycoside of *Amorpha fruticosa* L. fruits

fraction than in a purified rotenoid glycoside. The secretion of both cytokines, IL-6, and TNF- α was also higher in these samples, compared to the control. It could result in enhanced NK cell growth in which the amount of cytokines released is higher by adding a dichloromethane fraction. Based on these results, it could be said that both of the fractions and a rotenoid glycoside of *Amorpha fruticosa* L. have immune activation activities similar to those in *Rubus schizostylus* fruit (Lee et al. 2003) and *Dendropanax morbifera* leaf (Lee et al. 2002). However, generally speaking, the immune activation activities of the fractions seem to be better than those of a purified rotenoid glycoside. This could possibly be due to the synergetic effects of unknown compounds in the partitioned fractions. Among several fractions, dichloromethane fractions showed relatively higher activities, which could imply that this fraction contains more biologically active compounds than others. One could presume that this fraction and a new rotenoid glycoside have high potential to be developed for the use of immune activation activities.

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