Arch Pharm Res Vol 23, No 6, 592-598, 2000

Medicinal Chemistry & Natural Products

Comparative Study of Korean (*Viscum album* var. *coloratum*) and European Mistletoes (*Viscum album*)

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(Received September 14, 2000)

A lectin (agglutinin, VCA) from Korean mistletoe (*Viscum album* L. *coloratum*) was isolated by affinity chromatograpy on a asialofetuin-Sepharose 4B. The molecular weights of A- and B-chains of VCA were differenf from those of VAAS. The VCA recognized the antibody of VAAs in the Western blot analysis and ELLA system. We also investigated the synergistic effects of the components in mistletoe by dividing the extract into different molecular weight fractions.

Key words: Korean mistletoe, Lectin, Asialofetuin-Sepharose 4B, Western-blot, ELLA, Synergistic effect

INTRODUCTION

The extracts from white berry European mistletoe (*Viscum album* L.) have been widely used as unconventional cancer therapy (Büssing *et al.*, 1995, 1996, 1998). The clinical studies with mistletoe therapy have shown longer survival times, better quality of life or tumor regression. Recent scientific researches have confirmed that mistletoe induced apoptotic killing of cultured tumor cells and lymphocytes, stimulated the immune system (Hajto *et al.*, 1990, Schultze *et al.*, 1991, Müller *et al.*, 1990).

Among several components in mistletoe, it is considered that the cytotoxic and immunological properties of preparation are related to lectins. The European mistletoe lectins (*Viscum album* L. agglutinin, VAA-I, II, III) are D-galactose- and/or *N*-acetyl-D-galactosamine-specific (Franz *et al.*, 1986, Goldstein *et al.*, 1986), and have molecular weights between 55 and 63 kDa. The three VAAs have same N-terminal amino acid sequence (Dietrich *et al.*, 1992a, 1992b). The cloning of VAAs has recently revealed the presence of one gene only, and they can be distinguished from each other by the degree of glycosylation that is dependent on post-translational processing in the plant (Eck *et al.*, 1999a, 1999b). The VAAs are type-2 ribosome-inactivating proteins (RIPs) composed of two different subunits, A- and B-chain linked by a disulfide bridge. The A-chain is capable of inactivating the 60S ribosomal subunit of eukaryotic cells resulting in inhibition of protein synthesis. The B-chain, which binds galactose and saccharides containing non-reducing terminal -galactosyl units, is capable of binding to cell-surface glycoconjugates and thereby permits entry into the cell of the toxic subunit (Peumans *et al.*, 1996). Cell surface carbohydrate chains provide potential binding sites to endogeneous carohydrate binding proteins (Taylor-Papadimitriou *et al.*, 1994, Schumacher *et al.*, 1994, Goldstein *et al.*, 1986). The glycosylation of tumor cells can be different from that of the normal cells, since the changes in carbohydrate expression on tumor cells can alter cell adhesion. Thus, the cell-surface glycoconjugates can be suitable targets for therapeutic exploitation because the toxicity of VAAs can be specific for cancer cells (Schumacher *et al.*, 1995, Taylor-Papadimitriou *et al.*, 1994).

The yellow berry Korean mistletoe (*Viscum album* L. var. *coloratum*), a subspecies of European mistletoes, has been used traditionally as medicinal herbs. While the European mistletoe has been studied intensively, we know less about Korean mistletoe as therapeutic herbs, especially as a suggested anticancer drug. We isolated a cytotoxic lectin (*Viscum album* L. var. *coloratum* agglutinin, VCA) from Korean mistletoe. The VCA showed sugar specificity of D-galactose and N-acetylgalactosamine. The molecular weight of VCA (60 kDa) was similar to the molecular weight of VAA-II, but the molecular weights of A- and B-chains of VCA were different from those of VAAs (Park *et al.*, 1999a, 1997, 1998, 1999b). The N-terminal amino acid sequence of VCA has been reported

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to be different from those of VAAs (Yoon *et al.*, 1998, 1999).

It is obvious that mistletoe contains potent toxic protein that induces apoptosis, but the mechanisms of antitumor activity of other components remain to be clarified. Other components such as viscotoxins and polysaccharides have been also reported to be active components for the cancer treatment (Pfüller *et al.*, 1993, Ribereau-Gayon *et al.*, 1993, Park *et al.*, 1999). The experiments with extracts from Californian, European, and Korean mistletoe showed that Korean mistletoe was the most active in inhibiting the growth of leukemia cells as compared to the other extracts (Khwaja *et al.*, 1986). Similarly, alkaloidal fraction isolated from Korean mistletoe extract was the most active of all other fractions (Khwaja *et al.*, 1986). We reported recently that the methanolic extract of Korean mistletoe showed high activity, while the extract of European mistletoe showed lower cytotoxic activity against HL-60 cells (Park *et al.*, 1999a). Thus, we assumed that the low molecular weight components in the Korean mistletoe extract might play an important role for the cytotoxicity because the methanolic fraction does not contain lectin.

In this study, we report the biochemical properties of lectins and cytotoxicity of fractions with different molecular weight components of Korean and European mistletoes. We also investigated the synergistic effects of the components in mistletoe by dividing the extract into different molecular weight fractions.

MATERIALS AND METHODS

Purification of lectin

Viscum album L. var. *coloratum* growing on oak tree was collected in winter in Kangwon province, Korea. The botanical identity was established by Prof. Jong Suk Lee, College of Natural Sciences, Seoul Women's University. *Viscum album* L. growing on oak tree was collected in winter by Dr. Grazi of the Institute Hiscia, Arlesheim, Switzerland and delivered to Korea. Leaves, berries and 1- to 4- year old stems of the plants were sorted and stored at -70°C until use. The crude protein solution was prepared by binding the protein on the cation exchanger as described previously (Park *et al.*, 1999a). Briefly, 3.5 g of SP Sephadex C-50 (Pharmacia, Upsala, Sweden) was added to one liter of aqueous solution extracted from 100 g of air dried mistletoe and stirred at 4°C. The gel was filled into a chromatography column and washed with 0.1 M acetate buffer (pH 4.0) and the protein was eluted with the buffer solution (0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl). The eluate was applied to the column filled with asialofetuin-Sepharose 4B, and the column was washed with PBS buffer (pH 8.7). The absorbed protein was eluted with 0.2 M acetic acid and concentrated by ultralfiltration (MW=10 kDa, Amicon Corp, Danvers, MA, USA). The protein content was determined by the method of Lowry *et al* (Lowry *et al.*, 1951) using BSA (bovine serum albumin) as a standard. The hemagglutination and sugar specificity of lectin were measured as described previously (Park *et al*, 1997, 1998). The purified VAA-I, II and III from European mistleote were kindly provided by Prof. Pfüller (Institut für Phytochemie, Universität Witten/Herdecke, Germany).

SDS-PAGE and Western blot analysis

The purity check and the molecular weight were determined by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) as described previously (Park *et al*, 1997, 1998, 1999b). For Western-blot analysis purified lectin was separated by 12.5% SDS-PAGE, and electroblotted onto a nitrocellulose membrane (Amersham) using 25 mM Tris, 192 mM glycine, 20% methanol buffer (pH 8.3). The membrane was blocked with PBS containing 5% skimmed milk, and then incubated with rabbit polyclonal anti-VAA antibody (KOMA Biotech, Korea) at a room temperature. After washing with PBS-Tween 20 (0.5%), the membrane was incubated with a rabbit anti-mouse IgG-HRP (immunoglobulin G-horse raddish peroxidase) Ab (antibody) (Biorad, California, USA). The bound antibodies were detected by ECL detection system (Amersham, Braunachweig, Germany).

ELLA and ELISA

For ELLA (enzyme linked lectin assay), asialofetuin-1 (100 μl/well of 0.1 mg asialofetuin-1/ml in PBS) in a Nunclon microtiter plate (Nunc Immuno Plate Maxisorb, Nunc. Rosklide, Denmark) was incubated overnight, and washed with PBS-T. Blocking solution (200 μl/well of 1% BSA in PBS) was added, incubated, and washed. Then lectins or extracts at varied concentrations (100 ml/well of antigen in PBS) were added and incubated. The plate was washed with PBS-Tween 20. Primary antibody (a rabbit polyclonal anti-VAA antibody) solution was added, incubated, and washed. Secondary antibody (rabbit-anti-mouse-IgG-conjugated peroxidase) solution (100 μl/well) was added, incubated, and washed. Each washing process above repeated three times. Hydroperoxidase substrate ABTS (2,2'-azinobis [3-ethylbenzotiazoline-6-sulfonic acid]-diammonium salt (100 μl/well) solution was added and incubated for 20 min at RT in dark. The development was stopped with 100 μl/well of 1% sodium dodecyl sulfate. Absorbance was measured at 405 nm. For sandwich ELISA (enzyme linked immunosorbant assay), monoclonal antibodies of VAA-I (MNA9-TA5b) and VAA, II, III (CH12-H 11b) were kindly provided by Prof. Pfüller (Institut für Phytochemie, Universität Witten/Herdecke, Germany). A solution of monoclonal antibody (10 μg/ml PBS) in a Nunclon microtiter was incubated overnight and washed with PBS-T. Blocking solution (200 μl/well of 1% BSA in PBS) was

added, incubated, and washed. Antigen solution (100 μl/well of lectin in PBS) was added and incubated. After incubation, plates were washed and 100 μl per well of biotinylated antibodies (0.5 μg/ml) was added. The plates were incubated, washed, and a streptoavidin-peroxidase conjugate diluted 1:3000 was added at 100 μl per well. Visualization was achieved with *o*-phenylene diamine (OPD) in citrate-phosphate buffer. After developing color (20 min), the reaction was stopped, and the absorbance at 492 nm was recorded. Each assay was done in triplicate

Preparation of water extract and ultrafiltration

Ten gram of plant was crushed with 100 ml of saline between two rollers going in opposite directions in a vegetable juice miller (Angel Life Co., Korea), separated by filtration through a cheese cloth, and centrifuged at 12,000 rpm for 30 min. The concentration (mg/ml) of water extract of fresh plant was expressed as the amount of the fresh plant to prepare 1 ml of solution. One houndred milliliter of water extract was filtered through 10 kDa cut-off membrane with nitrogen gassing, and the filtrate was refiltered through 1 kDa cut-off membrane (Diaflo, Amicon Co., Danvers, MA, USA) using Amicon Standard UF-Cell 8050 (Amicon Co., Danvers, MA, USA). To eliminate the lower molecular weight components of each step, the remainder of filtration was washed with saline by filtering twice and adjusted the volume of each fraction to 100 ml with saline. The volume of filtrate through 1 kDa cut-off membrane was also adjusted to 100 ml with saline.

Cell lines and *in vitro* cytotoxic assay

The Molt-4 cells (acute human T-lymphoblastic, KCLB 21582) were provided from the Korean cell line bank (Seoul, Korea). The cells were maintained in suspension in RPMI 1640 (Flow Laboratories, Irvine, UK), supplemented with 10% fetal bovine serum (FBS, Hyclone) and with penicillin-streptomycin (10,000 unit/ml, Gibco) in 5 % CO_2, 95% air, at 37°C. The *in vitro* cytotoxic effect was determined by MTT (dimethylthiazol tetrazolium bromide) assay. The cell suspension was aliquoted into the 96-well microtiter plates (100 μl of cell suspension containing 1×10^5 cells/ml in each well). One hundred microliter of sample solution diluted with culture medium were added to the cell suspension, and the plate was incubated with 5% CO_2 at 37°C for 48 h. At the end of incubation the MTT solution was added and the plate was incubated for further 4 h and was detected with an ELISA plate reader at 570 nm. The cytotoxicity was determined by IC_{50} values (inhibitory concentration values, i.e. drug concentration required to inhibit viability by 50%) and each assay was done in triplicate.

Statistical analysis

All data represented means ± standard error. A Student's t-test was used for measuring the significance of a difference of means between control and experimental samples.

RESULTS AND DISCUSSION

Isolation and identification of lectins

In general, carbohydrate ligands for lectin purification can be immobilized to supports activated in the form of glycoproteins or sugar moieties. However, recognition of glycoconjugates by lectins is a highly complex phenomenon and is also controlled by their own glycan part. Accordingly, the use of different affinity ligands for purification of lectins has shown that such changes can yield qualitative and quantitative differences (Lee *et al.*, 1992). Thus, rational

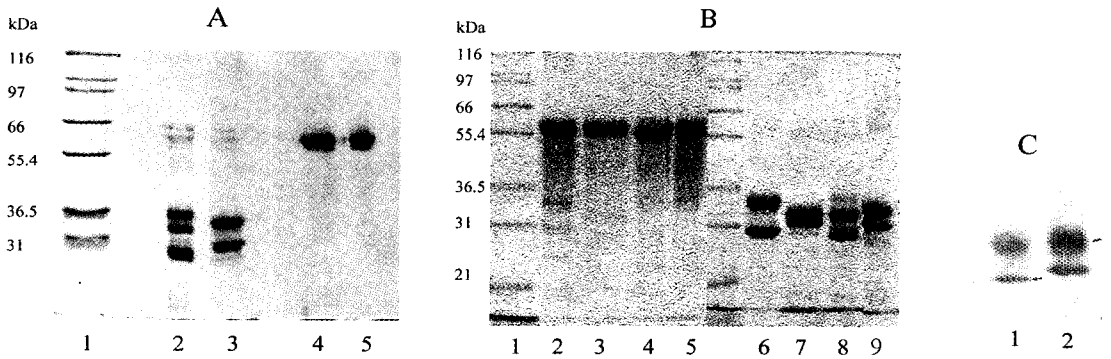

Fig. 1. SDS-PAGE (A, B) and Western blot (C) analysis of lectins in Korean and European mistletoes. (A) Lectins isolated by SP-50 Sephadex C-50 and asialofetuin-Sepharose 4B were separated by SDS-PAGE in the presence (lane 2, 3) and in the absence (lane 4, 5) of reducing agent. lane 1; molecular weight marker, lane 2, 4; VAAs, lane 3, 5; VCA. (B) Further purified VAA-I, II, III and the VCA isolated by asialofetuin-Sepharose 4B were separated by SDS-PAGE in the absence (lane 2~5) and in the presence (lane 6~9) of reducing agent. lane 1; molecular weight marker, lane 2 and 6; VAA-I, lane 3 and 7; VAA-II, lane 4 and 8; VAA-III, lane 5 and 9; VCA. (C) Western-blot analysis of purified lectin. Lectin was separated by 12.5% SDS-PAGE, electroblotted onto a nitrocellulose membrane, and probed with polyclonal rabbit anti-VAA antibody raised against VAA. The bound antibodies were detected ECL detection system. lane 1; VAA-I, lane 2; VCA isolated by asialofetuin-Sepharose 4B.

selection of the affinity ligands is a prerequisite for optimal lectin recovery. We reported the isolation of lectin from Korean mistletoe by acid-treated and lactose-Sepharose 4B, but the lectin was not isolated efficiently using those matrix (Park *et al.*, 1997, 1998). In this study, we prepared asialofetuin-Sepharose 4B for the isolation of lectin. The asialofetuin prepared by the removal of sialic acid from the fetuin is asialo-triantenary glycopeptide that contains three terminal galactose residues (Wu *et al.*, 1995). Lectin was more efficiently isolated from Korean mistletoe by asialofetuin-Sepharose 4B (120±25 μg from 1 g of mistletoe) than by former matrix used (10±5 μg from 1 g of mistletoe) (Park *et al.*, 1997, 1998). Less amount (55±12 μg) of lectin was isolated 4B from European mistletoe by asialofetuin-Sepharose 4B, indicating that the VCA may possess higher affinity to asialofetuin. Next, we investigated the patterns of lectins from Korean and European mistletoe by SDS-PAGE and Western blot analysis. The hololectins from both plants isolated by asialofetuin-Sepharose 4B showed identical patterns on the SDS-PAGE (Fig. 1, lane 4 and 5). In the presence of the reducing agent, however, the lectin from Korean mistletoe shows two bands (Fig. 1A, lane 3), whereas the lectins from European mistletoe show three bands. These patterns indicate that only one lectin is isolated from Korean mistletoe by asialofetuin-Sepharose 4B. On the other hand, different isolectins (VAAs) are isolated from European mistletoe (Fig. 1A, lane 2) by the same matrix, and the mixture of VAAs should be further purified by different steps (Franz *et al.*, 1995). Fig. 1B compares the VCA isolated by asialofetuin-Sepharose 4B with purified VAAs. The molecular weight of VCA (Fig. 1B, lane 5) was similar to the molecular weight of VAA-II (Fig. 1B, lane 3), but the molecular weights of A- and B-chains of VCA (Fig. 1B, lane 9) were different from those of VAAs (Fig. 1B, lane 6, 7, 8).

Identification of lectins by Western blot analysis, ELLA, and ELISA

In order to investigate homology between VCA and VAAs, we performed a Western blot analysis using a rabbit polyclonal antibody raised against VAAs. The antibody of VAAs cross-reacted with VCA, giving a strong signal at the both of subchains (Fig. 1C). These results demonstrate that the antigenic determinants of VCA are similar to those of the VAAs, although different amino acid sequence exists between two lectins. Next, we compared the lectins by ELLA and sandwich ELISA. In the ELLA based on lectin affinity to sugars, asialofetuin is used as coating antigen (Jäggy *et al.*, 1995). Accordingly, ELLA only determines sugar binding lectin. Compared with the result of VAA, the VCA highly recognized the polyclonal anti-VAA antibody by ELLA system (Fig. 2A). Considering the fact that the VCA was isolated more efficiently by asialofetuin-Sepharose 4B than the VAAs, the higher recognition of VCA in ELLA may come from higher affinity of VCA to asialofetuin. Further analysis was performed by sandwich ELISA with monoclonal antibody of VAAs. The VCA recognized MNA9-TA5b (monoclonal antibody of VAA-I) (Fig. 2B), but did not recognize CH12-H 11b (monoclonal antibody of VAA-II, III) (Fig. 2 C, D), indicating that the monoclonal antibodies MNA9-TA5b of VAA-I were derived from same epitopes as that of VCA, while the antibodies CH12-H 11b of VAA-II, III were derived from different epitopes from those of VCA. The cloning of VAAs has revealed the presence of one gene only, and they can be distinguished from each other by the degree of glycosylation that is dependent on post-translational processing in the plant (Eck *et al.*, 1999a, 1999b). At present, it cannot be concluded from our results whether the VCA and VAAs are expressed from same genes and differentiated by post-translational processing in the plants or not. Further investigation such as cloning of VCA gene will be necessary.

Cytotoxicity and lectin content of different fractions

Although mistletoe lectins are the focus of modern biomedical research, other components including visco-

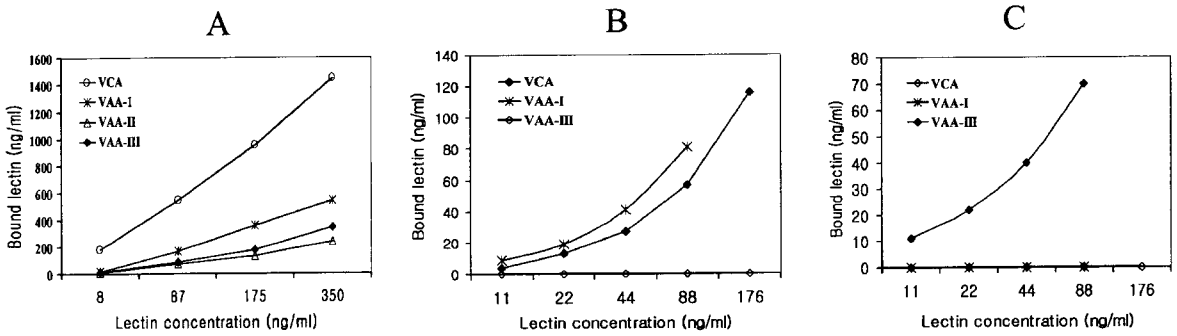

Fig. 2. ELLA (A) and ELISA (B, C) of VCA and VAAs. (A) ELLA: Asialofetuin/ lectin/ rabbit polyclonal anti-VAA Ab/ rabbit-antimouse-IgG-conjugated peroxidase/ ABTS. (B) ELISA of antibody of VAA-I: monoclonal antibody of VAA-I (MNA9-TA5b)/ lectin/ biotinylated Ab/ streptoavidin-peroxidase/ *o*-phenylene diamine (OPD). (C) ELISA of antibody of VAA-II, III: monoclonal antibody of VAA-I (CH12-H 11b)/ lectin/ biotinylated Ab/ streptoavidin-peroxidase/ *o*-phenylene diamine (OPD).

toxins, polysaccharides, and alkaloids have been also reported to be active components exerting anticancer acticvity (Pfüller *et al.*, 1993, Ribereau-Gayon *et al.*, 1993). Recently, the European mistletoe has been reported to show that the fraction of lower molecular weight components (MW<30 kDa) inhibited cancer cells, which was similar to the fraction of higher molecular weight components (Zarkovic *et al.*, 1998). However, the results are rather disputed because the fractions were prepared with 30 kDa cut-off membrane which pore size was not small enough for lectins not to flow out into the filtrate. It is thought that the lectin could be naturally reduced, flow out into the filtrate (MW<30 kDa) through 30 kDa cut-off membrane, and exert similar cytotoxic activity to the higher molecular weight fraction containing lectin and subchains. Accordingly, we fractionated the water extract with 10 kDa and 1 kDa cut-off membrane, and measured the lectin concentration of three fractions with different molecular weight components by ELLA. There was no significant difference of lectin content between two plant extracts. The lectin contents were 3.5 ± 1.0 ng/ml in 1 mg/ml of water extract of both plants. And the fraction 1 of both plants showed lower lectin content (2.3 ± 1.0 ng/ml in 1 mg/ml) than those of whole water extract. However, no lectin was detected in the fraction 2 and 3 of the plants, indicating that the 10 kDa membrane is small enough for lectins not to flow out into the filtrate. The cytotoxicity against cultured Molt-4 cells is one of the biological determination of mistletoe preparations in European countries (Ribereau-Gayon *et al.*, 1993). We investigated cytotoxicity against Molt-4 cells and the relevant lectin concentration of mistletoe extracts. The VCA (IC_{50}=1.2 ng/ml) showed similar cytotoxic activities to the VAA-I (IC_{50}=1.2 ng/ml) against Molt-4 cells (Fig. 3A). And the extract of Korean mistletoe (IC_{50}=2.5 μg/ml) was more cytotoxic to Molt-4 cells than the water extract of European mistletoe (IC_{50}=5.0 μg/ml) (Fig. 3B). The cytotoxicity of the extracts increased proportionally to the concentration of lectin. Compared with the activities (IC_{50}=2.5 -5.0 μg/ml) of whole extracts of both plants, the fraction 1 of both plants showed lower cytotoxicity (Korean mistletoe; IC_{50}=25 μg/ml, European mistletoe; IC_{50}=50 μg/ml). The fraction 2 and 3 of European mistletoe showed no cytotoxic activity against Molt-4 cells (Fig. 4B) while those of Korean mistleote showed slightly higher cytotoxicity than those of European mistletoe (Fig. 4A). Considering the presence of viscotoxins in the fraction 2, and the lower

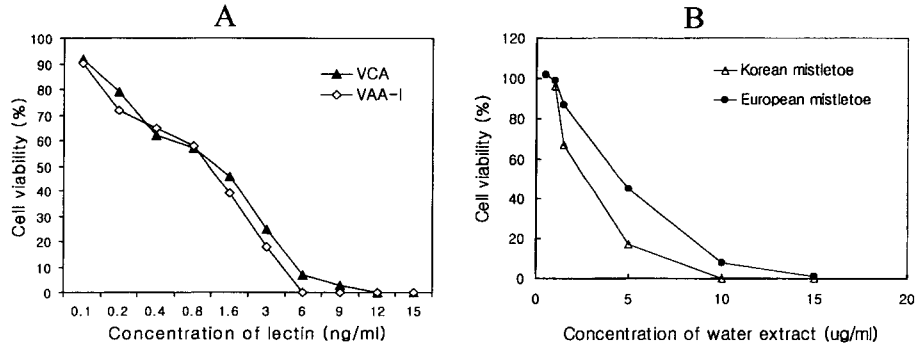

Fig. 3. Dose-dependent growth inhibition of Molt-4 cells by lectins (A) and water extract (B) of Korean and European mistletoe. The lectin concentration was determined by the method of Lowry *et al.* and the inhibition of cell growth was measured by MTT assay. The concentration of the water extract (μg/ml) was expressed as the weight of fresh plant for the preparation of 1 ml of aqueous extract. See materials and methods.

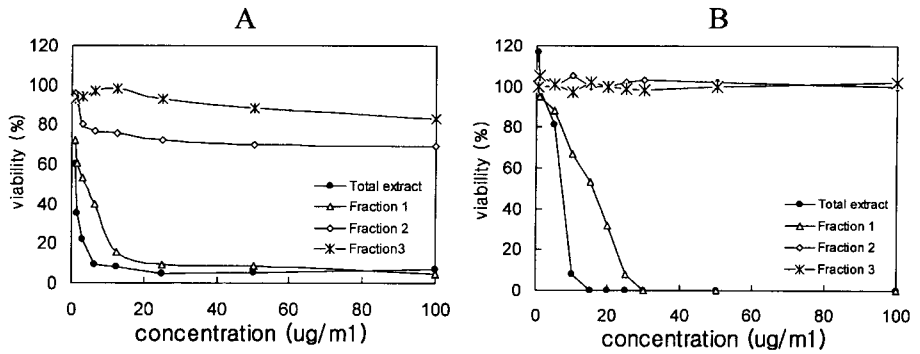

Fig. 4. Dose-dependent growth inhibition of Molt-4 cells by fractions with different molecules of water extract of Korean (A) and European (B) mistletoe. Fraction 1; MW ≥ 10 kDa, fraction 2; 1 kDa ≤ MW ≤ 10 kDa, fraction 3; MW ≤ 1 kDa. The inhibition of cell growth was measured by MTT assay.

molecular weight components such as alkaloids in the fraction 3, such components do not possess enough cytotoxic activity to exert cytotoxicity. Although the lectins of both plants are main components for exerting anticaner activity, however, the synergistic effects of other components such as viscotoxins and alkaloids can not be excluded in Korean mistletoe. We reported recently that the methanol extract of Korean mistletoe showed high activity, while the extract of European mistletoe showed low activity against Molt-4 cells (Park *et al.*, 1999a). The lower molecular weight components in the extract may play an important role for the cytotoxicity because the methanolic fraction of Korean mistletoe does not contain lectin (Park *et al.*, 1999a). Taking together, our results agree with the results of Khwaja *et al* (Khwaja *et al.*, 1980, 1986) that the alkaloidal fraction isolated from Korean mistletoe extract was the most active of all other mistletoes. Further study of other components such as viscotoxins and alkaloids will be necessary to elucidate the synergistic effects of the components in mistletoe extract.

ACKNOWLEDGEMENTS

This study was supported by research grant of Seoul Womens University in the program year of 1999.

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