Arch Pharm Res Vol 26, No 2, 157-161, 2003

Archives of Pharmacal Research
http://apr.psk.or.kr

Apoptosis Induction of Persicae Semen Extract in Human Promyelocytic Leukemia (HL-60) Cells

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(Received October 20, 2002)

The major ingredient of Persicae Semen is a cynogenic compound, amygdalin (D-mandelonitrile-β-gentiobioside). Controversial results on the anticancer activity of amygdalin were reported due to its conversion to its inactive isomer, neoamygdalin. In order to inhibit the epimerization of amygdalin, we used newly developed simple acid boiling method in preparation of Persicae Semen extract. HPLC analysis revealed most of amygdalin in Persicae Semen extract was active D-form. Persicae Semen extract was used to analyze its effect on cell proliferation and induction of apoptosis in human promyelocytic leukemia (HL-60) cells. Persicae Semen extract was cytotoxic to HL-60 cells with IC_{50} of 6.4 mg/mL in the presence of 250 nM of β-glucosidase. The antiproliferative effects of Persicae Semen extract appear to be attributable to its induction of apoptotic cell death, as Persicae Semen extract induced nuclear morphology changes and internucleosomal DNA fragmentation.

Key words: Persicae Semen, Amygdalin, Epimerization, Apoptosis

INTRODUCTION

A number of compounds purified from plant materials and their derivatives revealed anticancer activity. Some of them used in clinics include: paclitaxel, vincristine, podophyllotoxin and camptothecin which are derived from *Taxus brevifolia* L., *Catharanthus roseus* G. Don, *Podophyllum peltatum* L., and *Camptotheca acuminata* respectively (Lilenbaum and Green, 1993; Pezzuto, 1997; Bertrand and Sarié, 1999). More recently, cytotoxic constituents were purified from *Anthriscus sylvestris* (Lim *et al.*, 1999) and it is reported that a metabolite of ginseng saponin induced apoptosis via cytochrome c-mediated activation of caspase-3 protease (Lee *et al.*, 2000).

Amygdalin (D-mandelonitrile-β-gentiobioside), a cynogenic compound which is found in sweet and bitter almonds, Persicae Semen and Armeniacae Semen (Isozaki *et al.*, 2001; Dicenta *et al.*, 2002). Amygdalin is decomposed to mandelonitrile and glucose by β-glucosidase (Brimer *et al.*, 1996). Amygdalin has been subjected to considerable controversy in the treatment of cancer (Koeffler *et al.*, 1980; Newmark *et al.*, 1981; Moertel *et al.*, 1982). These controversial results seemed to result from that amygdalin is changed to its ineffective epimer, neoamygdalin (L-mandelonitrile-β-gentiobioside) in water (Takayama *et al.*, 1984). Recently, purification and determination methods for amygdalin epimers by using capillary electrophoresis were reported (Kang *et al.*, 2000; Isozaki *et al.*, 2001). However, these methods are not easily applicacble to amygdalin in natural products. Since it is reported that amygdalin conversion to neoamygdalin is inhibited by acidic boiling method (Hwang *et al.*, 2002), we prepared extracts from Persicae Semen by a simple acidic boiling method. The extract was analyzed for its amygdalin content and used to investigate its cytotoxic activity.

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MATERIALS AND METHODS

Preparation of plant extract

The outer shell of Persicae Semen (100 g) was removed and extracted by boiling in distilled water in the presence of 0.1% citric acid for 3 h. The extract was then filtered and the filtrate was concentrated with a vacuum rotary evaporator (Eyela, Japan) under low pressure. The residue was freeze-dried in a freezing-dryer (Ilsin, Korea) and stored at -70°C. The powder was dissolved in dimethyl sulfoxide and diluted

with phosphate buffered saline (PBS, pH 7.4) to give final concentrations of total extract ranging from 0.8 to 100 μg/mL. Amygdalin standard was obtained from Tokyo Kasei Chemical Co. (Tokyo, Japan). Methanol was purchased from Merck (Darmstadt, German). The other reagent and solvents used were of guaranteed or analytical grade. Distilled water was further purified by a water purification system (Pure power III, Taiwan).

The HPLC system consisted of a M 930 pump (Young Lin, Kyunggi, Korea), a M 720 UV detector (Young Lin, Kyunggi, Korea) set at 214 nm. The column was a Capcell Pak C18, Type UG120 (250 mm×4.6 mm, Shiseido, Japan) at 35°C contained in a CTS30 column oven (Young Lin, Kyunggi, Korea). Mobile phase was 25% methanol-water at 1 mL/min. A calibration curve was constructed using six different concentrations of standard solution containing 30, 60, 90, 120 and 150 μg/mL of amygdalin. Peak-area was calculated for the calibration plot.

Cell culture and cytotoxicity test

Human promyelocytic leukemia (HL-60, ATCC CCL240) cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids and antibiotics in a humidified incubator with 5% CO_2/95% air at 37°C. The trypan blue dye exclusion test was routinely used to assess cell viability. Exponentially growing HL-60 cells were seeded at 5×10^5 cells/well in a 96-well plate and treated with the indicated concentrations of extract or vehicle, as described in the figure legends. β-glucosidase was added to media to 250 nM when indicated. After various periods of exposure, the general viability of cultured cells was determined by assaying the reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan (Monks *et al.*, 1991). Experiments were performed in triplicate.

Nuclear staining with propidium iodide

Cells used in this study were constantly observed under an inverted phase-contrast microscope (Olympus, Japan). Cells were placed in a 60 mm culture plate at a concentration of 1.0×10^5 cells/mL. Sixteen hr after plating, cells were treated with various concentrations of extract with 250 nM of β-glucosidase. After 7 h, cells were harvested by centrifugation and washed with phosphate buffered saline (PBS) twice. After, cells were resuspended in PBS at a concentration of 5×10^5 cells/mL and placed onto a microscope slide using a cytospin (Hanil, Korea), which was left at room temperature for dryness. Cells were fixed with cold ethanol in the dark. The fixed cells were washed with PBS and stained with 2.5 mg/mL of propidium iodide and DNase-free RNase (100 mg/mL). The morphology of the stained cells was examined under a fluorescence microscope (Nikon E800, Japan).

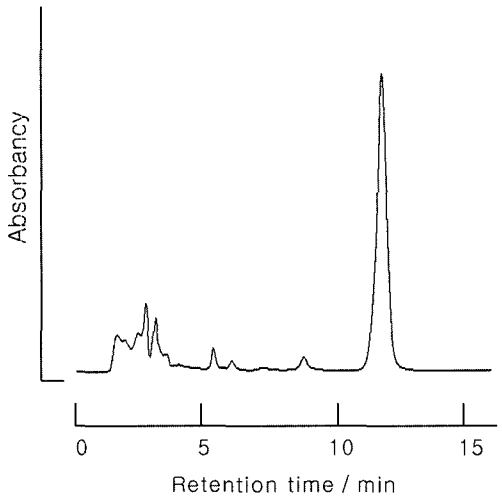

Fig. 1. Reverse-phase chromatogram of amygdalin from Persicae Semen.

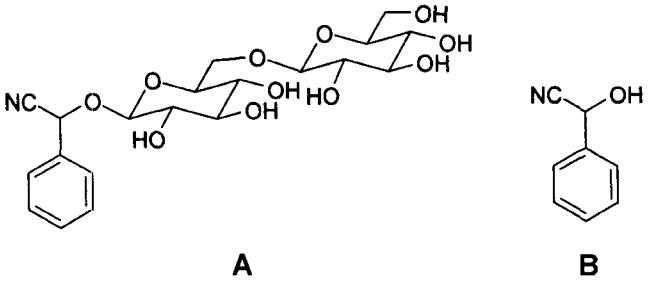

Fig. 2. Structure of amygdalin (**A**) and mandelonitrile (**B**). Amygdalin is hydrolyzed to mandelonitrile by β-glucosidase.

DNA fragmentation analysis

DNA was purified as described previously (Hyun *et al.*, 1997). Cells were grown at a density of 1×10^6 cells/mL and treated with various concentrations of Persicae Semen extract with 250 nM of β-glucosidase for 0 to 48 h, as described in the figure legends. The resulting purified DNA fragments were subjected to electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. The results shown are representative of those obtained in three independent experiments.

RESULTS

Analysis of extract prepared from Persicae Semen by HPLC

Fig. 1 is a chromatogram of amygdalin from Persicae Semen by reversed-phase separation with a 25% methanol as a mobile phase after extraction with water. The peak of amygdalin was completely separated in our method without any pretreatment. The structure of amygdalin is shown in Fig. 2. The calibration curve between peak area and the concentration of standard amygdalin showed excellent linearity ($r^2 = 0.9996$). The amygdalin content was 18% of

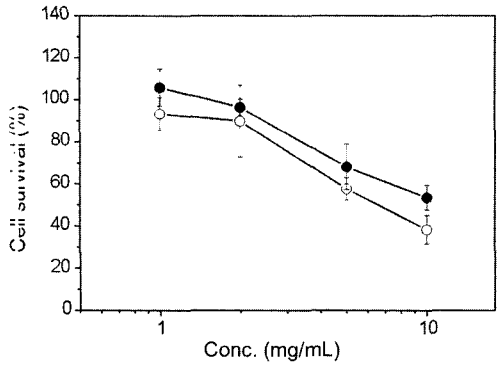

Fig. 3 Antiproliferative effect of Persicae Semen extract in HL-60 cells. Cells were incubated with 0, 1, 2, 5, or 10 mg/mL of Persicae Semen extract in the absence (●) or in the presence (○) of 250 nM of β-glucosidase for 48 h and cell survival was measured by the MTT assay.

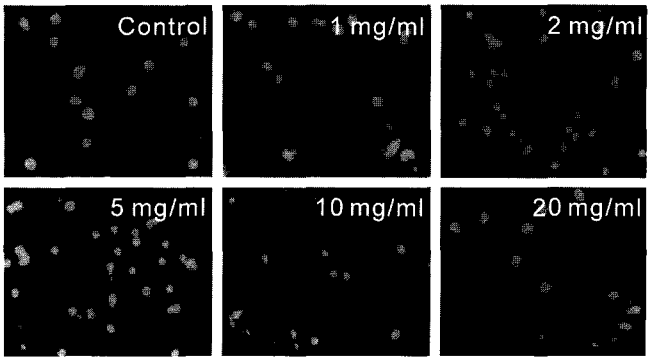

Fig. 4. Induction of apoptotic cells by Persicae Semen extract treatment. Cells were treated with 0, 1, 2, 5, 10 and 20 mg/mL of Persicae Semen extract with 250 nM of β-glucosidase for 7 h and cellular morphological changes were observed using a fluorescence microscope at the magnitude of 200x.

Persicae Semen extract (Fig. 1).

Cytotoxicity of Persicae Semen extract

The effect of Persicae Semen on cellular proliferation was evaluated using the MTT assay. A 48 h exposure to Persicae Semen dramatically decreased the proliferation of HL-60 cells in a dose-dependent manner (Fig. 3). The concentration of Persicae Semen extract required for inhibition of growth by 50% (IC_{50}) was approximately 11 mg/mL. In the presence of β-glucosidase, lower IC_{50} value, 6.4 mg/mL was obtained. Control cells treated with vehicle alone showed no changes in cell proliferation or viability. When we used purified amygdalin, IC_{50} obtained was approximately 18% of that of extract (data not shown), this is consistent with the content of amygdalin in Persicae Semen extract confirmed by HPLC analysis.

Induction of apoptosis by Persicae Semen extract

To determine whether the antiproliferative effect of Persicae Semen extract is associated with programmed cell death or apoptosis, we initially determined the morphological changes of HL-60 cells treated with Persicae Semen in the presence of β-glucosidase for 7 h. Cells exposed to Persicae Semen revealed characteristic apoptotic morphology including appearance of apoptotic bodies and condensed chromatin in the nuclei (Fig. 4).

In addition to morphological determination, apoptosis induction by Persicae Semen was ascertained by using an assay developed to measure DNA fragmentation, a biochemical hallmark of apoptotic cell death. As illustrated in Fig. 5, agarose gel electrophoresis of chromosomal DNA extracted from HL-60 cells treated with Persicae Semen in the presence of β-glucosidase revealed distinct internucleosomal DNA fragmentation into a ladder of 180 bp nucleosomes. The intensity of the DNA banding ladder progressively increased with in a time- and dose-dependent manner. The DNA fragmentation was first observed after 3 h incubation with 10 mg/mL and 7 h incubation with 5 mg/mL of Persicae Semen.

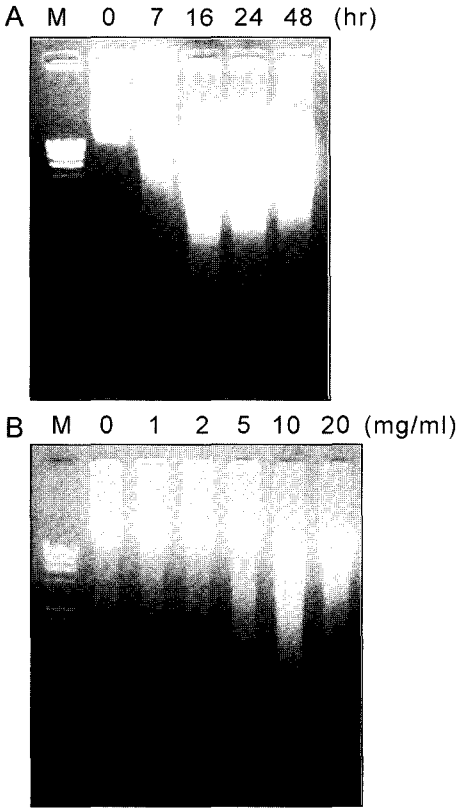

Fig. 5. Persicae Semen extract induces a time- and dose-dependent fragmentation of nuclear DNA. (**A**) HL-60 cells were incubated with 10 mg/mL of Persicae Semen extract with 250 nM of β-glucosidase for 0, 7, 16, 24 or 48 h, as indicated. (**B**) HL-60 cells were incubated with 0, 1, 2, 5, 10 and 20 mg/mL of Persicae Semen extract with 250 nM of β-glucosidase for 7 h, as indicated. DNA was isolated from the cells and analyzed on 1.5% agarose gel with by ethidium bromide staining, as described in Materials and Methods.

DISCUSSIONN

Amygdalin (D-mandelonitrile-β-gentiobioside), a major ingredient of Persicae Semen, has been studies for many years for its anticancer activity (Koeffler *et al.*, 1980; Moertel *et al.*, 1982; Syrigos *et al.*, 1998). Amygdalin is hydrolyzed by β-glucosidase to prunasin (D-mandelonitrile monoglucoside) and then, prunasin is again hydrolyzed to mandelonitrile by the same enzyme. The anticancer activity of the cynogenic compound, amygdalin has been controversial. However, it is reported that amygdalin in aqueous solution is epimerized to neoamygdalin (L-mandelonitrile-β-gentiobioside), which is inactive against cancer (Takayama and Kawai, 1984). We developed extraction method which inhibit the conversion of D-form to L-form (Hwang *et al.*, 2002) thus we employed this method to prepare Persicae Semen extract and used the extract for investigation of anticancer activity.

Persicae Semen extract reduced cell survival of human promyelocytic leukemia HL-60 cells. Lower IC_{50} value was obtained with co-treatment of Persicae Semen extract with β-glucosidase. To determine whether the growth inhibitory and antiproliferative effects of Persicae Semen are associated with programmed cell death or apoptosis, we initially examined the morphological changes induced by Persicae Semen treatment in HL-60 cells. Microscopic analysis of cells treated with Persicae Semen indicated that they had undergone gross morphological changes. Apoptosis is an active process that ultimately leads to the activation of endonucleases and cleavage of DNA into fragments of about 180-200 base pairs (Kerr *et al.*, 1994). Exposure to Persicae Semen fragmented cellular DNA in a pattern characteristic of apoptotic cell death. Pharmacological studies and various trials are carried out to use amygdalin as an anticancer agent (Strugala *et al.*, 1995; Llorens *et al.*, 1998; Syrigos *et al.*, 1998; Yuan *et al.*, 1999). Recently, amygdalin was used as a prodrug in antibody-directed enzyme prodrug therapy (Syrigos *et al.*, 1998). β-glucosidase was conjugated to a tumor-associated monoclonal antibody, thus the cytotoxic effect of amygdalin to cancer cells was highly increased. However, in order to develop Persicae Semen extract as a drug, or a prodrug, more mechanism and *in vivo* studies needed to be followed.

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