Anticancer Effects of *Annona glabra* Plant Extracts in Human Leukemia Cell Lines

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Abstract. *Annona glabra (pond apple), a tropical tree growing wild in the Americas and Asia, is used in traditional medicine against several human ailments, including cancer. To validate the ethnopharmacological claims against cancer, the anticancer effects of alcoholic extracts prepared from pond apple leaves, pulp and seed, were investigated in human leukemia cell lines. The alcoholic extracts were not cytotoxic to normal human lymphocytes. However, extracts were highly cytotoxic to drug sensitive (CEM) and multidrug-resistant leukemia (CEM/VLB) cell lines. The seed extract was more potent than leaf and pulp extracts, and the cytotoxicity values were significantly lower than that for adriamycin. The seed extract caused a concentration-dependent increase in the percentage of the sub G_0/G_1, as well as G_0/G_1 cell population, contributing to the cytotoxicity. The sub G_0/G_1 population increased from 2.2 to 7.0% in CEM and from 1.0 to 10.7% in CEM/VLB cell lines, when the cells were treated with 0-10 Bg/ml seed extract. Treatment of CEM and CEM/VLB cells with seed extract induced apoptosis and necrosis in both sensitive and resistant leukemia cells in a concentration-dependent manner. The seed extract at 2 and 5 Bg/ml enhanced cellular daunorubicin accumulation, indicating the competitive P-glycoprotein binding ability and drug-resistance reversal effect. Treatment of CEM and CEM/VLB cells with seed extracts also up-regulated the expression of cyclin kinase inhibitor (WAF1/p21) contributing to the arrest of cells at the G_0/G_1 phase of the cell cycle. These results support the traditional use of A. glabra and the alcoholic seed extract is a potent source of anticancer compounds that could be utilized pharmaceutically.*

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Plant-derived compounds and their semi-synthetic, as well as synthetic analogs, serve as major source of pharmaceuticals for human diseases. It is estimated that approximately 25% of prescriptions handled in United States contain a plant-derived natural product and 74% of the 119 most important drugs currently contain ingredients from plants used in traditional medicine (1). Hence, for the treatment of disease states wherein drug therapy is a rational approach, plant materials represent legitimate starting materials for the discovery of new agents. In the case of human cancers, thus far, nine plant-derived compounds have been approved for clinical use as anticancer drugs in the United States. They are vinblastine (Velban), vincristine (Oncovin), vinorelbine (Navelbine®), etoposide (VP-16®), teniposide (VM-26®), paclitaxel (Taxol®), docetaxel (Taxotere®), topotecan (Hycamptin®), and irinotecan (Camptosar®) (2-4).

In the cancer drug discovery program, a paradigm based on ethnobotanical and ethnopharmacological data would be more economical and beneficial for identifying potential anticancer molecules than mass screening of plant species. *Annona glabra* L., commonly known as pond apple, is a tropical wild tree native to the Americas and Asia. The leaves and bark of pond apple are used traditionally as an insecticide and parasiticide (5). In China, pond apple leaves are used to treat chronic bronchitis and the whole plant is used as an anticancer drug (6). Additionally some compounds with anticancer properties have been isolated from this plant, although none has reached the stage of clinical trials. One of the first cytotoxic compounds isolated from *A. glabra* is the alkaloid liriodenine from stem bark extract which displayed activity against human nasopharyngeal carcinoma (7).

A. glabra belongs to the *Annonaceae* family from which a group of compounds collectively called acetogenins have been isolated over the last 26 years. Acetogenins possess potential cytotoxic activity and are one of the most potent inhibitors of mitochondrial complex I (8). Some of these compounds are able to inhibit P-glycoprotein and hence are useful against multidrug-resistant cancer (9-12).

The pharmaceutical effects of crude extracts from *A. glabra* seed, pulp or leaves, commonly used in traditional systems of medicine in Mexico, China and Japan, have not been evaluated *in vitro* or *in vivo* to validate the Complementary and Alternative Medicine (CAM) practice. In this paper, we report the biorational screening of *A. glabra* leaf, fruit pulp, and seed extracts to determine the cytotoxicity against a human leukemia cell line and its multidrug-resistant-derived cell line. The mechanism of action of these extracts on cell cycle, apoptosis, and multidrug resistance is also presented.

Materials and Methods

Extract preparation. The leaves and fruits of *A. glabra* were collected from the campus of Florida International University, Miami, FL, USA. A voucher specimen was placed in the Florida International University Herbarium (FIU Voucher No.8003). The seeds, leaves and fruit pulp were frozen to –80˚C for 24 hours and then lyophilized with a freeze-drying system (Labconco Corporation, Kansas City, MO, USA) for 24 hours. The frozen leaves, pulp and seeds were ground separately into a powder using a grinder. Crude extracts were prepared by placing 0.5 g of the powdered plant material into a 15 ml centrifuge tube and adding 5 ml of absolute ethanol. The tubes were placed on a rocker and extraction carried out for 24 hours. Following extraction, the solution was allowed to settle by gravity and the supernatant was removed with a pipette and filtered through a 0.22 μm syringe filter (Costar). Serial dilutions ($1.0x10^{-1}$ to $1.0x10^{-5}$) of the filtered crude extract stock were prepared in 50% ethanol and stored at room temperature in the dark. The average yields for fruit, seed and leaf extracts were 1.6%, 1.5% and 4.8%, respectively on a dry weight basis. The microgram level of extract used in the study is based on the original 0.5 g plant material used for extraction.

Cell lines and normal lymphocytes. The human drug-sensitive leukemia (CEM) and its multidrug-resistant-derived (CEM/VLB) cell lines were used in all assays. These cell lines were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37˚C in an incubator.

Peripheral adult human blood samples were drawn for isolation of normal human lymphocytes. Blood specimens were diluted 1:1 with phosphate-buffered saline (PBS) and normal lymphocytes separated with Histopaque 1077 (Sigma Chemical Co, St. Louis, MO, USA) gradient centrifugation (13). Normal lymphocytes were resuspended in RPMI medium with 10% fetal bovine serum and used for cytotoxicity analysis.

Analysis of cytotoxicity. Cytotoxicity assays of drug-sensitive leukemia cell line (CEM), multidrug-resistant leukemia cell line (CEM/VLB) and normal human lymphocytes against *A. glabra* extracts were performed using the cell proliferation kit I (MTT) from Roche Biochemicals (Indianapolis, IN, USA). The cells were plated in 96-well plates at 10^4 cells/100 Bl/well for CEM and CEM/VLB, and $8x10^4$ cells/100 Bl/well for the normal lymphocytes. The leaf, pulp and seed extracts (0.01 to 250 μg/ml) were added in triplicates. The plates were incubated at 37˚C for 48 hours and the MTT assay was performed according to the manufacturer's protocol. The percentage of surviving cells based on control treatment (untreated) was calculated and plotted against extract concentrations. The concentration of *A. glabra* extracts inducing 50% inhibition of cancer cells (IC_{50} values) was calculated from the cytotoxicity curves. The values from three separate experiments were averaged and the mean IC_{50} as well as standard deviation values were calculated for each extract (14).

Cell cycle analysis. Following extract exposure (0, 0.05, 1 and 10 Bg/ml) for 48 h, cells (10^6) were centrifuged and resuspended in propidium iodide(PI)-hypotonic citrate solution for 1 h before flow cytometric analysis of the cell cycle distribution (15)

Apoptosis assay. CEM and CEM/VLB cells (10^5 cells/ml) were treated with *A. glabra* seed extracts (0, 0.05, 1 and 10 Bg/ml) at 37˚C for 48 h in an incubator maintained at a humidified atmosphere of 5% CO_2. The apoptosis induced by the extract was analyzed using Annexin-V-EGFP Apoptois Detection Kit (Medical & Biological Laboratories Co. Ltd., Woburn, MA, USA). Tumor cells were centrifuged and resuspended in 500 Bl of 1X binding buffer containing 1 Bl of Annexin-V-EGFP and 1 Bl of PI. The cells were incubated in the dark at room temperature for 5 min and analyzed in a Coulter Elite flow cytometer. Annexin-V-EGFP binding was detected using a FITC signal detector (FL1) and PI staining by the phycoerythrin emission signal detector (FL2). Percentages of FITC/PI-positive cells (apoptotic) were calculated from flow cytograms (16, 17).

Cellular daunorubicin (DNR) accumulation. Log-phase cells ($1x10^6$) were incubated with 3.5 BM (2 Bg/ml) DNR in the presence or absence of *A. glabra* seed extract (2 or 5 Bg/ml) for 1 h at 37˚C. Cellular DNR fluorescence was analyzed in a Coulter Elite flow cytometer. Fluorescence emissions (above 530 nm) from at least 10,000 cells were collected, amplified and scaled to generate single parameter histograms (15).

Analysis of p21 protein expression. CEM and CEM/VLB cells ($5x10^6$) were incubated with *A. glabra* seed extract (0.01-10 Bg/ml) for 48 h at 37˚C in a CO_2 incubator. Cells were centrifuged, washed once with PBS and total cellular proteins were isolated using the antigen extraction reagent supplied with *WAF1/p21* ELISA kit (Calbiochem, San Diego, CA, USA). Protein concentration of the extract was quantified using the Bradford assay and 100 Bg protein was used for *WAF1/P21* ELISA according to the manufacturer's instructions. P21 amounts were plotted against seed extract concentrations.

Statistical analysis. Microsoft Office Excel was used for calculation and plotting of mean and standard deviation estimates in the graphs. Mean IC_{50} values were compared by Student's *t*-test and significance levels calculated.

Results

Cytotoxicity. Figure 1A-C show the cytotoxicity curves of leaf, pulp and seed extracts in human leukemia cell lines in comparison with normal human lymphocytes; the IC_{50} values are presented in Table I. While not all extracts were

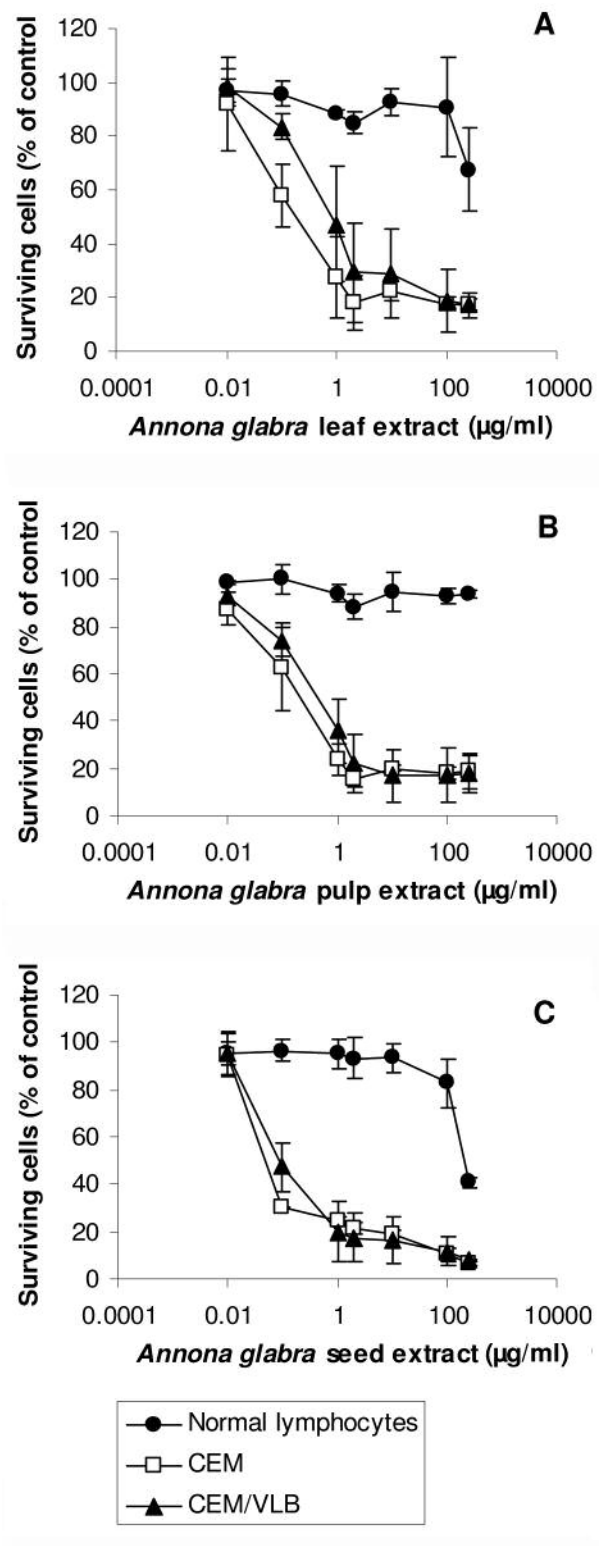

Figure 1. *Cytotoxicity curves of normal human lymphocytes and leukemia (CEM and CEM/VLB) cells lines against total ethanolic extracts of A. glabra leaf (A), pulp (B) and seed (C). Cells were treated with extracts for 48 h and cytotoxicity analyzed using the cell proliferation kit (MTT) from Roche Biochemicals, Indianapolis, IN, USA.*

Table I. *Cytotoxicity of normal lymphocytes and human leukemia cell lines using alcoholic extracts of leaf, pulp and seed from Annona glabra.*

Cells	IC_{50} values of *A. glabra* extract (µg/ml)		
	Leaf	Pulp	Seed
CEM	0.30±0.02	0.35±0.02	0.07±0.00
CEM/VLB	1.00±0.07**	0.65±0.06**	0.10±0.01*
Human lymphocytes	>250.00±9.23***	>250.00±0.06***	>250.00±9.23***

*$p<0.05$ and **$p<0.01$ CEM *vs*. CEM/VLB; ***$p<0.001$ human lymphocytes *vs*. CEM or CEM/VLB.

cytotoxic to human lymphocytes (IC_{50} values >250 Bg/ml), they caused significant cell death in both sensitive as well as resistant human leukemia cell lines. The IC_{50} values for sensitive cell line (CEM) were lower than CEM/VLB with approximately 1.4-, 1.86- and 3.3-fold resistance for seed, pulp and leaf extracts, respectively in CEM/VLB cells. When the alcoholic extracts of leaf, pulp and seed are compared, the seed extract appeared to be more potent with IC_{50} values of 0.07 and 0.1 µg/ml for CEM and CEM/VLB cell lines, respectively.

Cell cycle. Since the seed extract appeared to be more potent, we concentrated on it for further studies. The effect of seed extract on the cell cycle in human leukemia cell lines is given in Figure 2. In both sensitive and resistant cell lines, a concentration-dependent increase in the percentage of the sub G_0/G_1 as well as G_0/G_1 cell population was quite evident with increasing concentrations of seed extract. The sub G_0/G_1 population increased from 2.2% to 7.0% in CEM and from 2.0% to 10.7% in CEM/VLB cell lines when they were treated with 0-10 µg/ml seed extract. Similarly, cells in the G_0/G_1 phase increased from 56.9% to 73.9% in CEM and from 53.7% to 62.5% when tumor cells were treated with 0-10 µg/ml seed extract.

Apoptosis. Flow cytometric analysis of apoptosis induced by increasing amounts of *A. glabra* seed extract in CEM and CEM/VLB cell lines is presented in Figure 3. The cells in the upper and lower right quadrants represent late apopotic/necrotic and early apoptotic cells, respectively. The percentage of early apoptotic and late aoptotic/necrotic cells induced by seed extract (0-10 µg/ml) ranged from 1.4% - 17.7% and from 0.6% - 27.9%, respectively in CEM cell line. The seed extract-induced early apoptotic and late aopototic/necrotic cells ranged from 3.3% - 34.0% and from 0.6% - 20.3%, respectively, in CEM/VLB cell line.

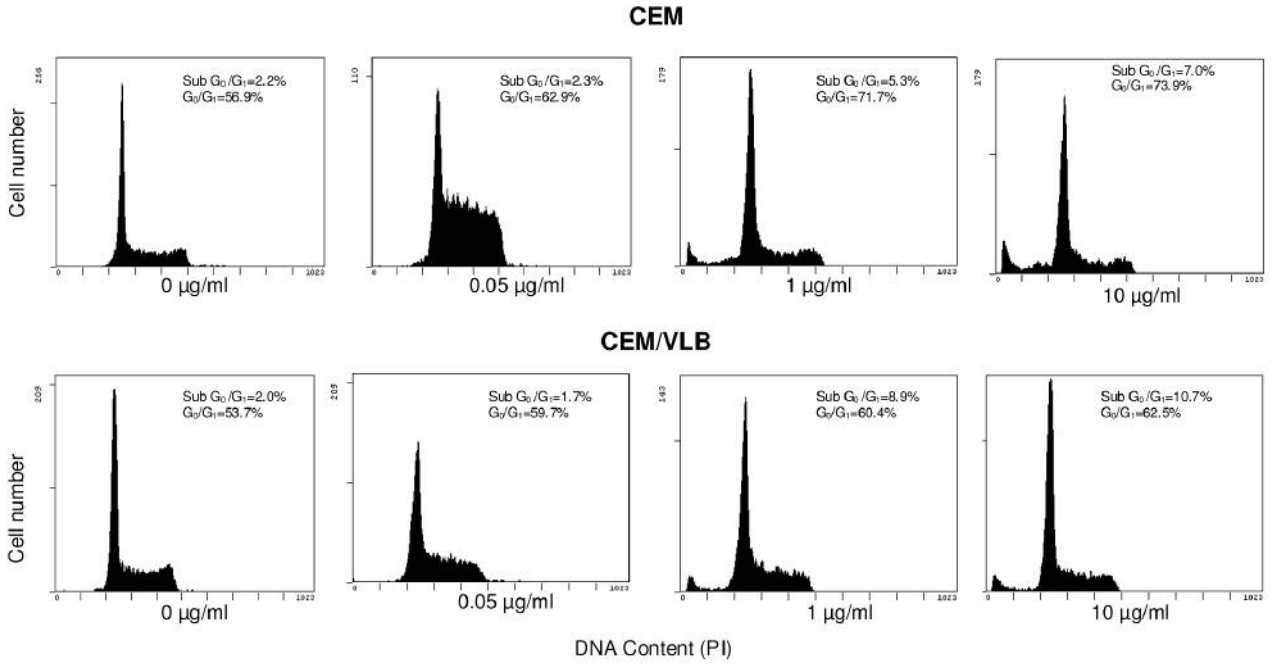

Figure 2. *Cell cycle analysis of CEM and CEM/VLB human leukmemic cell lines treated with A. glabra seed extract for 48 h. An increase in G_0/G_1 and subG_0/G_1 cell population was found with increasing concentrations of seed extract.*

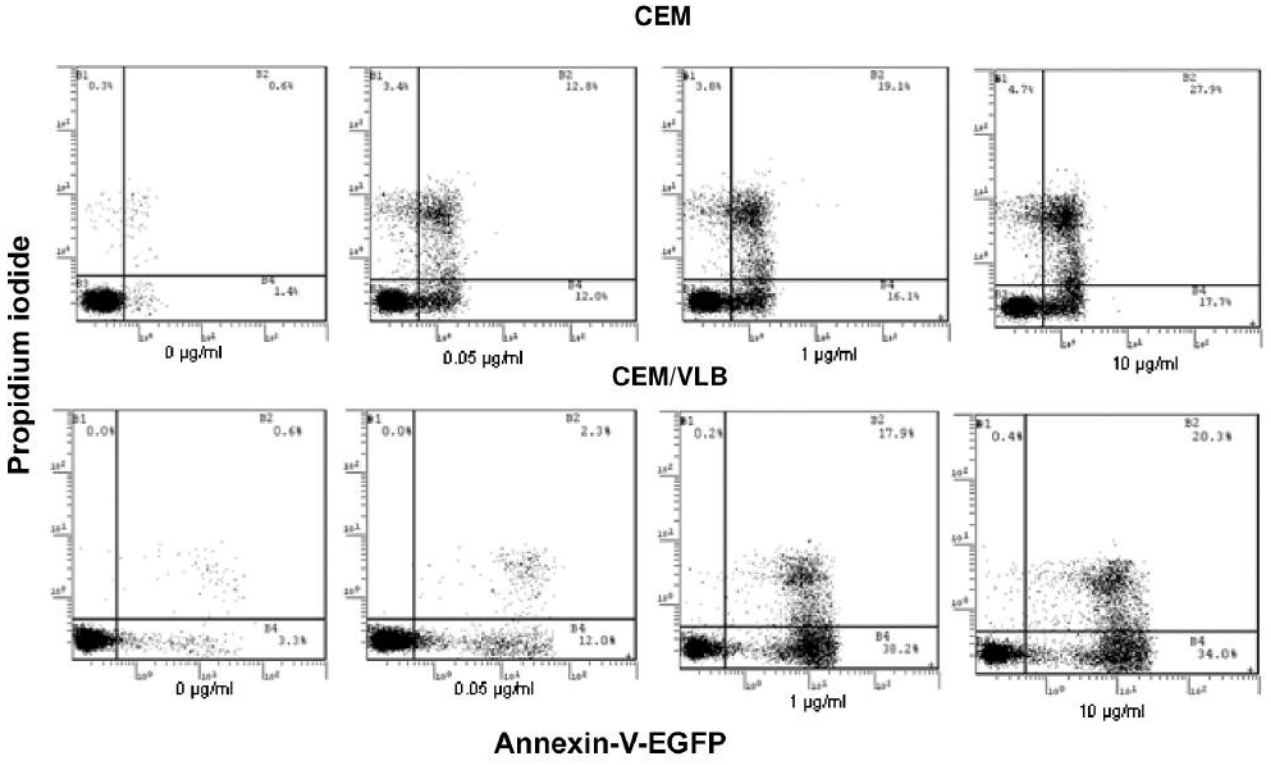

Figure 3. *Analysis of apoptosis induced by A. glabra seed extract in CEM and CEM/VLB leukemia cell lines. Cells treated with seed extract for 48 h were stained with propidium iodide and Annexin-V-EGFP using the reagent kit from MBL International, MD, USA.*

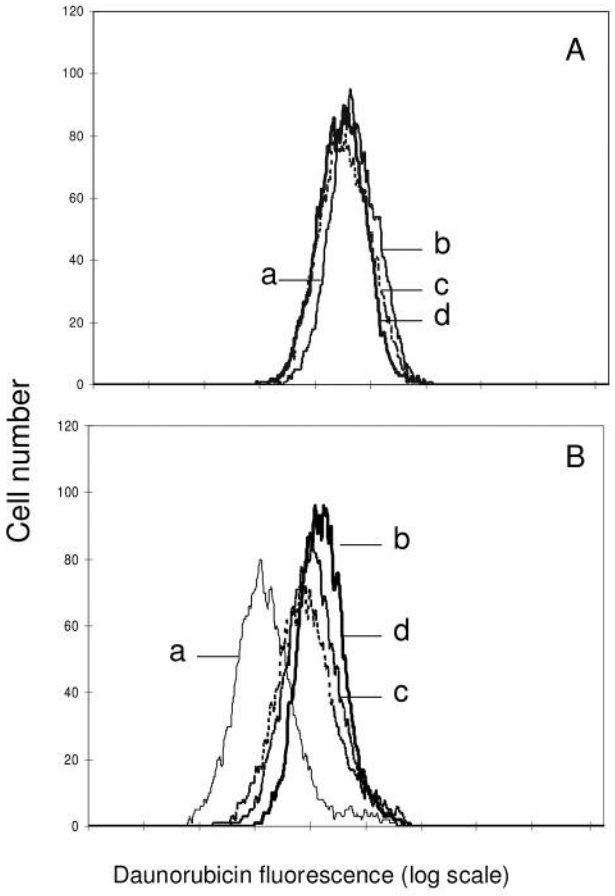

Figure 4. *Effect of A. glabra seed extract on daunorubicin (DNR) accumulation in CEM (A) and CEM/VLB (B) cell lines. Cells were incubated with DNR in the presence or absence of A. glabra seed extract and cellular fluorescence analyzed by flow cytometry. a, daunorubicin alone; b, daunorubicin + 10 BM verapamil (positive control); c, daunorubicin + 2 Bl of A. glabra seed extract; d, daunorubicin + 5 Bl of A. glabra seed extract.*

Effect of seed extract on cellular DNR accumulation. DNR uptake and accumulation is an functional assay of MDR1/P-glycoprotein. CEM/VLB is a multidrug-resistant (MDR) cell line abundantly expressing P-glycoprotein having an active efflux pump showing a reduced level of DNR retention compared to CEM (Figure 4). Flow cytometric analysis of DNR accumulation with and without *A. glabra* seed extract in CEM and CEM/VLB cell lines is given in Figure 4A and B, respectively. The sensitive CEM cells lacking MDR1/P-glycoprotein expression when incubated with DNR in the absence (a) or presence of verapamil (b) did not increase cellular DNR accumulation. Similarly, 2 μg/ml or 5 μg/ml *A. glabra* seed extract failed to modulate DNR in sensitive cells. On the other hand, the CEM/VLB cell line expressing MDR1/P-glycoprotein showed modulation of DNR accumulation in the presence of both verapamil and *A. glabra* seed extracts. A concentration-dependent increase in DNR accumulation was observed between 2 and 5 μg/ml *A. glabra* seed extracts.

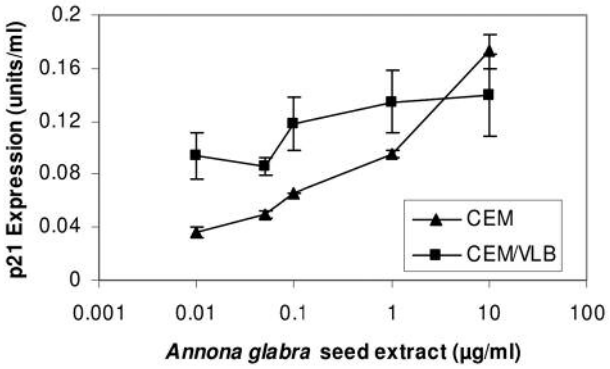

Figure 5. *Effect of A. glabra seed extract on p21 expression in CEM and CEM/VLB cells analyzed using an ELISA protocol.*

p21 protein expression. p21 is a cyclin kinase inhibitor having a direct role in cell cycle phase transition from G_0/G_1 to S-phase. Since we observed G_0/G_1 cell cycle phase-arrest with *A. glabra* seed extract, the expression of p21 expression in seed extract-treated CEM and CEM/VLB cells was analyzed by ELISA (Figure 5). In both tumor cell lines, we observed an elevation of *p21* expression with increasing concentrations of seed extract (0-10 μg/ml). In CEM cells, *p21* protein increased approximately 4-fold between 0-10 Bg/ml *A. glabra* seed extract concentrations. However, in CEM/VLB cells, *p21* increase with seed extract treatment was smaller than in CEM cells.

Discussion

The leaves and bark of pond apple are used in Chinese medicine against cancer and other ailments (6). This plant also has been used lately for identification of pharmaceutically important compounds. Although some compounds have been identified as possessing medicinal properties, none of these compounds has ever reached clinical trials. Moreover, the anticancer effects of total extract of leaf, pulp and seed have not been validated *in vitro* to date based on their use in Chinese or other systems of medicine. Beyond this objective, the present investigation was also undertaken to identify the most potent plant part that could be used to isolate pharmaceutically important molecules from pond apple.

The cytotoxicity estimates of total leaf, pulp and seed extracts indicate that *A. glabra* is a repository for anticancer compounds and the fact that the IC_{50} values are significantly lower than adriamycin (CEM=0.13 Bg/ml and CEM/VLB=13.4 Bg/ml)

indicates its potential for cancer drug discovery programs (18). Furthermore, the data confirm the CAM use of whole plant parts for cancer treatment as practiced in South Asia, especially China. If we compare the leaf, pulp and seed extracts, it is apparent that seed extracts are more potent than other extracts. Unfortunately seed has not been thoroughly exploited for the isolation of anticancer compounds from *A. glabra*. Therefore, pharmaceutical companies may have better success for isolation of novel and potent cancer drugs using seed as starting material; Li *et al.* (6, 19) isolated and characterized four cytotoxic cytopeptides (glabrin A, B, C and D) from seeds.

Flow cytometric analysis of the DNA content of *A. glabra* extract-treated cells has shown that the extracts caused a G_0/G_1 phase arrest and induction of the sub-G_0/G_1 population. Incidentally, some of the compounds isolated from *A. glabra* leaf such as cunabic acid and ent-kauran-19-al-17oic acid have been reported to induce accumulation of cells at G_0/G_1 phase by previous investigators (20). To test the mechanism behind this G_0/G_1 phase arrest, we analyzed the p21 expression in *A. glabra* seed extract-treated CEM and CEM/VLB cells. *P21* is a cdk1 inhibitor that is usually expressed in the G_0/G_1 phase of the cell cycle (21, 22). We found an up-regulation of *p21* expression in CEM and CEM/VLB cells treated with *A. glabra* seed extract, although the effect in the former was significantly higher than in the latter cell line. *WAF1/p21* has been shown to inhibit a wide range of Cdk/cyclin complexes, which are involved in phosphorylation of Rb protein. Phosphorylation of Rb allows E2F to dissociate and switch on the genes required for the progression from the G_1- to the S-phase. Therefore, up-regulation of *p21* protein with *A. glabra* extracts will adversely affect *Rb* phosphorylation and the progression of cells from the G_1- to the S-phase, resulting in G_0/G_1 phase arrest and apoptosis (23-25).

Our results also showed that the total ethanolic extract of *A. glabra* seeds also induced apoptosis when analyzed by Annexin-V staining. This data correlated with the appearance of the subG_0/G_1 population observed in the cell cycle analysis. A concentration-dependent increase in the percentage of apoptotic cells was observed with increasing concentrations of extract. The percentage of apoptotic cells increased up to 27.9% and 20.3% in CEM and CEM/VLB cells respectively at 10 Bg/ml of seed extract. Several previous investigators working with isolated diterpenoid compounds from *A. glabra* have noticed that the apoptosis is associated with cytotoxicity of those compounds in tumor cells (20, 26).

Total extracts of *A. glabra* seed also appeared to modulate daunorubicin accumulation within MDR cells unlike drug sensitive CEM cells. This observation is quite interesting in the fact that the extract contains compounds that might be substrates for MDR1/P-glycoprotein (27). Both 2 Bg/ml and 5 Bg/ml of total extract modulated daunorubicin uptake, the latter modulating to the same level as the positive control, verapamil, in CEM/VLB cells. Firstly, this may indicate that the extract may contain compounds that may be potent modulators of MDR1/P-glycoprotein which could be isolated for overcoming drug resistance in tumor cells. Secondly, the extracts may contain anticancer compounds that might be substrates for P-glycoprotein which could be combined with other cancer drugs as an adjuvant agent.

The results from the present study clearly indicated the anticancer potential of *A. glabra* extract, validating its CAM (Complementary and Alternative Medicine) use. The cytoxicity estimates of *A. glabra* leaf, pulp and seed extracts are significantly better than important cancer drugs, such as anthracyclines. Although each extract may contain several compounds, it certainly has potent anticancer compounds which could be isolated and characterized further. The cytotoxic effect of these extracts on cancer cells is through up-regulation of *p21* protein and blockage of cell cycle at G_0/G_1 phase, leading to apoptosis. *A. glabra* seed extract also modulated multidrug-resistance in cancer cells and can be used as an adjuvant with other MDR drugs. Further, *A. glabra* seed extract can be developed as a botanical drug initially in cancer patients before it can be thoroughly analyzed for the isolation, characterization and testing of anticancer compounds.

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