Discovery of Water-Soluble Anticancer Agents (Edotides) from a Vegetable Found in Benin City, Nigeria

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Cancer claims the lives of more than six million people each year in the world. About 1,268,000 new cancer cases, and 553,400 deaths were reported in the United States in 2001. Current treatment approaches have yielded significant progress in the fight against cancer, but the incidence of developing certain types of cancer continues to rise. This is especially true in the African-American communities. African Americans are about 33% more likely to die of cancer than are whites and more than twice likely to die of cancer as are Asian-Islander, American-Indians, and Hispanics. This increase coupled with the harsh side effects of some of the cancer chemotherapies have led to the search for more natural biological products, especially those derived from plant products, currently known as herbal medicine. There is a need for a continued search for novel natural products that may be used as cancer chemopreventive and/or chemotherapeutic agents. The objective of this study was to evaluate the effect(s) of a novel water-soluble leaf extract of *Vernonia amygdalina* (VA) on human breast cancer cell DNA synthesis. MCF-7 cell line, considered a suitable model, was used in this study. Treatment of cells with physiologically relevant concentrations of water-soluble VA extract potently inhibited DNA synthesis in a concentration-dependent fashion both in the absence and presence of serum. Fractions of VA extract separated using preparative reverse-phase chromatography also inhibited DNA synthesis ($P < 0.005$). These results suggest that VA vegetable, if incorporated in the diet, may prevent or delay the on-set of breast cancer. Exp Biol Med 228:293–298, 2003

Key words: human breast cancer; DNA synthesis; *Vernonia amygdalina* extract

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The use of herbal products for medicinal benefits has played an important role in nearly every culture on earth. Herbal medicine was practiced by ancient people in Africa, Asia, Europe, and the Americas (1). During the past decade, use of herbs and related products has increased from 34% in 1990 to 42% in 1995, with related out-of-pocket costs of about $27 billion (2). It is estimated that 50% of breast cancer and 37% of prostate cancer patients use herbal products (2). The recent increase in the use of herbal products (3) is associated with the belief that herbs can provide some benefits over and above allopathic medicine and allows users to feel that they have some control in their choices of medications. In the United States, one of every four deaths is caused by cancer (4). In 2001, about 1,268,000 new cancer cases were reported in the United States. Prostate cancer ranked first (198,100) followed by breast (193,700) and lung cancer (169,500) (4). An estimated 553,400 Americans died of cancer in 2001, which is more than 1500 deaths daily. Consequently, there are significant needs to improve current cancer therapies and to search for novel therapies. Plant-derived products are excellent sources for the discovery and development of new cancer chemotherapies (5). After all, many cancer chemotherapies in use today trace their origins to plant; example: Taxol, Oncovin, Navelbine, and Vumon (6).

Studies show an inverse relationship between consumption of plant products, such as fruits and vegetables, and risk for developing cancer of numerous sites. Fruits and vegetables contain chemoprotective and chemotherapeutic agents that may reduce the risk of developing cancer (7, 8). In response to the quest for search for novel cancer chemopreventive agents, studies were designed to investigate the effect(s) of *Vernonia amygdalina* (VA) leaf extract, a vegetable in the diets of many Nigerians and other West Africans (9, 10), on MCF-7 cell growth. This human cancer cell line is considered a suitable model for cancer biology and related studies (11). First, studies were conducted to test the effect(s) of VA extract on DNA synthesis in MCF-7 cells. DNA synthesis is required for cancer cell growth; hence, DNA synthesis inhibition may be considered an anticancer

activity. In this present study, it is demonstrated that concentrations (3–200 μg/ml) of VA extract potently inhibited DNA synthesis in a concentration-dependent fashion in the absence or presence of serum. So far, similar findings have started to emerge in other cancer cell lines, thus suggesting that VA extract may be active in multiple cancer cell lines. These data further suggest that VA extract is a promising agent that may be used to prevent or delay the on-set of breast cancer.

Materials and Methods

Human breast tumor cell line (Passage #165) was a generous gift from Dr. Adrian Senderowicz, NIDCR/ National Institutes of Health, who purchased them from ATCC lot #2431315 starting at the 149th Passage with a 90–100% viability. Fetal bovine serum (FBS), RPMI 1640 medium, and phosphate-buffered saline (PBS) were purchased from Gibco BRL (Grand Island, NY). BCA protein assay kits were obtained from Pierce (Rockford, IL). [^{3}H]thymidine 1 mCi/ml was purchased from ICN Pharmaceutical (Irving, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

VA Extract Preparation. Pesticide-free fresh VA leaves, collected in Benin City, Nigeria, were rinsed with cold distilled water. The leaves were soaked in cold water (1:1) overnight at 4°C before crushing leaves by a gentle means to a mixture. The mixture was then filtered through clean white gauze to remove particulate matter before filtration through a 0.45-μm filtration unit for sterilization. The resulting solution was lyophilized (500 mg) and stored at −20°C.

Preparative Reverse-Phase Chromatography (PRPC) of VA Extract. The lyophilized VA extract was dissolved in 10 ml of TRIS-HCL buffer, pH 7.0. Nine hundred milliliters of ethanol was added and then the proteins were precipitated by incubating the solution at −20°C for 24 hr. The protein pellets were collected by centrifugation at 755*g* using a Sorvall SS-34 rotor in a Sorvall RC-5B centrifuge (at −20°C for 35 min). The pellets (210 mg) were washed twice with methanol:chloroform (9:1) mixture. The pellets were then dissolved in 1 ml of 4 M guanidine HCl. Samples of the protein solution were loaded on a C_{18} column (25 mm × 250 mm), 300 Å, 5 μM, purchased from Vyadac Company (Herperia, CA) and separated by PRPC. The fractions were eluted with a linear gradient of Buffer A [0.1% trifluoroacetic acid: water (1:1000)] and Buffer B [0.1% trifluoroacetic acid in acetonitrile (1:1000)]. The gradient progressed from 5% Buffer B to 70% Buffer B in 60 min (at 10 ml/min flow rate). The mixture was then held at 70% Buffer B for an additional 6 min. A total of 22 fractions were collected between 0 and 60 min, with samples taken at 3-min intervals. The fractions were then lyophilized and kept at −20°C. The fractions were reconstituted by adding water and concentrations determined by a spectrophotometer before use.

Ion Exchange Chromatography (IEC) of PRPC Fractions. Selected samples, specifically fractions 17 (10 mg) and 19 (25.5 mg) were diluted to 125 ml with 20 mM Tris-buffer, pH 8.5, and loaded separately on a 25 mm × 150 mm TSK-DEAE (Diethylaminoethyl) column. The column was then washed with 250 ml of starting buffer, i.e., 20 mM Tris, pH 8.5. The column was next developed with an eluting buffer having a linear concentration gradient beginning with 20 mM Tris and ending with 20 mM Tris + 800 mM KCL, pH 8.5, over 55 min. The fractions were collected in 5-min intervals. This resulted in 10 subfractions for PRPC Fraction 19, and there were no detectable subfractions for Fraction 17.

Reverse-Phase Chromatography (RPC) of IEC Subfractions. Each of the 10 subfractions was individually loaded on a 10 mm × 250 mm C18 (Vydac Company). The columns were washed with 25 ml of 0.1% trifluoroacetic acid:5% acetonitrile:water (1:50:949). The columns were developed with a eluting solution with a linear concentration gradient beginning with 0.1% trifluoroacetic acid:5% acetonitrile:water and ending with 0.1% trifluoroacetic acid:80% acetonitrile:water. For each of the 10 IEC subfractions, 35 RPC subfractions were obtained. The resulting 350 RPC fractions were lyophilized and stored at −20°C before use.

Cell Culture. Human breast tumor cells (MCF-7) were seeded at a density of 4×10^4 cells in 35-mm diameter tissue culture plate and propagated in RPMI 1640 medium containing 10% FBS and 1% pen/strep/fungisome mixture and grown in a humidified incubator under an atmosphere of 95% air and 5% CO_2 at 37°C to subconfluence. Fresh medium was supplied every 48 hr. We have previously determined the doubling time of these cells to be 18 hr by growth curve studies.

DNA Synthesis Assay. DNA synthesis was determined by [^{3}H]thymidine incorporation assays as we have previously described (12, 13) in the absence and presence of 10% FBS (serum). For DNA synthesis determination in the absence of serum, cells were grown to subconfluence in medium supplemented with serum and 1% pen/strep/ fungisome mixture before overnight serum starvation for synchronization. Fresh serum-free medium was provided and then cells were treated with either different concentrations of VA extract or chromatographic fractions for 18 hr before 1 μCi/ml [^{3}H]thymidine was added to each 35-mm diameter dish for an additional 6-hr period. In contrast, in the DNA synthesis determination experiments in the presence of serum, overnight serum-starved cells were co-stimulated with fresh serum supplemented medium and VA extract. For the concentration and time-dependent effects of VA extract on DNA synthesis in the presence of serum experiments, cells were seeded at the indicated density and grown to subconfluence. Log-phase proliferating cells were then treated with different concentrations of VA extract (0–200 μg/ml) for 24, 48, 72, and 96 hr (6-hr pulse + 18, 42, 66, and 90 hr, respectively) and incubated with

1 μCi/ml [^{3}H]thymidine/35-mm diameter dish for an additional 6-hr period. All incubations were terminated by aspirating the culture medium and doing sequential washes (three times) with cold PBS, followed by the addition of 2 ml/35-mm dish ice-cold 10% TCA for 20 min at 4°C. After washing the cells three times with ice-cold water, cells were solubilized with 1 ml of 0.5 M NaOH/35-mm diameter dish at 37°C for 30 min. Upon solubilization, contents were transferred to scintillation vials, 5 ml of scintillation cocktail was added to each vial and radioactivity was determined by a scintillation counter.

Cytotoxicity Assay. Medium supplemented with 10% FBS and 1% pen/strep/fungisome mixture was aspirated from MCF-7 cells grown to about 90% confluence. Cells were washed with PBS, trypsinized, counted with a hemocytometer, and diluted with RPMI 1640 to 2.8 × 10^4 cells/ml. One hundred eighty microliters of the cell suspension (≈5 × 10^3 cells/well) was placed in 96-well microtiter tissue culture plates. Cells were incubated for 24 hr at 37°C in a 5% CO_2 incubator. Fresh medium was added after 24 hr before treatment. Twenty microliters of serial dilutions of VA extract (0, 3, 6, 12.5, and 25 μg/ml) were added column wise to the 96-well microtiter tissue plates and incubated for additional 48 hr. Cell viability assays were performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method as described by Mosmann (14). The absorbance was read at a wavelength of 550 nm using a microtiter plate reader (Bio-Tek Instruments Inc.).

Determination of IC_{50} Concentration of VA Extract. The absorbance values obtained per treatment were converted to percentage cell viability. Regression analysis was performed on the cell viability data and the resulted equation was used to compute the inhibition concentration required to produce a 50% reduction in cell viability (IC_{50}) was 5.68 ± 0.2 μg/ml using the MTT assay data and confirmed by trypan blue exclusion assay data.

Statistical Analysis. Results are expressed as the mean ± SD of values obtained in triplicate from at least three different experiments. Differences between groups were compared by Student's *t* test; *P* values less than 0.05 were considered significant. When more than two means were compared, significance was determined by one-way analysis of variance followed by multiple comparisons using the Student–Neuman–Keul's test.

Results

Cytotoxicity Assay. The result of the cytotoxic effect of VA extract on MCF-7 cells after 48-hr exposure by MTT assay is shown in Figure 1. The percentages of cell viability were 100 ± 5, 60.9 ± 7, 37.3 ± 9.2, 23.7 ± 9.5, and 19 ± 5% for 0, 3, 6, 12.5, and 25 μg/ml VA extract, respectively. The concentration of VA extract required to produce a 50% reduction in cell viability (IC_{50}) was computed by a regression analysis to be 5.68 ± 0.2 μg/ml using data from MTT assays (Fig. 1). The trypan blue exclusion stud-

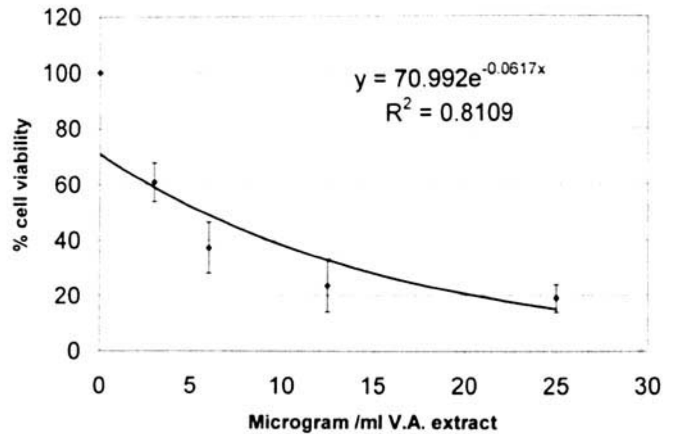

Figure 1. MTT assay. Cells were treated with various concentrations (0, 3, 6, 12.5, and 25 μg/ml) of VA for 48 hr. Cell viability was determined by MTT assay as described under the Materials and Methods section. Each data point represents the mean ± SD of results from eight individual measurements.

ies not only confirmed the IC_{50} value, but showed that while total cell number changed among treatments (0, 3, 6, 12.5, and 25 μg/ml VA) the viable cell, dead cell ratio remained constant (96–98% viability) (data not shown). Therefore, these differences in cell number were interpreted to be due to proliferation.

Inhibition of DNA Synthesis by VA Extract in the Absence of Serum. DNA synthesis is required for cancer cell growth; hence, DNA synthesis inhibition may be considered an anticancer activity. In the absence of serum intended for synchronization of cells, VA extract inhibited DNA synthesis by MCF-7 cells in a concentration-dependent fashion (3–200 μg/ml). One hundred μg/ml caused a 10-fold reduction in DNA synthesis ($P < 0.01$) whereas 200 μg/ml concentration completely inhibited DNA synthesis ($P < 0.005$; Fig. 2). To test whether VA extract could block serum-stimulated DNA synthesis, cells

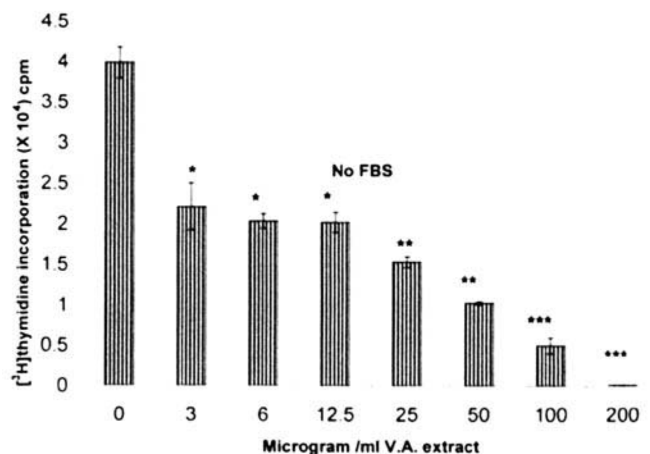

Figure 2. Inhibition of DNA synthesis by VA extract in the absence of serum. Subconfluent cells were serum-starved overnight then treated with various concentrations of VA as described under the Materials and Methods section. Each data point represents the mean ± SD of three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.

were co-stimulated with various concentrations (0–200 μg/ml) of VA in the presence of serum VA for 24 hr.

Inhibition of Serum-Stimulated DNA Synthesis by VA Extract. Serum alone markedly stimulated DNA synthesis ($P < 0.01$), but the stimulation was blocked in a concentration-dependent fashion by VA extract. Two hundred μg/ml treatment of VA completely blocked serum-stimulated DNA synthesis ($P < 0.005$; Fig. 3). To test the combined effects of VA extract concentration and time on DNA synthesis, studies were conducted to evaluate the following concentrations: 0, 3, 6, 12.5, 25, 50, 100, and 200 μg/ml for 24-, 48-, 72-, and 96-hr periods in the presence of 10% serum.

Concentration-Dependent DNA Synthesis Inhibitory Activity of VA Extract in the Presence of Serum. As shown in Figure 4, and consistent with the serum-free medium data, VA extract inhibited DNA synthesis ($P < 0.05$) in a concentration but not time-dependent fashion in the presence of serum compared to untreated cells. The magnitude of DNA synthesis inhibition was similar in all time periods studied. To identify the active ingredient(s) of the VA extract mediating these inhibitory effects on cells, crude VA extract was separated into two fractions: protein and nonprotein. Both fractions were tested for activity. The protein fraction was further fractionated by PRPC after determination that the protein fraction retained biological activity.

Selected PRPC Fractions of VA Extract Inhibit DNA Synthesis. DNA synthesis was inhibited in cells treated with PRPC fractions of VA extract compared to untreated cells. Ten fractions (8, 9, 10, 14, 15, 16, 17, 18, 19, and 20) of the 22 PRPC fractions were biologically active (DNA synthesis inhibition; data not shown). To determine the potencies of the fractions, MCF-7 cells were exposed to equal amount (100 μg/ml) of selected PRPC fractions for 24 hr. DNA synthesis inhibitory activities of the fractions were determined to be in the following order of potency (strongest to weakest) fraction 17 > 19 > 16 > 15 > 18 (Fig. 5). Fractions 17 and 19 were selected for further separation for two reasons: they were relatively most potent and adequate compared to other fractions. Fraction 19, separated by IEC followed by RPC, resulted in 350 subfractions. Fraction 17 did not yield detectable subfractions.

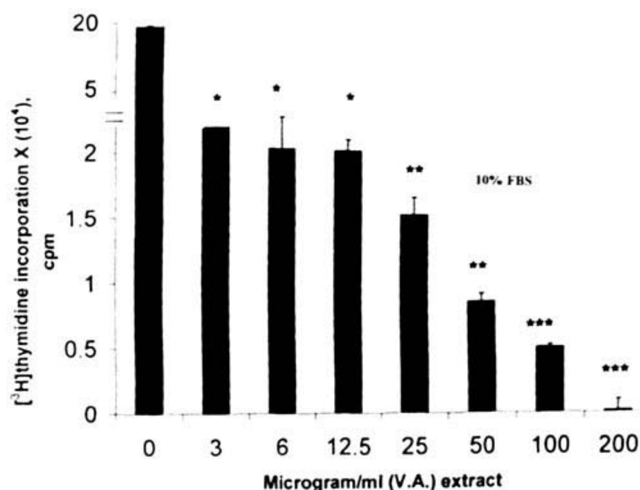

Figure 3. Inhibition of serum-stimulated DNA synthesis by VA extract. Subconfluent cells were serum-starved overnight then treated with various concentrations of VA in the presence of serum for 18 hr as described under the Materials and Methods section. Each data point represents the mean ± SD of three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.

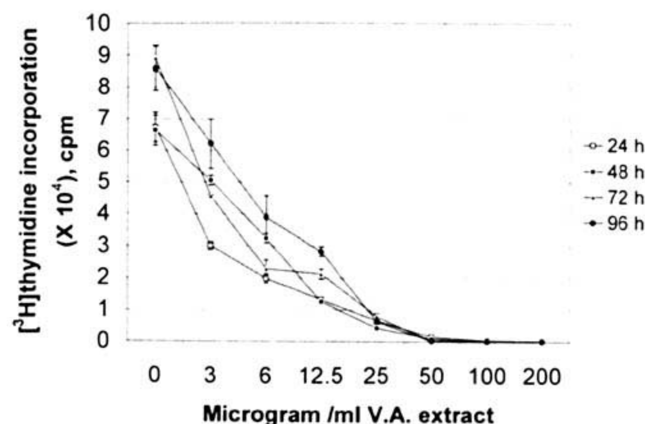

Figure 4. Concentration-dependent anticancer activity of VA extract in the presence of serum. Proliferating cells at log-phase were treated with various concentrations (0, 3, 6, 12.5, 25, 50, 100, and 200 μg/ml of VA extract in the presence of 10% serum for 24, 48, 72, and 96 hr, as described under the Materials and Methods section. The results represent the mean ± SD of three independent experiments.

RPC Fractions (Edotides) of VA Extract Inhibit DNA Synthesis. Of the 350 RPC fractions generated from fraction 19, three fractions (19-5-13, 19-5-15, and 19-5-16) now named Edotides possessed DNA synthesis inhibitory effects at 100 ng/ml concentrations which are very potent (30,000-fold) compared with more than 300 μg/ml amounts previously reported for purified plant extract fraction, deguelin (17). These three fractions inhibited DNA synthesis by 10-fold, 4-fold, and 4-fold respectively (Fig. 6).

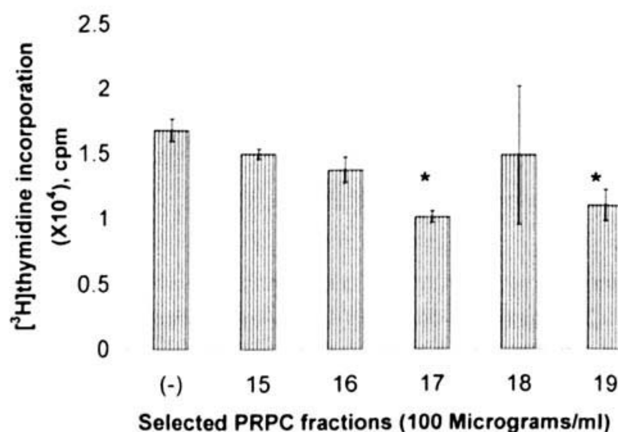

Figure 5. Selected PRPC fractions of VA extract inhibit DNA synthesis. Subconfluent cells were serum-starved overnight and then treated with selected PRPC fractions of VA extract for 18 hr, as described under the Materials and Methods section. The results represent the mean ± SD of three independent experiments. (-) untreated; *$P < 0.05$.

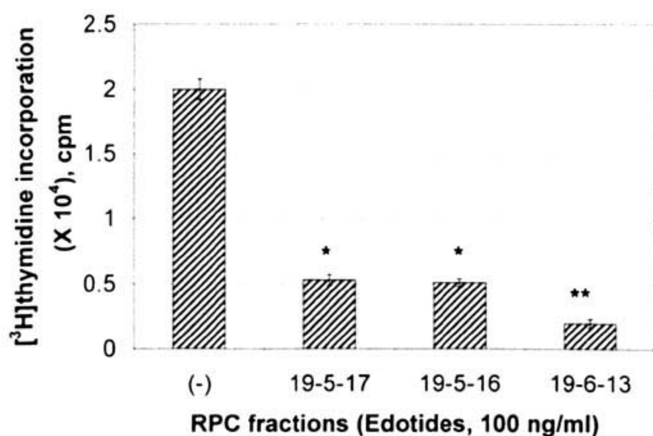

Figure 6. RPC fractions (Edotides) of VA extract inhibit DNA synthesis. Subconfluent cells were serum starved overnight and then treated with three RPC (19-5-16, 19-5-17, 9-5-13) subfractions of VA extract for 18 h, as described under the Materials and Methods section. The results represent the mean ± SD of three independent experiments. (-) untreated; $^{*}P < 0.05$; $^{**}P < 0.01$.

Discussion

The present studies demonstrate anticancer activities of both crude and fractions of a water-soluble leaf extract of VA, a vegetable consumed in large amounts by Nigerians and other West Africans (9, 10). Earlier investigators have shown that purified fractions of chloroform extract of VA elicited anticancer effects in human carcinoma of the nasopharynx (15). The process began with chloroform extraction of VA dried leaves to generate fractions A and B. Purification of A between 10% aqueous methanol and petroleum ether yielded an aqueous methanol fraction D. Fractionation of fraction D with silicic acid chromatography resulted in two cytotoxic fractions called F and H. Further chromatography of fraction H produced a colorless oil called vernodaline, while rechromatography of the cytotoxic fraction F yielded two similar crystalline compounds, vernolide and vernomygdine. These three pure fractions elicited cytotoxic effects in human carcinoma nasopharynx cells with IC_{50} values of 1.8, 2.0, and 1.5 μg/ml, respectively. The investigators concluded that the activities of these three compounds were dependent on their possessions of the α-methyl-γ-lactone group as part of their structures. Jisaka and colleagues (16) also showed that vernodaline and vernolide elicited antitumoral effects in leukemia cells P-388 and L-1210 with IC_{50} values of 0.11 and 0.17 μg/ml for vernodaline and 0.13 and 0.11 μg/ml for vernolide, respectively. The water-soluble crude extract and its sub-fractions being reported here are different from the chloroform-extracted fractions described by Kupchan and co-workers (15). In the water-soluble extraction method, lyophilized water-soluble protein fraction of VA extract separated by PRPC and followed by IEC and RPC, resulted in three fractions, edotides, which are peptides whose sequences are being determined. In comparison with vernodaline, vernolide, and vernomygdine which are toxic to 50% of the cells at approximately 2.0 μg/ml, water-soluble VA extract showed no significant cytotoxicity effects at a concentration range of 3–25 μg/ml as shown in the MTT studies (Fig. 1). Although treatment of cells with 25 μg/ml VA induced a 5-fold inhibition of cell proliferation compared to untreated cells as determined by MTT studies. This 5-fold difference may be explained by the proliferation of the untreated cells while the 25 μg/ml VA-treated cells were growth-arrested. Furthermore, percent cell viabilities were similar among treatments in trypan blue exclusion assays which suggest that 3–25 μg/ml VA treatment did not change the viable: nonviable cell ratio. Concentrations of VA higher than 25 μg/ml probably induced cytotoxic actions or apoptosis to explain the 10-fold or greater inhibition of cell proliferation shown at 100 and 200 μg/ml VA treatment in the [^{3}H]thymidine incorporation studies. The MTT assays showed that the concentration of VA extract required to inhibit the growth of 50% of the cell population (IC_{50}), computed using a regression analysis, was 5.68 ± 0.2 μg/ml, which is greater than 1400 times (>8 mg/ml) more efficacious than other plant extracts previously reported (18). These results suggest that VA extract elicited a cytostatic effect on cells *in vitro*, which may imply tumor stabilization, or preventive effects *in vivo*.

Mitogen-activated proteins kinases (MAPKs), also known as extracellular signal-regulated kinases, are serine/threonine protein kinases that are rapidly activated upon stimulation by a variety of cell surface receptors (19, 20). They function to convert extracellular stimuli to intracellular signals regulating the expression of genes important for many cellular processes, including cell growth and differentiation (21). MAPK has been implicated in ligand-independent activation of estrogen receptors (ERs), resulting in the cross-talk between growth factors and ER-mediated signaling (22). Atanaskova and co-workers (22) showed that expression of constitutively activated MAPKs activator (MEK1) in ER-positive MCF-7 cells (MEK1/MCF-7) increased ER-α-stimulated transcriptional activation and tumor growth. This suggests that activation of ER-α by MAPK was not only crucial for breast tumor growth but represents a key regulation point for tumor growth. Furthermore, the antiestrogen, breast cancer drug (tamoxifen) in use for the past decade modulates MAPK activity (22, 23). Interestingly, we now have preliminary data to show that VA is a potent inhibitor of MAPK activity (data not shown). Work is currently underway in our laboratory to further test the effects of VA on other checkpoint enzymes of the cell cycle, such as cyclin-dependent kinases and their inhibitors. In summation, these data demonstrate potent cytostatic effects of VA on human breast cancer cells. Our collaborators have also observed VA-induced cell growth inhibition in prostate cancer cell line (PC-3) and no effect on normal human peripheral blood mononuclear cells (PBMC) (data not shown). Thus, VA extract has a great potential to be used as cancer prevention agent(s).

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