# A Novel Natural Inhibitor of Extracellular Signal-Regulated Kinases and Human Breast Cancer Cell Growth

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**Water-soluble extracts of edible *Vernonia amygdalina* leaves were recently reported as potent inhibitors of cultured MCF-7 cells. The mechanism by which *V. amygdalina* inhibits MCF-7 cell growth has not been previously studied. The objective of this study was to evaluate the effects of *V. amygdalina* on the activities, DNA synthesis, and subsequent cell growth of extracellular signal-regulated protein kinases 1 and 2 (ERKs 1/2). Treatment of cells with various concentrations (3–100 μg/ml) of water-soluble *V. amygdalina* extract potently inhibited ERK activities, DNA synthesis ($P < 0.005$), and cell growth ($P < 0.01$) in a concentration-dependent fashion, both in the absence and presence of serum. The growth rate of cells pretreated with 10 μg/ml *V. amygdalina* for 48 hrs before transfer to *V. amygdalina*-free medium was not significantly different ($P > 0.05$) from untreated cells. These results suggest that *V. amygdalina*, at least at concentrations up to 10 μg/ml, exhibits cytostatic action to retard the growth of human breast cancer cells. In addition, the ERK signaling pathways may be one or more of the intracellular targets for *V. amygdalina* antineoplastic actions.** Exp Biol Med 229:163–169, 2004

**Key words:** human breast cancer; DNA synthesis; ERKs activities; *V. amygdalina* extract

Breast cancer is the most commonly diagnosed cancer in women, representing approximately 30% of all types of cancer in women (1). One out of every eight women will be diagnosed with breast cancer in her lifetime. It is estimated that in the United States in 2002, breast cancer will have accounted for more than 15% of all new cancer cases and 7% of cancer-related deaths (1). Overall, cancer mortality rates in several locations have improved in the past decade, but the incidence for some types of cancer among certain ethnic groups continues to rise. Conventional methods of treatment have yielded some benefits, but novel chemotherapeutic agents are needed to improve cancer incidence and survival rates. Plant-derived products are considered excellent sources for the discovery and development of such novel cancer chemoprotective and chemotherapeutic agents (2). During the past decade, the number of people using herbs and related products in the United States has increased from 34% in 1990 to 42% in 1995, with related out-of-pocket costs of about $27 billion (3).

Several factors have contributed to the increase in the use of herbal products. First, results from cell culture and animal model experiments suggest that some herbal products have potential for use as chemotherapeutic or chemopreventive agents for cancer (4–29). Second, the deregulating effect of the 1994 Dietary Supplement Health and Education Act, which weakens the Food and Drug Administration's ability to regulate "dietary supplements," also contributed to the increase in herbal product use. Third, the belief that herbs can provide some benefits over and above allopathic medicine also allows users to believe that they have some control in their choice of medications (30). As a result, it is estimated that 50% of patients with breast cancer use herbal products (3). A survey conducted by the World Health Organization revealed that about 80% of the world's population, in nearly all countries, still depend on herbal medicine as their main source for therapy (31).

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Herbal medicinal use is popular in Nigeria, where there are anecdotal reports on the efficacies of many herbal products against many illnesses. One such herb, *Vernonia amygdalina*, is purported to have several health benefits. More than 30 years ago, Kupchan and colleagues (32) first demonstrated that purified subfractions of organic solvent extracts of *V. amygdalina* were cytotoxic to human carcinoma cells of the nasopharynx. Twenty-four years later, Jisaka *et al.* (33) reported similar anticancer activities of purified organic solvent fractions of *V. amygdalina* against other cancerous cell lines. In the same year, Obaseiki-Ebor *et al.* (34) reported that organic solvent extracts of *V. amygdalina* inhibited ethyl methanesulfonate-induced mutations in *Salmonella typhimurium*, thus suggesting that antimutagenic activity represents one mechanism used by the organic solvent extracts of *V. amygdalina* to elicit their anticancer benefits. By the end of 2002, only two studies (32, 33) showed anticancer activities of organic solvent extracts of *V. amygdalina* or its fractions, and only one study (34) suggested a possible mechanism to explain the cancer protective effects.

The effects of water-soluble extracts of *V. amygdalina* were unknown before Izevbigie (35) reported that *V. amygdalina* inhibited the growth of human MCF-7 breast cancer cells in vitro. The latter findings may have more relevant clinical implications because water-soluble extract preparations represent the traditional method by which *V. amygdalina* is administered to patients by herbalists. In addition, mechanisms to explain the antiproliferative actions of *V. amygdalina* remain largely uninvestigated. Because many anticancer agents, including tamoxifen, share the attenuation of extracellular signal-regulated protein kinases 1 and 2 (ERKs 1/2; Refs. 36–39) activities, we sought to investigate the effects of *V. amygdalina* on ERK activities.

In this study we demonstrate that concentrations (3–100 μg/ml) of *V. amygdalina* potently inhibited DNA synthesis in a concentration-dependent fashion in the absence or presence of serum. The inhibitory effects of 10 μg/ml *V. amygdalina* were reversed by switching cells to *V. amygdalina*-free medium. We also observed that ERK activities were markedly decreased in cells exposed to *V. amygdalina* compared to those of unexposed cells, thus suggesting ERK-dependent cytostatic actions of *V. amygdalina*. These data further suggest that *V. amygdalina* shows promise for use as an agent to prevent or delay the onset of breast cancer.

## Materials and Methods

Human breast tumor cell line MCF-7 (passage #169) was a generous gift from Dr. Adrian Senderowicz, National Institute of Dental and Craniofacial Research, who purchased them from American Type Culture Collection 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 Pharmaceuticals (Irving, CA). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

***V. amygdalina* Preparation.** Pesticide-free fresh *V. amygdalina* leaves, collected in Benin City, Nigeria, were rinsed with cold, distilled water. The leaves were soaked in cold water (1:1 w/v) overnight at 4°C before being crushed 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 (5 g) and stored at −20°C.

**Cell Culture.** MCF-7 cells were seeded at a density of $4 \times 10^4$ cells in 35-mm-diameter tissue culture plates and propagated in RPMI 1640 medium containing 10% FBS and 1% pen/strep/fungisome mixture. They were then 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 hrs.

**DNA Synthesis Assay.** DNA synthesis was determined by [$^3$H]thymidine incorporation assays as we previously described (35), 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 different concentrations (3–100 μg/ml) of *V. amygdalina* for 18 hrs before 1 μCi/ml [$^3$H]-thymidine was added to each 35-mm-diameter dish, and the cells were grown for an additional 6-hr period. In contrast, for the DNA synthesis determination experiments in the presence of serum, log-phase proliferating cells were treated with different concentrations of *V. amygdalina* (3–100 μg/ml) for 18 hrs, and incubated with 1 μCi/ml [$^3$H]-thymidine/35-mm dish for an additional 6-hr period. All incubations were terminated by aspirating the culture medium, doing three sequential washes with cold PBS, followed by the addition of 2 ml/35-mm dish of ice-cold 10% TCA for 20 mins 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 dish at 37°C for 30 mins. Upon solubilization, contents were transferred to scintillation vials, 5 ml scintillation cocktail was added to each vial, and radioactivities were determined with a scintillation counter.

**Cell Proliferation Studies.** Log-phase proliferating cells were treated with different concentrations (12.5–100 μg/ml) of extracts with the appropriate controls. Twenty-four hours following treatment, triplicate 100-mm wells/treatment were randomly selected for cell number determination. The medium was aspirated from cell monolayers and washed with PBS pH 7.4 to more easily detach the cells from the substratum of the culture. The resulting

cell monolayers were treated with 1 ml trypsin/100-mm well and incubated briefly at 37°C. Cells were viewed microscopically to ensure a complete cell detachment. Cells were then resuspended in RPMI 1640 medium and counted with a hemocytometer. For the studies on cytostatic inhibition of cell growth, following cell exposure to *V. amygdalina* for 48 hrs with unexposed cells as controls, medium removal, PBS washing, trypsinization, and cell number determination, equal number of cells ($300 \times 10^3$)/100 mm plate of either exposed or unexposed cells were plated. At 24 and 48 hrs, triplicate plates per treatment were harvested and counted.

**Determination of $IC_{50}$ Concentration of *V. amygdalina*.** The absorbance values obtained per treatment were converted to percentage cell viability. Regression analysis was performed on the cell viability data and the resulting equation was used to compute the inhibition concentration required to produce a 50% reduction ($IC_{50}$) in cell viability.

**Mitogen-Activated Protein Kinase (MAPK) Assays.** Cells at approximately 80% confluence were serum-starved overnight, stimulated with different concentrations (12.5–100) μg/ml of *V. amygdalina*, and incubated at 37°C for 10 mins. After incubation, the culture medium was aspirated, cells were washed with cold PBS, and lysed in a buffer containing 20 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM ethyleneglycotetraacetic acid, 1 mM β-glycerophosphate, 1% Triton X-100, 2.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM sodium pyrophosphate, and 10 μg/ml leupeptin. Cells were scraped, transferred to Eppendorf tubes, and centrifuged. Two-hundred microliters containing 200 μg of total protein were immunoprecipitated with immobilized phospho-p44/42 kinase (Thr202/Tyr204) monoclonal antibody with gentle rocking overnight at 4°C. Pellets were recovered and washed twice with lysis buffer and twice more with kinase buffer containing 25 mM Tris pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium vanadate, and 10 mM $MgCl_2$. The pellets were suspended in 50 μl of kinase buffer supplemented with 200 μM ATP and 2 μl ELK-1 fusion protein (substrate for MAPK) and incubated for 30 mins at 37°C. The reactions were terminated by the addition of 15 μl 5× Laemmli buffer. Samples were then boiled and electrophoresed in 12% polyacrylamide gel electrophoresis. The resulting gels were transferred onto a nitrocellulose membrane in buffer containing 25 mM Tris base, 0.2 M glycine, 25% methanol pH 8.5 at 70 mA overnight.

**Western Immunoblotting.** After transfer, the membrane was washed with Tris-buffered saline (TBS) for 5 mins at room temperature, followed by incubation in blocking buffer for 2 hrs at room temperature. The membrane was then incubated with primary antibody (ELK-1 at 1:1000 dilution) in antibody dilution buffer containing TBS, 0.1% Tween-20, and 5% bovine serum albumin, with gentle agitation overnight at 4°C. The membrane was washed three times for 5 mins each with TBS-Tween (TBST), followed by incubation with a secondary antibody conjugated to horseradish peroxidase at 1:2000 dilution in blocking buffer containing TBS, 0.1% Tween, and 5% w/v nonfat dry milk, with gentle agitation for 1 hr at room temperature. Finally, each membrane was washed with TBST three times for 5 mins at room temperature.

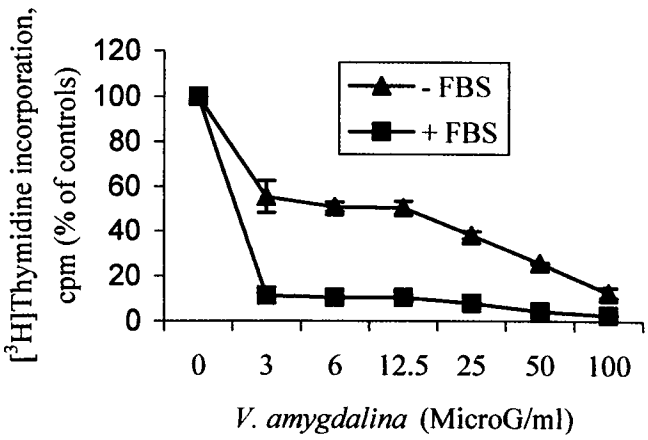


**Figure 1.** *V. amygdalina* inhibits DNA synthesis in the absence or presence of serum. Either subconfluent cells were serum-starved overnight for DNA synthesis in the absence of serum experiments or log-phase proliferating cells in the presence of serum were treated with various concentrations of *V. amygdalina* as described in "Materials and Methods." Each data point represents the mean ± SD of three independent experiments.

**Detection of Phospho ELK-1 (Serine 383).** According to the manufacturer's instructions, the membrane was incubated with 10 ml of LumiGLO (chemiluminescent reagent) with gentle agitation for 1 min at room temperature. The membrane was drained of excess developing solution, wrapped in Saran wrap, and exposed to Kodak X-OMAT AR film. Phosphorylated ELK-1 fusion protein was visualized by autoradiography and quantitated by densitometry.

**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 $< 0.05$ 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

***V. amygdalina* Inhibits DNA Synthesis in the Absence or Presence of Serum.** *V. amygdalina* exposure markedly inhibited DNA synthesis by MCF-7 cells in a concentration-dependent fashion (3–100 μg/ml) in the absence or presence of serum. The highest concentration used (100 μg/ml) caused a 7-fold growth inhibition in the absence of serum ($P < 0.01$) and 20-fold growth inhibition ($P < 0.005$) in the presence of serum compared to and expressed as percentages of controls (Fig. 1). Two different controls were used in these experiments; one received serum

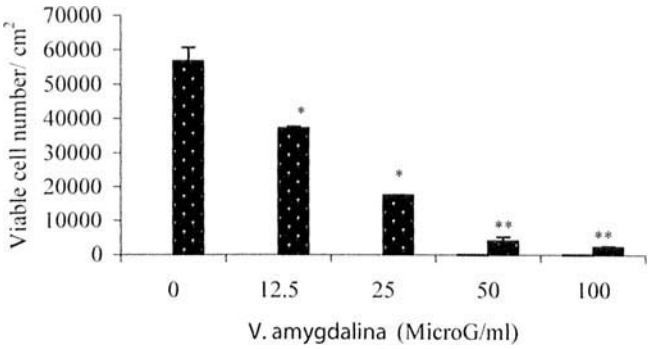


**Figure 2.** *V. amygdalina* inhibits mitosis. Log-phase proliferating cells were treated for DNA synthesis in the presence of serum with various concentrations of *V. amygdalina* as described in "Materials and Methods." Each data point represents the mean ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

(serum positive) and the other did not (serum negative). The average DNA synthesis for the serum-positive controls was approximately 5-fold that of serum-negative controls. Therefore, DNA synthesis inhibition by *V. amygdalina*, expressed as percentages of controls, were higher in the presence of serum. To confirm the DNA synthesis data presented in Figure 1, cell count studies, using a hemocytometer to determine cell number, were carried out.

***V. amygdalina* Inhibits Cell Growth.** Consistent with the DNA synthesis data shown in Figure 1, treatment of cells with *V. amygdalina* inhibited cell proliferation in a concentration-dependent fashion. Twelve-and-a-half microgram/milliliter concentration produced a 40% decrease in cell proliferation ($P < 0.05$) compared to that of untreated cells. The highest concentration of 100 μg/ml resulted in an approximately 15-fold decrease in cell proliferation ($P < 0.005$; Fig. 2).

**Effects of *V. amygdalina* Pretreatment on MCF-7 Cell Growth.** Although the DNA synthesis (Fig. 1) and cell count studies (Fig. 2) have shown cell growth inhibitory (anticancer) effects of *V. amygdalina*, we wanted to know whether such inhibition was reversible or irreversible upon drug withdrawal. Because Izevbigie (35) had previously reported the $IC_{50}$ value of *V. amygdalina* in these cells to be 5.68 ± 0.2 μg/ml, in the present studies we almost doubled the $IC_{50}$ value (10 μg/ml) to determine the effects of *V. amygdalina* pretreatment and withdrawal on cell growth.

Table 1 shows that pretreatment of cells with 10 μg/ml *V. amygdalina* for up to 48 hrs before returning cells to *V. amygdalina*-free medium did not affect subsequent cell growth ($P > 0.05$) for either 24 or 48 hrs of growth compared to that of untreated cells, thus suggesting cytostatic actions of *V. amygdalina*, at least up to 10 μg/ml. These findings are corroborated by the cytotoxicity studies previously reported by Izevbigie (35).

So far, in our estimation, *V. amygdalina* has yielded some encouraging data on the inhibition of DNA synthesis and subsequent cell growth, cytotoxicity, and mechanisms of inhibition, and thus further investigation was warranted.

**Table 1.** Effect of *V. amygdalina* Pretreatment on MCF-7 Growth[ab]

| Group | 0 | 24 hours | 48 hours |
|---|---|---|---|
| Control | 300 ± 0 (100)[c] | 351 ± 11.7 (117)[c] | 783 ± 43.6 (261)[cd] |
| *V. amygdalina* (10 μg/ml) | 300 ± 0 (100)[c] | 347 ± 14.1 (116)[c] | 770 ± 31.5 (256)[cd] |

[a] The values represent the average number of cells ± SD of three independent experiments.
[b] Values to be multiplied by $10^3$.
[c] Percent of untreated cells (control) or *V. amygdalina*-treated cells plated.
[d] $P < 0.05$ compared with either control or log-phase proliferating cells in the presence of serum. Experiments were treated with various concentrations of *V. amygdalina*, with untreated cells as controls described in "Materials and Methods." Each data point represents the mean ± SD of three independent experiments.

Next, we sought to determine the intracellular targets mediating *V. amygdalina* actions. Because the inhibition activities of ERKs 1/2 is a common characteristic shared by many anticancer agents including tamoxifen (36), paclitaxel, and estramustine (37), we chose to investigate the effects of *V. amygdalina* on ERK activities.

***V. amygdalina* Vitiates ERK Activities.** As shown in Figure 3, exposure of cells to different concentrations (12.5–100 μg/ml) of *V. amygdalina* vitiated ERK activities in a concentration-dependent fashion. The concentration of 12.5 μg/ml elicited about 40% inhibition of ERK activities. The inhibition remained steady at 25 μg/ml but became stronger at 50 μg/ml (6-fold inhibition) and again remained steady at 100 μg/ml.

**Comparison of *V. amygdalina* to Some Previously Reported Anticancer Plant Extracts Against MCF-7 Cells.** An exhaustive review of the literature revealed that about 14 crude plant extracts (excluding the purified fractions) have been studied in these cells (human breast cancerous cells or MCF-7). We thought that it would be of interest to present, in a tabular form, the efficacies ($IC_{50}$) of the 14 anticancer plant extracts previously reported by others (40) in comparison to our extract (*V. amygdalina*; Ref. 35).

Table 2 shows that the $IC_{50}$ values for 14 previously reported plant crude extracts with human breast cancerous cell growth inhibitory properties ranged from the least potent (>1.0 mg/ml; *Ligustrum lucidum*, *Taraxacum mongolici*, *Sarcandra glabra*) to the most potent of 0.1 mg/ml (*Anemarrhena asphodeloides*, *Artemisia argyi*, *Rubia cordiforlia*). In comparison, *V. amygdalina* is more than 17-fold more potent than the most potent and more than 170-fold more potent than the least potent of the plant extracts previously reported by others (40).

## Discussion

There are anecdotal reports of the biological activities of *V. amygdalina* against a wide range of human diseases. But evidence to support these health benefit claims is still

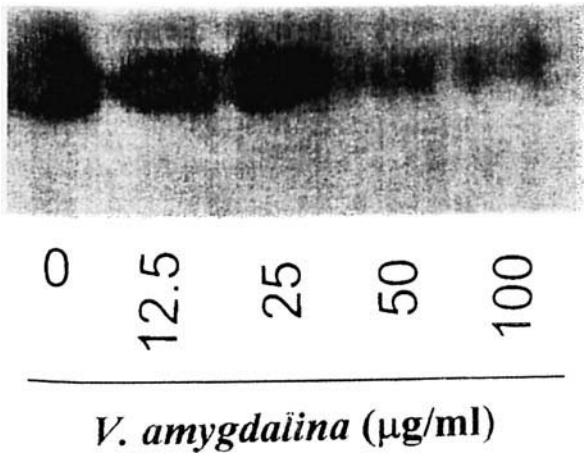


**Figure 3.** *V. amygdalina* vitiates ERK activities. Cells propagated to subconfluence were serum-starved overnight and treated with various concentrations of *V. amygdalina* (12.5–100 μg/ml) and incubated at 37°C for 10 mins. After incubation, MAPK assays were performed as described in "Materials and Methods." The result shown here is representative of at least three independent experiments.

**Table 2.** Comparison of *V. amygdalina* Anticancer Activity (35) to Some Previously Reported Plant Extracts Against MCF-7 Cells (40)

| Extract | $IC_{50}$ (mg/ml) |
|---|---|
| *Ligustrum lucidum* | >1.0 |
| *Taraxacum mongolici* | >1.0 |
| *Sarcandra glabra* | >1.0 |
| *Duchesnea indica* | >1.0 |
| *Gleditsia sinensis* | >0.8 |
| *Commiphora myrrha* | >0.7 |
| *Uncaria rhycophylia* | >0.5 |
| *Rheum palmatum* | >0.5 |
| *Salvia chinensis* | >0.5 |
| *Trichosanthes kirilowii* | >0.3 |
| *Vaccaria segetalis* | >0.2 |
| *Anemarrhena asphodeloides* | >0.1 |
| *Artemisia argyi* | >0.1 |
| *Rubia cordiforlia* | 0.1 |
| *Vernonia amygdalina* | 0.0056 |

scanty. The anticancer activity of organic extracts of *V. amygdalina* was first reported for human carcinoma cells of the nasopharynx 34 years ago by Kupchan and colleagues (32). Twenty-four years later, Jisaka and others (33) showed that components of organic extracts of *V. amygdalina*, vernodaline and vernolide, retarded the growth of P-388 and L1210 leukemia cells with $IC_{50}$ values of 0.11 and 0.17 μg/ml for vernodaline and 0.13 and 0.11 for vernolide, respectively. Obaseiki-Ebor *et al.* (34) reported an antimutagenic activity of organic solvent extracts of *V. amygdalina*. These investigators observed that *V. amygdalina* prevented the development of ethyl methansulfonate–induced mutations in *Salmonella typhimurium*. Most recently, Izevbigie (35) reported anticancer activities of *V. amygdalina* with an $IC_{50}$ value of 0.0056 ± 0.0002 mg/ml. These extracts represent the first crude extracts, with this magnitude of potency against human breast cancerous cells, reported to date.

We have now advanced our understanding of how *V. amygdalina* extracts inhibit cell growth: by actions that were reversed by the replacement of *V. amygdalina*-containing medium with *V. amygdalina*-free medium. Hence, cells exposed to 10 μg/ml of this extract for up to 48 hrs grew at the same rate after withdrawal of extracts compared to the rate of unexposed cells. This suggests that *V. amygdalina*, at least up to a concentration of 10 μg/ml, uses cytostatic actions to inhibit cell growth.

To gain insights on the intracellular targets of *V. amygdalina* extracts, we chose to study their effects on ERK activities because many breast cancer chemotherapeutic drugs modulate their activities to elicit their antineoplastic actions (36–39). ERKs are serine/threonine kinases that are rapidly activated upon stimulation of a variety of cell surface receptors (41, 42). They function to convert extracellular stimuli to intracellular signals regulating the expression of genes that are important for many cellular processes, including cell growth and differentiation (43). These kinases play a central role in mitogenic signaling, as impediment of their functions may prevent agonist-induced cell proliferation. Evidence shows that elevated ERK expression or activities are associated with breast tumorigenesis (39). In tissue samples from three groups of patients: (i) human breast tumors and their matched adjacent normal breast tissue, (ii) breast tumors and their matched lymph node metastases, and (iii) breast tumors from patients who later proved to be tamoxifen-sensitive or resistant, Adeyinka *et al.* (39) reported positive associations between ERK expression, breast tumorigenicity, and metastases compared to those of control normal cells. These finding were corroborated by reports by Wang *et al.* (44) based on studies using tumor specimens from 48 patients. Taken together, these findings suggest that cancer chemotherapeutic drugs may elicit their antineoplastic actions by attenuating ERK expression or activities, and this characteristic is shared by the *V. amygdalina* extracts.

Other investigators have also reported MAPK inhibitory activities of other natural products such as catechins, one of the active ingredients in green tea (45) and resveratrol (18). Thus, MAPKs appear to be a critical component of growth-promoting pathways.

Hitherto, a commercially available drug (PD098059), to the best of our knowledge, was the only MEK1-specific inhibitor used to block the ERK pathway for research purposes only. We now have preliminary data (not shown) to show that on an equal concentration basis, *V. amygdalina* extracts are more potent inhibitors of ERK activities than PD098059. Even more interesting, *V. amygdalina* is edible (34, 46), which makes the use of this extract as a cancer chemopreventive or a chemotherapeutic agent (or both) very

likely in the very near future. Work is currently ongoing in our laboratory to examine the effects of *V. amygdalina* on other crucial cell cycle regulators such as the cyclin-dependent kinases and their inhibitors.

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