JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antiproliferative and Apoptotic Effects of Chamomile Extract in Various Human Cancer Cells

JANMEJAI K. SRIVASTAVA[†] AND SANJAY GUPTA*[,†,‡]

Department of Urology, Case Western Reserve University, Cleveland, Ohio 44106, University Hospitals Case Medical Center, Cleveland, Ohio 44106, and Case Comprehensive Cancer Center, Cleveland, Ohio 44106

Chamomile (*Matricaria chamomilla*), a popular herb valued for centuries as a traditional medicine, has been used to treat various human ailments; however, its anticancer activity is unknown. We evaluated the anticancer properties of aqueous and methanolic extracts of chamomile against various human cancer cell lines. Exposure of chamomile extracts caused minimal growth inhibitory responses in normal cells, whereas a significant decrease in cell viability was observed in various human cancer cell lines. Chamomile exposure resulted in differential apoptosis in cancer cells but not in normal cells at similar doses. HPLC analysis of chamomile extract confirmed apigenin 7-*O*-glucoside as the major constituent of chamomile; some minor glycoside components were also observed. Apigenin glucosides inhibited cancer cell growth but to a lesser extent than the parent aglycone, apigenin. Ex vivo experiments suggest that deconjugation of glycosides occurs in vivo to produce aglycone, especially in the small intestine. This study represents the first reported demonstration of the anticancer effects of chamomile. Further investigations of the mechanism of action of chamomile are warranted in evaluating the potential usefulness of this herbal remedy in the management of cancer patients.

KEYWORDS: Prostate cancer; cancer chemoprevention; chamomile; apigenin; apigenin 7-*O*-glycoside; apoptosis

INTRODUCTION

Chamomile (*Matricaria chamomilla*) has been one of the most widely used and well-documented medicinal plants for centuries (*1*, *2*). As a traditional medicine, it is used to treat wounds, ulcers, eczema, gout, skin irritations, neuralgia, sciatica, rheumatic pain, hemorrhoids, mastitis, and other ailments (*3*–*5*). Externally, chamomile has been used to treat diaper rash, cracked nipples, chicken pox, poison ivy, and conjunctivitis (*6*, *7*). On the basis of its broad-spectrum anti-inflammatory, antioxidant, and mild astringent properties, German Commission E has approved chamomile for use for inflammation of the skin and mucous membranes and for various bacterial infections of the skin, oral cavity, gums, and respiratory tract (*8*). In recent years, chamomile has become increasingly popular in a form of tea which is consumed at a rate of more than 1 million cups per day. Chamomile, in the form of aqueous extract, has been frequently used as a mild sedative to calm nerves and reduce anxiety and to treat hysteria, nightmares, insomnia, and other sleep problems (*9*). Chamomile has been valued as a digestive relaxant and has been used to treat various gastrointestinal disturbances, including flatulence, indigestion, diarrhea, anorexia, motion sickness, nausea, and vomiting (*4*, *10*). Other purported actions of this herb include antiulcer, antibacterial, liver stimulatory, and antimycotic effects (*11*–*13*). In children, chamomile has been used to treat colic, croup, and fevers. In women, it has been used as an emmenagogue and a uterine tonic. Chamomile's essential oil is also used as a treatment for malaria and parasitic worm infections, cystitis, colds, and flu (*14*, *15*).

The amount of research into the utility of chamomile continues to increase because of its reported biological activities, which appear to be related to its content of several classes of bioactive compounds. The demonstrable effects of chamomile are caused by the presence of both lipophilic and hydrophilic compounds. Aqueous standardized extract of chamomile flowers contains approximately 1.2% apigenin and 0.5% essential oils. Other major constituents of the flowers include several phenolic compounds, primarily the flavonoids apigenin, quercetin, and patuletin as glucosides and various acetylated derivatives (*16*, *17*). The principal components of the essential oil extracted from the chamomile flowers are the terpenoids α-bisabolol and its oxide, azulenes, including chamazulene and acetylene derivatives (*18*). To date, most of the studies on chamomile have focused on the characterization and assessment of the biological activity of individual extractable components. For example, flavonoids isolated from chamomile, such as apigenin and luteolin, have been shown to possess anti-inflammatory, anti-

* To whom correspondence should be addressed: Department of Urology, Case Western Reserve University, University Hospitals Case Medical Center, 10900 Euclid Ave., Cleveland, OH 44106. Phone: (216) 368-6162. Fax: (216) 368-0213. E-mail: sanjay.gupta@case.edu.

[†] Case Western Reserve University and University Hospitals Case Medical Center.

[‡] Case Comprehensive Cancer Center.

10.1021/jf071953k CCC: $37.00
Published on Web 10/17/2007

carcinogenic, carminative, antispasmodic, and mild sedative properties. Terpenoids, bisabolol, and chamazulene have been shown to possess anti-inflammatory, antiallergic, antispasmodic, antibacterial, antipyretic, ulcer-protective, and antifungal properties (*9*, *19*). Several studies of chamomile have shown that it has anti-inflammatory and antimutagenic effects, and cholesterol-lowering activities (*20–24*). However, the anticancer properties of chamomile have not been evaluated. In this study, we investigated the anticancer properties of aqueous and methanolic extracts derived from *M. chamomilla* using cell lines derived from various human cancers. Our study is the first to demonstrate that chamomile plant extract suppresses growth of human cancer cells and causes apoptosis.

MATERIALS AND METHODS

Chemicals. Dry chamomile flowers were purchased from Baroody Imports Inc. (Clifton, NJ). Cell culture medium, RPMI 1640, DMEM and keratinocyte serum free medium, fetal bovine serum, penicillin–streptomycin cocktail, and phosphate-buffered saline were purchased from Cellgro Mediatech, Inc. (Herndon, VA). All other reagents were purchased from Sigma (St. Louis, MO) and were of analytical reagent grade or HPLC grade where applicable. Apigenin, apigenin 7-*O*-glucoside, apigenin 7-*O*-neohesperidoside, and apiin (>95% pure) were obtained from Sigma. Tris-EDTA buffer and TBE buffer were procured from Fisher Scientific (Pittsburgh, PA).

Preparation of Extracts. Dry chamomile flowers were weighed and crushed to powder with a marble pestle and mortar, and a 5% (w/v) suspension was prepared in a flask by adding hot boiled water. The flask was then placed on a shaker (200 rpm) for 4 h, and the temperature was maintained at 37 °C. After being shaken, the flask was brought to room temperature, and the suspension was filtered through a series of Whatman filters and finally passed through a 0.22 μm filter (Millipore, Billerica, MA). The filtered aqueous extract was freeze-dried and stored at −20 °C until it was used. For cell culture studies, the material was weighed and dissolved in culture medium. Similarly, powdered chamomile was also soaked in methanol [5% (w/v)] for 4 h on a shaker, filtered, and evaporated at room temperature in Petri dishes. The dried material was retrieved and stored in tubes at −20 °C until it was used. Methanolic extract was dissolved in dimethyl sulfoxide (DMSO) to prepare a 100 mg/mL stock solution; this was later mixed with the culture media to achieve the desired concentration.

Cell Lines and Culture. Human prostate cancer cells derived from different metastatic sites were studied, including androgen-responsive LNCaP (originally obtained from metastatic prostate cancer in a supraclavicular lymph node), androgen-refractory PC-3 (originally obtained from metastatic prostate cancer in bone), and DU145 (originally obtained from metastatic prostate cancer in brain). In addition, virally transformed PZ-HPV-7 cells were studied, derived from normal tissue of the peripheral zone of the prostate, and immortalized by transfection with HPV 18 virus; these were obtained from American Type Culture Collection (Manassas, VA). The LNCaP cells were cultured in RPMI 1640 medium with 10% FBS and 1% penicillin–streptomycin cocktail; DU145 and PC3 cells were cultured in RPMI 1640 medium with 5% FBS and 1% penicillin–streptomycin cocktail at 37 °C in a humidified atmosphere of 5% CO_2. The PZ-HPV-7 cells were cultured in keratinocyte serum free medium supplemented with 5 ng/mL human recombinant EGF and 0.05 mg/mL bovine pituitary extract (Gibco, Carlsbad, CA). Other non-prostate cancer cell lines, viz., HeLa (cervical adenocarcinoma), T-47D (breast carcinoma), RKO (colon carcinoma), and HT 1080 (fibrosarcoma), were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin–streptomycin cocktail.

Proliferation Assay. The effect of chamomile on the viability of cells was determined with a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, the cells were plated at a density of 1×10^4 cells/well in 200 μL of complete culture medium containing 1000, 2000, and 4000 μg/mL freeze-dried aqueous extract in 96-well microtiter plates. A stock solution of methanolic extract was prepared in DMSO and diluted with the culture media to achieve final concentrations of 100, 200, and 400 μg/mL. The concentration of DMSO remained within the maximum permissible concentration of 0.1% in both control and treated samples. Each concentration of chamomile was repeated in 10 wells. After incubation for the desired period of time at 37 °C in a humidified incubator, cell viability was assessed. MTT (50 μL, 5 mg/mL in phosphate-buffered saline stock, diluted to a working strength of 1 mg/mL with media) was added to each well and incubated for 2 h, after which the plate was centrifuged at 600*g* for 5 min at 4 °C. The MTT solution was removed from the wells by aspiration. After careful removal of the medium, 0.1 mL of buffered DMSO was added to each well, and plates were shaken. The absorbance was recorded on a microplate reader at a wavelength of 540 nm. The effect of chamomile on growth inhibition was assessed as percent cell viability where vehicle-treated cells were taken to be 100% viable. Pure apigenin and its analogues, viz., apigenin 7-*O*-glucoside, apigenin 7-*O*-neohesperidoside, and apiin, were also evaluated for their efficacy at doses ranging from 50 to 250 μM for 24 h.

DNA Fragmentation Assay. Fragmentation of chromatin to units of single or multiple nucleosomes that form the nucleosomal DNA ladder in agarose gel is an established hallmark of programmed cell death or apoptosis (*25*). Briefly, the cells were grown to 70% confluence and treated with 2000 μg/mL aqueous and 200 μg/mL methanolic chamomile extracts for 48 h. Following this treatment, the cells were washed twice with phosphate-buffered saline [10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, and 0.5% Triton X-100], left on ice for 15 min, and pelleted by centrifugation (14000*g*) at 4 °C. The pellet was incubated with DNA lysis buffer [10 mM Tris (pH 7.5), 400 mM NaCl, 1 mM EDTA, and 1% Triton X-100] for 30 min on ice and then centrifuged at 14000*g* and 4 °C. The supernatant that was obtained was incubated overnight with RNase (0.2 mg/mL) at room temperature and then with proteinase K (0.1 mg/mL) for 2 h at 37 °C. DNA was extracted using a phenol/chloroform mixture (1:1) and precipitated with 95% ethanol for 2 h at −80 °C. The DNA precipitate was centrifuged at 14000*g* and 4 °C for 15 min, and the pellet was air-dried and dissolved in 20 μL of Tris-EDTA buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. The total amount of DNA was resolved over a 1.5% agarose gel, containing 0.3 mg/mL ethidium bromide in 1× TBE buffer [89 mM Tris (pH 8.3), 89 mM boric acid, and 2 mM EDTA] (BioWittaker, Inc., Walkersville, MD). The bands were visualized under an UV transilluminator followed by digital photography.

Cell Death Detection Assay. Following chamomile treatment, the extent of apoptosis was determined by a Cell Death Detection ELISA^{PLUS} assay (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's protocol. Briefly, the cells were harvested after treatment with 2000 μg/mL aqueous and 200 μg/mL methanolic chamomile extracts for 48 h, incubated on ice for 30 min in Tris lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 0.5% NP-40, and 1% Triton X-100] containing fresh protease inhibitors (5 μg/mL aprotinin, 10 μg/mL phenylmethanesulfonyl fluoride, and 10 μg/mL sodium vanadate), and then centrifuged at 14000*g* for 10 min at 4 °C. The total cell lysate was used for the assessment of protein by the DC Bio-Rad protein assay. The lysate (30 μg of total protein) were added to lysis buffer and pipetted on a streptavidin-coated 96-well microtiter plate to which immunoreagent mix was added and incubated for 2 h at room temperature with continuous shaking at 600*g*. The wells were then washed with washing buffer; the substrate solution was added, and the color that developed (10–20 min) was read at 405 nm against a blank, reference wavelength of 490 nm. The enrichment factor (total amount of apoptosis) was calculated by dividing the absorbance of the sample (A_{405}) by the absorbance of the controls without treatment (A_{490}) which was equal to 1.

Detection of Apoptosis by Fluorescence Microscopy. Cells were grown in eight-chamber slides (Nunc-Labtek, Nunc, Naperville, IL) and were treated with 2000 μg/mL aqueous and 200 μg/mL methanolic chamomile extracts for 24 h. Following treatment, the cells were washed with PBS and processed for detection of apoptosis by using Cytokeratin 18 (CK18) monoclonal antibody, which is an early marker for apoptosis. The fluorescence analysis was performed under the BX51 Olympus microscope by using an M 30 assay kit (Roche Applied Sciences, Mannheim, Germany), according to the vendor's protocol.

HPLC Analysis. All standards and all aqueous and methanolic extracts of chamomile were analyzed on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) using a C-18 column. The mobile phase consisted of acetonitrile and water as the isocratic solvent (30:70, v/v) maintained at a flow rate of 1 mL/min with an injection volume of 5 μL and a run time of 8 min. Data were collected at 335 nm (λ_{max} for the majority of the apigenin glucosides).

Mass Spectrometric Analysis of Apigenin and Its Derivatives. Electrospray ionization tandem mass spectrometry was used to identify apigenin and its derivatives in HPLC fractions. In brief, HPLC fractions were dissolved in 50% methanol and introduced onto a Quattro Ultima triple-quadruple mass spectrometer (Micromass, Inc., Beverly, MA) at a rate of 50 μL/min and analyzed using electrospray ionization in both negative and positive ion modes. Apigenin and its derivatives were identified using both full and product scans. The capillary and cone voltages were set at 3.5 kV and 50 V, respectively. The desolvation and cone temperatures were set at 250 and 120 °C, respectively. The nitrogen gas flow rates for desolvation and cone were 600 and 80 L/h, respectively. Collision-induced dissociation was achieved using argon gas.

Ex Vivo Conversion of Apigenin 7-*O*-Glucoside into Apigenin. To assess the conversion of chamomile glucosides to aglycone by mammalian glucosidase enzyme, this ex vivo experiment was performed. Normal mouse intestine and liver were excised and flushed with cold HBSS. Several pieces (1 cm × 1 cm) of small intestine and liver were chopped and individually dipped into Petri dishes containing HBSS supplemented with 100 μg/mL methanolic extract of chamomile and incubated at 37 °C in a cell incubator. For assessment of conversion, 200 μL of buffer medium was collected from each Petri dish at 0, 1, 2, and 3 h. Similarly, the chopped tissues were collected at time intervals, washed with fresh HBSS, and homogenized in PBS. A portion of homogenate was aliquoted, deproteinized via addition of 0.4 mL of methanol, vortex-mixed for 60 s, and centrifuged at 2200*g* for 15 min at 4 °C. The supernatant was collected and subjected to HPLC analysis.

Statistical Analysis. The values are expressed as means ± the standard error. The significance between the control and treated groups was determined by Student's *t* test, and *p* values of less than 0.05 were taken to be significant in the experiments.

RESULTS

In this study, we first evaluated the growth inhibitory effects of chamomile in virally transformed normal human prostate epithelial PZ-HPV-7 cells and various human prostate cancer cells, viz., LNCaP (androgen-responsive; p53 wild type), DU145 (androgen-refractory; p53 mutant), and PC-3 (androgen-refractory; p53 null) cells, which represent various stages of disease progression. Exposure of PZ-HPV-7 cells to chamomile resulted in a modest decrease in cell viability. As shown in **Figure 1A**, exposure to chamomile at the largest doses of 4000 μg/mL aqueous extract resulted in a 5.6–9.7% decrease in cell viability between 24 and 72 h, whereas a 7.8–10.4% decrease in cell viability between 24 and 72 h was observed at 400 μg/mL methanol extract. In sharp contrast, all three cancer cell lines that were tested were more sensitive to chamomile-mediated loss of cell viability which occurred with smaller doses and was much more pronounced than the effects observed in PZ-HPV-7 cells. Aqueous chamomile treatment for 24 h resulted in a dose-dependent reduction in cell viability which ranged from 9.8 to 37.2% in LNCaP cells, from 6.7 to 35.2% in DU145 cells, and from 8.6 to 33.4% in PC-3 cells at concentrations of 1000–4000 μg/mL. The IC_{50} values in these cell lines after they had been exposed to aqueous chamomile extract for 72 h ranged from 2000 to 3000 μg/mL. Similar growth inhibitory responses in all three prostate cancer cells were noted for methanolic extract, but at lower concentrations. A decrease in cell viability which ranged from 25.4 to 61.9% in LNCaP cells, from 14.7 to 47.5% in DU145 cells, and from 16.3 to 55.6% in PC-3 cells was observed at concentrations of 100 to 400 μg/mL. The IC_{50} values in these cell lines range from 100 to 200 μg/mL. More pronounced growth inhibitory effects were noted 48 and 72 h after chamomile treatment in all three prostate cancer cell lines. To evaluate the anticancer activity of chamomile in other types of cancer, we next evaluated the antiproliferative effects on various human cancer cell lines, viz., HeLa (cervical adenocarcinoma), HT1080 (fibrosarcoma), RKO (colon carcinoma), and T-47D (breast carcinoma) (**Figure 1B**). The IC_{50} values after exposure for 72 h to aqueous chamomile extract ranged from 1650 to 4000 μg/mL and from 165 to 300 μg/mL for methanolic extract. The order of IC_{50} values was as follows: T-47D > HT1080 > HeLa > RKO. Overall, these observations suggest that chamomile causes differential cell growth inhibitory responses in cancer versus normal cells.

We next investigated whether chamomile-mediated loss of cell viability in human prostate cancer cells is a result of apoptosis. We first evaluated the induction of apoptosis by chamomile via a classical DNA ladder assay. Compared to vehicle-treated controls, exposure of LNCaP, DU145, and PC-3 cells to 2000 μg/mL aqueous and 200 μg/mL methanolic chamomile extract for 48 h resulted in induction of apoptosis as evidenced by the formation of internucleosomal DNA fragments (**Figure 2A**). Importantly, it was noted that chamomile exposure did not result in the formation of a DNA ladder in PZ-HPV-7 cells, conforming to a selective dose–response effect compared to prostate cancer cells (data not shown). To demonstrate that chamomile exposure causes apoptosis in cancer cells, we performed cell death detection with an ELISA. Compared to vehicle-treated controls, 3.1-, 2.5-, and 2.3-fold increases in the level of induction of apoptosis were observed with aqueous chamomile, whereas 2.8-, 2.7-, and 3.0-fold increases in the level of apoptosis were noted with methanolic extract in LNCaP, DU145, and PC-3 cells, following treatment for 48 h (**Figure 2B**). To further investigate the induction of apoptosis by chamomile, fluorescence microscopy was employed after the cells had been labeled with M30 CytoDEATH, an antibody that binds to a caspase-cleaved epitope of the cytokeratin 18 cytoskeletal protein as a marker for apoptosis. As shown in **Figure 2C**, exposure to 2000 μg/mL aqueous and 200 μg/mL methanolic chamomile extract for 24 h resulted in apoptosis in all three prostate cancer cell lines, compared to vehicle-treated controls. These results suggest that chamomile causes apoptosis in cancer cells and spares normal cells.

Next we performed HPLC analyses of the aqueous and methanolic extracts of chamomile. Previous studies have identified most of the characteristic constituents of chamomile which include volatile oil, sesquiterpene lactones, and phenolics, including flavonoids (*15*, *16*, *26*). We performed a HPLC scan at wavelengths ranging from 200 to 590 nm to determine whether various constituents were present (**Figure 3A**). Altogether, a total of 10 peaks were recorded in aqueous chamomile extract during the scanning process, most of which correspond to water soluble flavonoids. Since the major flavonoids previously identified in chamomile were apigenin and their derivatives, we narrowed the wavelength scan between 335 and 360 nm, which identified two major peaks with retention times of 1.15 min (27.7%) and 1.52 min (63.3%) and five other minor peaks which together constitute 9% of the total flavonoids. In a comparison with the standard flavonoids (apigenin, apigenin 7-*O*-glucoside, apigenin 7-*O*-neohespridoside, and apiin), the two major peaks in the aqueous chamomile extract correspond to apigenin 7-*O*-neohespridoside (27.7%) and apigenin 7-*O*-glucoside (63.3%) (**Figure 3B**). Similarly, the methanolic

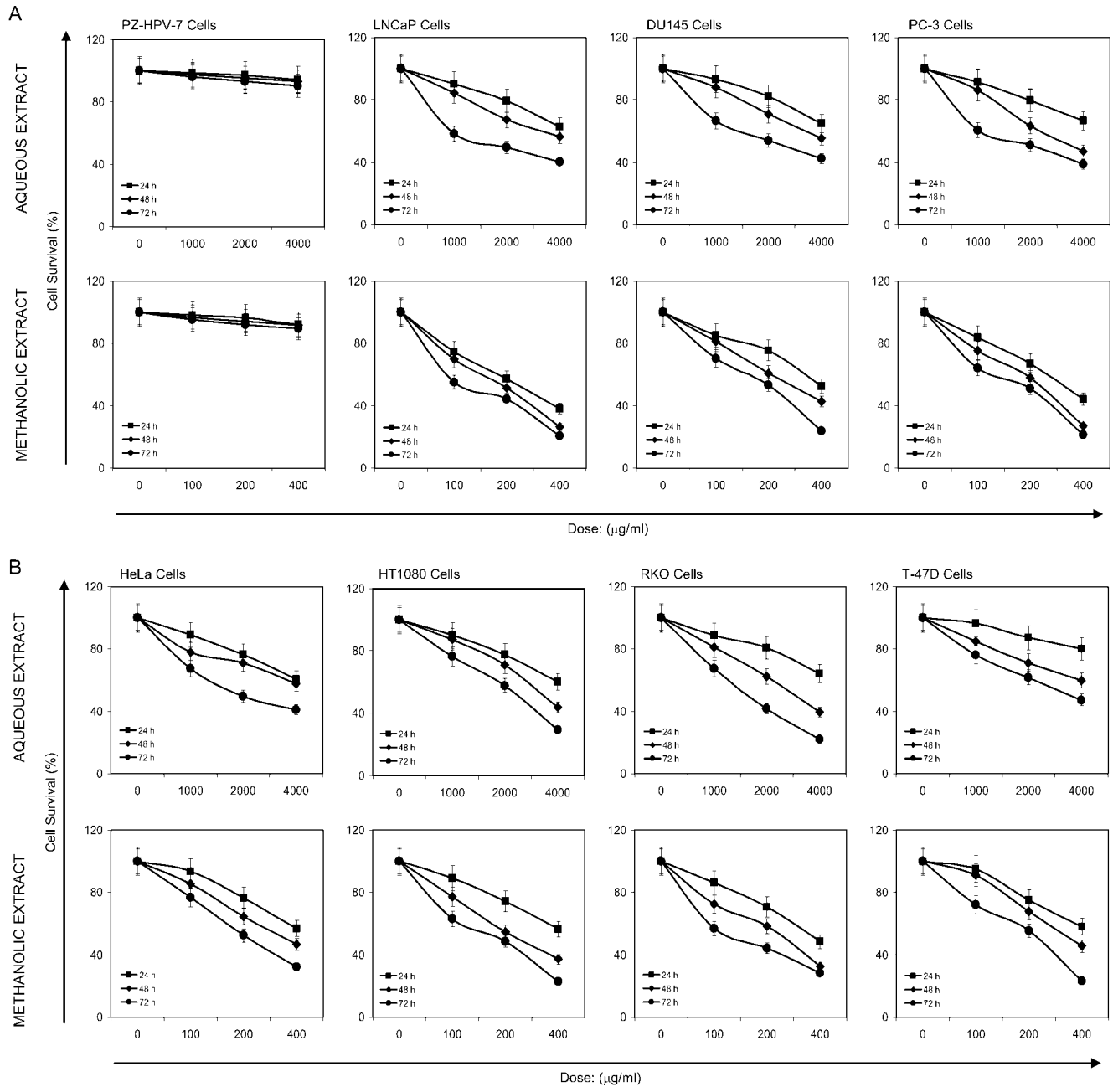

Figure 1. Effect of aqueous and methanol extracts of chamomile on cell viability: (**A**) virally transformed normal human prostate epithelial PZ-HPV-7 cells and human prostate cancer LNCaP, DU145, and PC-3 cells and (**B**) HeLa (cervical adenocarcinoma), HT1080 (fibrosarcoma), RKO (colon carcinoma), and T-47D (breast carcinoma) cells. The cells were exposed to the specified concentration of chamomile extracts for various time intervals, and the viability of the cells was determined by the MTT assay. Cell viabilities are shown as percentages, and the untreated cells were regarded as 100% viable. Data represent the means of the three experiments conducted in triplicate. Details are given in Materials and Methods.

chamomile extract yielded a total of 21 peaks between 200 and 590 nm. It is apparent that the use of methyl alcohol as a solvent extracted both hydrophilic and lipophilic constituents. Further scanning of the extract between 335 and 360 nm resulted in the detection of nine peaks. The two major peaks with retention times of 1.183 and 1.587 min correspond to apiin (24.6%) and apigenin 7-*O*-glucoside (56.8%), respectively, whereas the seven remaining minor peaks constitute 18.6% of the unidentified flavonoids and their derivatives (**Figure 3A,B**).

To further characterize the presence of various apigenin glucosides, we performed LC–MS analyses of both aqueous and methanolic extracts of chamomile. Both aqueous and methanolic chamomile extracts showed the presence of a mixture of several apigenin glucosides and parent aglycone as previously demonstrated (*15*, *16*). The most notable were apigenin 7-*O*-glucoside and apigenin 7-*O*-neohespiridoside in both chamomile fractions (**Figure 4B,C**). Since the major constituents identified in the aqueous chamomile extracts are apigenin 7-*O*-glucoside (63.3%) and apigenin 7-*O*-neohespridoside (27.7%) and the major constituents in methanolic extracts are apigenin 7-*O*-glucoside (56.8%) and apiin (24.6%), we evaluated the growth inhibitory effects of these individual constituents on various human prostate cancer cell lines. As shown in **Figure 5A**, treatment of all three cell lines with apigenin, apigenin 7-*O*-glucoside, apigenin 7-*O*-neohespridoside, and apiin for 24 h resulted in dose-dependent reductions in cell viability. Treatment of LNCaP cells with apigenin for 24 h produced the greatest dose-dependent response, from 67.6 to 98.0% cell growth inhibition at concentrations ranging from 50 to 250 μM followed by those of apigenin 7-*O*-glucoside (from 25.8 to 67.4% inhibition), apigenin 7-*O*-

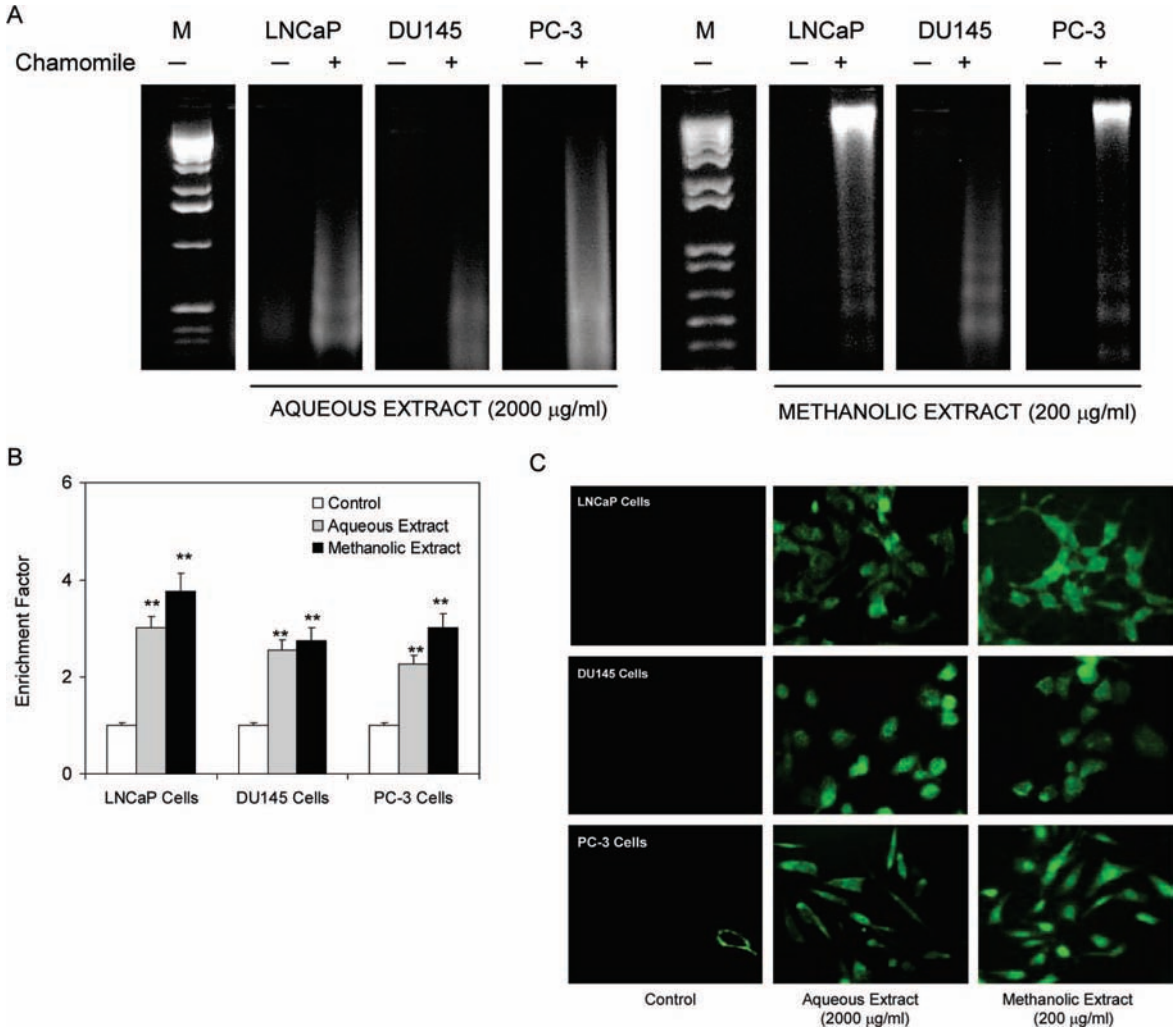

Figure 2. Effect of aqueous and methanol extracts of chamomile on induction of apoptosis in human prostate cancer LNCaP, DU145, and PC-3 cells. (**A**) DNA fragmentation assay. The cells were treated without or with 2000 μg/mL aqueous and 200 μg/mL methanolic chamomile extract for 48 h, collected for DNA isolation, and subjected to agarose gel electrophoresis, followed by visualization of bands and polaroid photography. (**B**) Apoptosis determined by a Cell Death ELISA, according to the vendor's protocol, after exposure of cells for 48 h to chamomile extract. Data are expressed as enrichment factor. Values represent means $\pm$ the standard error of three different assays in duplicate ($p < 0.001$). (**C**) Immunofluorescence detection of apoptosis by M30 CytoDEATH antibody that binds to the caspase-cleaved epitope of the cytokeratin 18 cytoskeletal protein, a marker of apoptosis. A marked increase in M30 fluorescence was observed in the cells exposed to chamomile for 24 h. A representative figure from each group at a magnification of 80$\times$ is shown here. Details are given in Materials and Methods.

neohespridoside (from 12.4 to 36.7% inhibition), and apiin (from 9.8 to 41.3% inhibition). In DU145 cells, treatment with these constituents resulted in levels of cell growth inhibition ranging from 65.5 to 98.2% with apigenin followed by 35.4 to 77.2% with apigenin 7-*O*-glucoside, 22.7 to 44.4% with apigenin 7-*O*-neohespridoside, and 11.2 to 37.8% with apiin. In PC-3 cells, the level of cell growth inhibition observed with apigenin ranged from 42.6 to 97.6%, followed by 13.6 to 65.5% with apigenin 7-*O*-glucoside, 10.2 to 37.3% with apigenin 7-*O*-neohespridoside, and 5.7 to 34.0% with apiin at concentrations ranging from 50 to 250 μM for 24 h. The cell growth inhibition with these constituents at 150 μM correlated with induction of apoptosis: apigenin exhibited the highest levels of apoptosis induction of all three prostate cancer cell lines after exposure for 48 h (**Figure 5A,B**). Since all the test agents contain apigenin bound to glucoside moiety, these results suggest that cell growth inhibition and apoptosis observed in cancer cells exposed to chamomile extract might be due to the presence of apigenin, which has been shown to possess anticancer activity (*12*).

Flavonoid glucosides are mostly unmodified during extraction, although studies have shown that aglycone is released by fermentation, autolysis, and deglycosylation via β-glucosidase (*27*). β-Glucosidase is an enzyme that can effectively deglycosylate various flavonoid glycosides. This enzyme is abundant in the mammalian liver, kidney, and small intestine (*28*). Bearing this in mind, next we conducted experiments to evaluate whether various apigenin glucosides may be converted after intestinal absorption into apigenin, the active flavonoid identified in the systemic circulation (*29*). For these studies, we dissected small portions of small intestine and liver from mice and incubated these tissue samples with aqueous and methanol extracts of chamomile for up to 3 h along with phosphate buffer, HBSS. As shown in **Figure 6A**, a representative chromatogram from 0 and 3 h with methanolic chamomile extract incubated with portions of small intestine demonstrated the conversion of apigenin 7-*O*-glucoside to apigenin by the hydrolysis of the β-glucoside bond. Approximately 49.9% conversion of apigenin 7-*O*-glucoside was noted in the small intestine homogenate after incubation with methanolic extract. In addition, 4.2% conversion was also observed in the buffer media probably due to the release of the enzyme. A lower rate of deglycosylation was observed with liver portions incubated with methanolic cham-

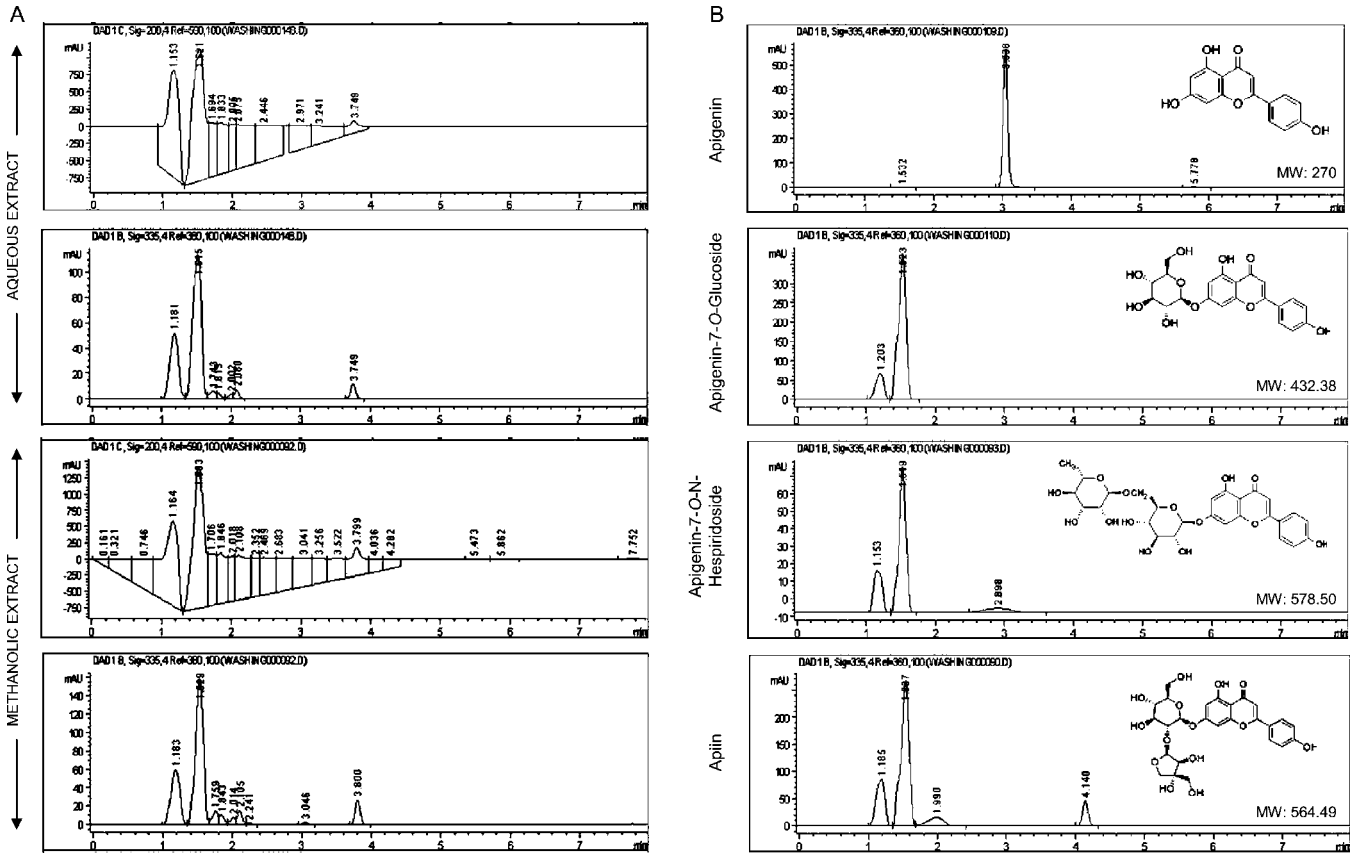

Figure 3. HPLC chromatograms of the (**A**) aqueous and methanol extracts of chamomile demonstrating the presence of various constituents and (**B**) standards of apigenin, apigenin 7-*O*-glucoside, apigenin 7-*O*-neohespridoside, and apiin monitoring the relative absorbance at 335 nm on the same scale. The major constituents observed in aqueous chamomile extracts are apigenin 7-*O*-glucoside and apigenin 7-*O*-neohespridoside and in methanolic extracts apiin and apigenin 7-*O*-glucoside.

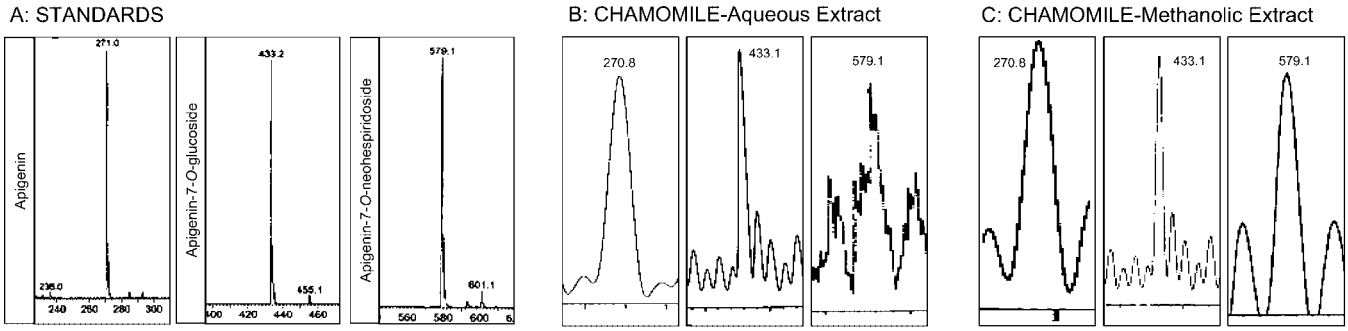

Figure 4. Electrospray product ion mass spectra. (**A**) Apigenin (MW = 270; precursor ion *m*/*z* 271), apigenin 7-*O*-glucoside (MW = 432; precursor ion *m*/*z* 433), and apigenin 7-*O*-neohespridoside (MW = 578; precursor ion *m*/*z* 579). (**B**) Flavonoids extracted from the aqueous chamomile. (**C**) Methanolic extract of chamomile confirming the presence of the most abundant protonated molecular ion in the extracts.

omile extracts (data not shown). The rate of glycosylation in these samples suggests that the small intestine is more active in this hydrolysis than the liver (**Figure 6B**). These results suggest that the alimentary canal and specifically the intestinal mucosa play an important role in the deglycosylation of flavonoids and their conversion into aglycone which is bioactive and exerts anticancer activity.

DISCUSSION

The rationale for selection of chamomile for evaluation of its anticancer activity is threefold. (i) It has been used widely for many years as a traditional folk medicine, and it has been shown to exert effects on various human illnesses. (ii) It is present in many components of a healthy diet that is rich in fruits and vegetables and in a commonly consumed beverage. (iii) It contains several classes of plant flavonoids and phenols which have been suggested to play preventive roles in cancer (*30*, *31*). To date, many bioactive compounds have been identified that show antioxidant, anti-inflammatory, and in some instances anticancer effects (*32*). In our studies, the aqueous and methanolic extracts of chamomile rich in flavonoids cause cell growth inhibition and induce apoptosis differentially in cancer cells. Apoptosis is a well-identified biological response exhibited by cells after suffering DNA damage and is a useful marker for screening compounds for subsequent development as possible anticancer agents. We exposed human prostate cancer cells and cell lines from other types of cancer to either chamomile extracts or standard apigenin glucosides. The cell growth inhibitory effects of these plant extracts support the notion that glucosides must be converted to aglycone to exert

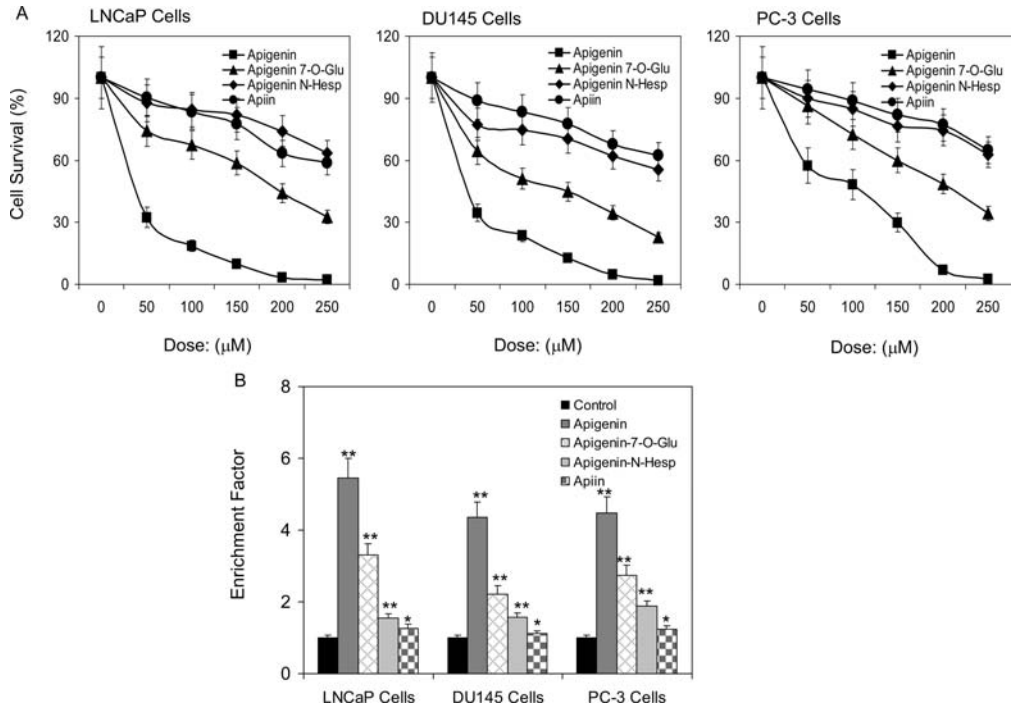

Figure 5. Effect of apigenin, apigenin 7-*O*-glucoside, apigenin 7-*O*-neohespridoside, and apiin on (**A**) cell viability and (**B**) apoptosis in human prostate cancer LNCaP, DU145, and PC-3 cells. The cells were exposed to the specified concentration of these compounds for 24 h, and the viability of the cells was determined by the MTT assay. Apoptosis was assessed by an ELISA after the cells had been exposed to concentrations of these agents of 150 μM for 48 h. Values represent means $\pm$ the standard error of three different assays in duplicate (two asterisks, $p < 0.001$; and one asterisk, $p < 0.05$). Details are given in Materials and Methods.

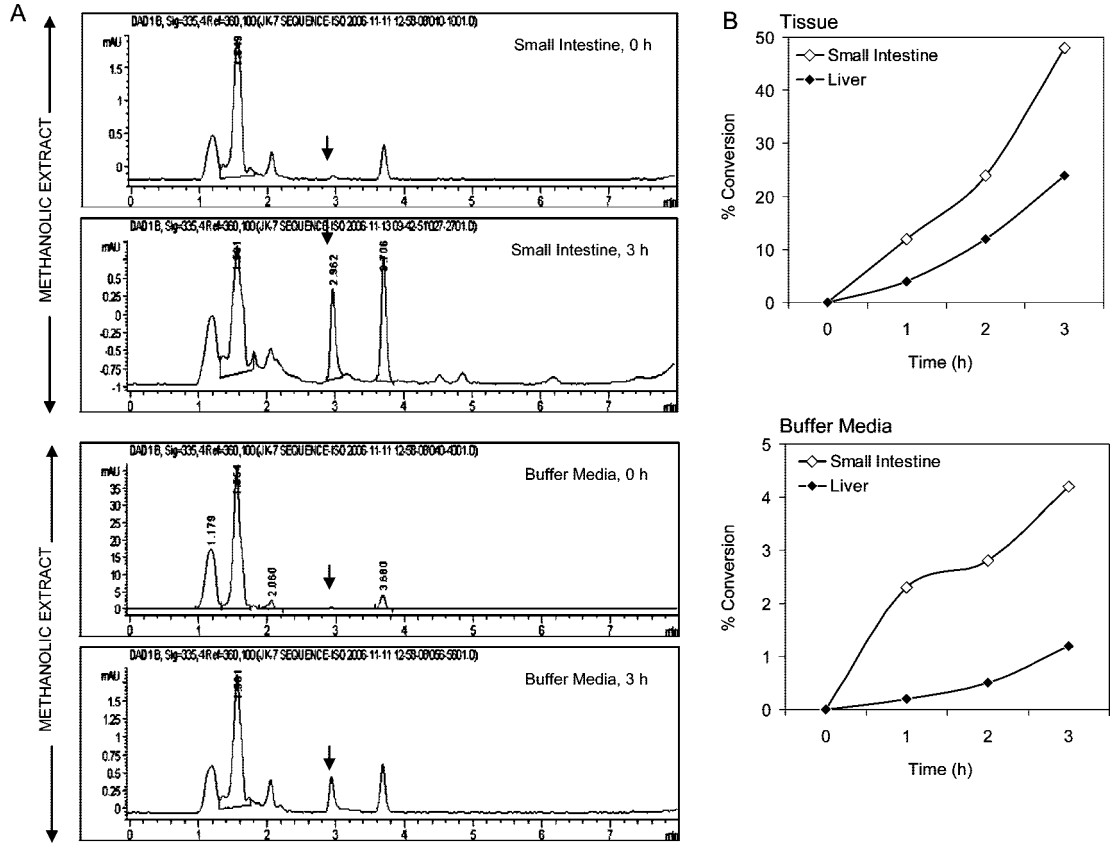

Figure 6. HPLC chromatogram monitoring (**A**) the relative absorbance at 335 nm of methanol extract of chamomile after incubation with small intestine and liver of mouse at 37 °C for 0 and 3 h for conversion to aglycone in tissue and buffer media (the arrow denotes the aglycone) and (**B**) the time course for conversion to aglycone in tissue and buffer media as percent conversion.

their anticancer effects, a response demonstrated after exposure of various human prostate cancer cells to various apigenin glucosides. These studies and our previous investigations have demonstrated that the aglycone, apigenin, possesses significant

anticancer properties and causes selective growth inhibition and apoptosis in cancer cells.

Flavonoid glycosides are mostly unmodified by various cooking methods, and the food that is consumed has a low level of aglycone compared to the glycosides. Our studies have demonstrated that aglycone possesses high anticancer activity compared to glycosides. Since aglycone is likely to have a greater biological effect than glycoside, deglycosylation via β-glucosidase activity might play an important role in metabolism. Our ex vivo studies provide evidence that the small intestine and liver both have β-glucosidase capable of efficiently hydrolyzing naturally occurring flavonoid glucosides. After absorption, the flavonoids are further metabolized by the phase II drug-metabolizing enzymes into sulfate and glucuronate conjugates of the parent aglycone (*33*). Parsley, chamomile, apples, tea, and some varieties of millets in which apigenin is exclusively present as glucosides are the most important dietary sources of this flavone. Previous studies have demonstrated that the rate of uptake of flavonoids is higher if they are present as glycosides; furthermore, the biological activity is profoundly dependent on the presence or absence of the glycoside residue (*34*). Studies have also shown that the position and nature of the sugar residue may modify the uptake of the compound in the small intestine (*34*). A recent study evaluating the metabolic profile after chamomile tea ingestion in humans demonstrates a significant alteration in biological responses impacting overall health which was further enhanced after prolonged tea ingestion (*35*).

Studies aimed at evaluating the major constituents present in chamomile confirm that apigenin is an important constituent and that it possesses anticancer activity. We observed the growth inhibitory effects on several human cancer cells at aqueous concentrations of 1000–4000 and 100–400 μg/mL of methanol extracts of chamomile, which are well within the reported in vitro concentration ranges of other plant extracts. In our experiments, we cannot attribute the differences in biological effects observed between the methanolic and aqueous chamomile extract, because many additional phenolic compounds were noted in methanolic extract. Our study investigates the anticancer effects of chamomile extract as a whole. Extracts derived from other plants such as licorice root extract, soy extract, green tea extract, Chinese herbal extract, and white and red wine extracts have also demonstrated anticancer properties, findings that are in agreement with our results. Currently available data suggest that some extracts may show greater effects than the individual constituent, implying that combinations of constituents present in plant extracts may be highly important in the ultimate biological activity. In addition, different fractions of the herbs (aqueous or organic) contain compounds with different chemistry and therefore may exhibit different types of activities.

On the basis of the findings in this investigation, our long-term goal is to characterize the active constituents in the chamomile extract. We are currently undertaking extensive experiments to identify compounds and their anticancer effects on prostate cancer cells, as well as experiments to delineate their mechanisms of action on various signal transduction pathways. An important goal of our future studies will be to investigate the possibility that synergistic combinations of biologically active agents may exist naturally in plants, and to investigate whether such combinations may be clinically useful as cancer chemopreventive agents. Moreover, our findings support the concept of conducting clinical trials using chamomile extract in the prevention and/or treatment of various human cancers.

ABBREVIATIONS USED

DMSO, dimethyl sulfoxide; TBE, Tris-borate-EDTA; HBSS, Hank's buffered salt solution; PBS, phosphate-buffered saline, HPLC, high-performance liquid chromatography.

LITERATURE CITED

(1) Weiss, R. F. *Herbal Medicine*; Arcanum, A. B., Ed.; Beaconsfield Publishers: Beaconsfield, U.K., 1988; pp 22–28.

(2) Hansel, R.; Sticher, O. *Pharmakognosie, Phytopharmazie*; Springer-Verlag: Heidelberg, Germany, 2002.

(3) Rombi, M. *Cento Piante Medicinali*; Nuovo Instituto d'Arti Grafiche: Bergamo, Italy, 1993; pp 63–65.

(4) Tyler, V. E. *The honest herbal*, 3rd ed.; George, F., Ed.; Stickley Co.: Philadelphia, 1993.

(5) Medicine Plus-Herbs and Supplements: Chamomile (*Matricaria recutita*, *Chamaemelum nobile*): A service of the U.S. National Library of medicine and National Institutes of Health. http://www.nlm.nih.gov/medlineplus/druginfo/natural/patient-chamomile-.html (accessed January 23, 2007).

(6) Martens, D. Chamomile: The herb and remedy, power. *The Journal of the Chiropractic Academy of Homeopathy* **1995**, *6*, 15–18.

(7) Newall, C. A.; Anderson, L. A.; Phillipson, J. D. *Herbal medicine: A guide for health care professionals*; Pharmaceutical Press: London, 1996; Vol. ix, p 296.

(8) Blumenthal, M.; Busse, W. R.; Goldberg, A. *The Complete German Commission E Monographs: Therapeutic Guide to Herbal Medicines*; American Botanical Council: Austin, TX, 1998.

(9) LHTP publication on chamomile, in depth monograph and Clinician Information Summary on Chamomile (*Matricaria recutita*, *Anthemis nobilis*). http://www.longwoodherbal.org/chamomile/chamomile.pdf, 2002.

(10) Forster, H. B.; Niklas, H.; Lutz, S. Antispasmodic effects of some medicinal plants. *Planta Med.* **1980**, *40*, 309–319.

(11) Achterrath-Tuckermann, U.; Kunde, R.; Flaskamp, E.; Isaac, O.; Thiemer, K. Pharmacological investigations with compounds of chamomile. V. Investigations on the spasmolytic effect of compounds of chamomile and Kamillosan on the isolated guinea pig ileum. *Planta Med.* **1980**, *39*, 38–50.

(12) Gupta, S.; Afaq, F.; Mukhtar, H. Involvement of nuclear factor-κB, Bax and Bcl-2 in induction of cell cycle arrest and apoptosis by apigenin in human prostate carcinoma cells. *Oncogene* **2002**, *21*, 3727–3738.

(13) Al-Jubouri, H. H. F.; Al-Jalil, B. H.; Farid, I.; et al. The effect of chamomile on hyperlipidemias in rats. *J. Fac. Med., Baghdad* **1990**, *32*, 5–11.

(14) Anderson, C.; Lis-Balchin, M.; Kirk-Smith, M. Evaluation of massage with essential oils on childhood atopic eczema. *Phytother. Res.* **2000**, *14*, 452–456.

(15) Avallone, R.; Zanoli, P.; Corsi, L.; et al. Benzodiazepine-like compounds and GABA in flower heads of *Matricaria chamomilla*. *Phytother. Res.* **1996**, *10*, S177–S179.

(16) Svehlikova, V.; Bennett, R. N.; Mellon, F. A.; Needs, P. W.; Piacente, S.; Kroon, P. A.; Bao, Y. Isolation, identification and stability of acylated derivatives of apigenin 7-O-glucoside from chamomile (*Chamomilla recutita* [L.] Rauschert). *Phytochemistry* **2004**, *65*, 2323–2332.

(17) Avallone, R.; Zanoli, P.; Puia, G.; Kleinschnitz, M.; Schreier, P.; Baraldi, M. Pharmacological profile of apigenin, a flavonoid isolated from *Matricaria chamomilla*. *Biochem. Pharmacol.* **2000**, *59*, 1387–1394.

(18) Ganzera, M.; Schneider, P.; Stuppner, H. Inhibitory effects of the essential oil of chamomile (*Matricaria recutita*) and its major constituents on human cytochrome P450 enzymes. *Life Sci.* **2006**, *78*, 856–861.

(19) McKay, D. L.; Blumberg, J. B. A review of the bioactivity and potential health benefits of chamomile tea (*Matricaria recutita* L.). *Phytother. Res.* **2006**, *20*, 519–530.

(20) Amirghofran, Z.; Azadbakht, M.; Karimi, M. H. Evaluation of the immunomodulatory effects of five herbal plants. *J. Ethnopharmacol.* **2000**, *72*, 167–172.
(21) Middleton, E.; Drzewiecki, G. Effects of flavonoids and transitional metal cations on antigen-induced histamine release from human basophils. *Biochem. Pharmacol.* **1982**, *31*, 1449–1453.
(22) Kobayashi, Y.; Nakano, Y.; Inayama, K.; et al. Dietary intake of the flower extracts of German chamomile (*Matricaria recutita* L.) inhibited compound 48/80-induced itch-scratch responses in mice. *Phytomedicine* **2003**, *10*, 657–664.
(23) Gomes-Carneiro, M. R.; Dias, D. M.; De-Oliveira, A. C.; Paumgartten, F. J. Evaluation of mutagenic and antimutagenic activities of α-bisabolol in the *Salmonella*/microsome assay. *Mutat. Res.* **2005**, *585*, 105–112.
(24) McAnlis, G. T.; McEneny, J.; Pearce, J.; Young, I. S. The effect of various dietary flavonoids on the susceptibility of low density lipoproteins to oxidation in vitro using both metallic and non-metallic oxidising agents. *Biochem. Soc. Trans.* **1997**, *25*, 142S.
(25) Bortner, C. D.; Oldenburg, N. B.; Cidlowski, J. A. The role of DNA fragmentation in apoptosis. *Trends Cell Biol.* **1995**, *5*, 21–26.
(26) Carnat, A.; Carnat, A. P.; Fraisse, D.; Ricoux, L.; Lamaison, J. L. The aromatic and polyphenolic composition of Roman camomile tea. *Fitoterapia* **2004**, *75*, 32–38.
(27) Lambert, N.; Kroon, P. A.; Faulds, C. B.; Plumb, G. W.; McLauchlan, W. R.; Day, A. J.; Williamson, G. Purification of cytosolic β-glucosidase from pig liver and its reactivity towards flavonoid glycosides. *Biochim. Biophys. Acta* **1999**, *1435*, 110–116.
(28) Day, A. J.; DuPont, M. S.; Ridley, S.; Rhodes, M.; Rhodes, M. J.; Morgan, M. R.; Williamson, G. Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver β-glucosidase activity. *FEBS Lett.* **1998**, *436*, 71–75.
(29) Kroon, P. A.; Clifford, M. N.; Crozier, A.; Day, A. J.; Donovan, J. L.; Manach, C.; Williamson, G. How should we assess the effects of exposure to dietary polyphenols in vitro. *Am. J. Clin. Nutr.* **2004**, *80*, 15–21.
(30) Dashwood, R. H. Frontiers in polyphenols and cancer prevention. *J. Nutr.* **2007**, *137*, 267S–269S.
(31) Soobrattee, M. A.; Bahorun, T.; Aruoma, O. I. Chemopreventive actions of polyphenolic compounds in cancer. *Biofactors* **2006**, *27*, 19–35.
(32) Ogasawara, M.; Matsunaga, T.; Suzuki, H. Differential effects of antioxidants on the in vitro invasion, growth and lung metastasis of murine colon cancer cells. *Biol. Pharm. Bull.* **2007**, *30*, 200–204.
(33) Chen, J.; Lin, H.; Hu, M. Metabolism of flavonoids via enteric recycling: role of intestinal disposition. *J. Pharmacol. Exp. Ther.* **2003**, *304*, 1228–1235.
(34) Hollman, P. C.; de Vries, J. H.; van Leeuwen, S. D.; Mengelers, M. J.; Katan, M. B. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am. J. Clin. Nutr.* **1995**, *62*, 1276–1282.
(35) Wang, Y.; Tang, H.; Nicholson, J. K.; Hylands, P. J.; Sampson, J.; Holmes, E. A metabonomic strategy for the detection of the metabolic effects of chamomile *(Matricaria recutita* L.) ingestion. *J. Agric. Food Chem.* **2005**, *53*, 191–196.

Received for review June 30, 2007. Revised manuscript received September 18, 2007. Accepted September 19, 2007. This work was supported by U.S. Public Health Service Grants RO1 AT002709 and RO1 CA108512.

JF071953K