Growth inhibitory and chemo-sensitization effects of naringenin, a natural flavanone purified from *Thymus vulgaris*, on human breast and colorectal cancer

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Abstract

**Background:** Natural products with diverse bioactivities are becoming an important source of novel agents with medicinal potential. Cancer is a devastating disease that causes the death of millions of people each year. Thus, intense research has been conducted on several natural products to develop novel anticancer drugs.

**Methods:** Chromatographic and spectral techniques were used for the isolation and identification of naringenin (Nar). MTT, flow cytometry, western blotting, Real Time PCR were used to test anticancer and chemosensitization effects of Nar, cell cycle, apoptosis, and expression of cell cycle, apoptosis, pro-survival and anti-survival-related genes.

**Results:** In the present study, *Thymus vulgaris* ethanol extract was purified repeatedly to produce several compounds including the known flavanone, Nar which was identified using different spectral techniques. Nar was shown to inhibit both human colorectal and breast cancer cell growth in a dose- and time-dependent manner through cell cycle arrest at S- and G₂/M-phases accompanied by an increase in apoptotic cell death. Additionally, Nar altered the expression of apoptosis and cell-cycle regulatory genes by down-regulating Cdk4, Cdk6, Cdk7, Bcl2, x-IAP and c-IAP-2 and up-regulating p18, p19, p21, caspases 3, 7, 8 and 9, Bak, AIF and Bax in both colorectal and breast cancer cells. Conversely, it diminished the expression levels of the cell survival factors PI3K, pAkt, pIkBα and NFκBp65. Moreover, Nar enhanced the sensitivity of colorectal and breast cancer cells to DNA-acting drugs.

**Discussion:** These findings provide evidence that Nar’s pro-apoptotic and chemo-sensitizing effects are mediated by perturbation of cell cycle, upregulation of pro-apoptotic genes and down-regulation of anti-apoptotic genes and inhibition of pro-survival signaling pathways.

**Conclusion:** In conclusion, Nar might be a promising candidate for chemoprevention and/or chemotherapy of human cancers. However, further studies exploring this therapeutic strategy are necessary.

**Keywords:** Naringenin, Colorectal and breast cancers, Anticancer effects, Chemo-sensitization, Molecular mechanisms
Background
Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries [1]. The burden of cancer is increasing in economically developed countries as a result of population aging and growth and, increasingly, an adoption of cancer-associated lifestyle choices, such as smoking, physical inactivity, and unhealthy food.

Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cancer cases and 608,700 deaths estimated to have occurred in 2008 [2]. Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths in 2008 [2].

Cancer is the second most common cause of death in Kuwait after cardiovascular diseases [3]. It has been noted that the age-adjusted incidence rates (AAIR) of colorectal cancer among Kuwaiti males has increased by approximately 5-fold over the last 33 years and ranked the most frequent site for the years 2003–2007. For Kuwaiti females, breast cancer has the highest incidence among the Kuwaiti population; breast cancer incidence has increased by 3-fold over the last 33 years. Conversely, the incidence of colorectal cancer has increased by approximately 4-fold. [3].

Cancer survival rates tend to be poorer in developing countries, most likely because of a combination of a late diagnosis and limited access to timely and standard treatment. Cancer can be treated by chemotherapy, radiation, surgery, monoclonal antibody therapy, and other methods. However, the effectiveness of chemotherapy is often limited by their serious side effects [4]. Radiation may also affect normal tissues. Therefore, complete removal of cancerous tissue without affecting normal tissue is the major goal of treatment. Approximately 50-60% of cancer patients in the United States utilize agents derived from different parts of plants or nutrients (complementary and alternative medicine), exclusively or concurrently with a traditional therapeutic regimen such as chemotherapy and/or radiation therapy. The need for new drugs has promoted studies evaluating possible anticancer agents in fruits, vegetables, herbs, and spices.

In recent years, dietary phytochemicals have received the attention of various scientists for their action in treating various cancers [5]. Several studies have documented that naturally occurring dietary phytochemicals inhibit the growth of various cancer cells through the inhibition of cell proliferation and the activation of apoptosis [6]. In particular, flavonoids, polyphenolic compounds that occur naturally in the plant kingdom, display a wide range of pharmacological properties, including antioxidant and anti-carcinogenic activities [6-9].

Thyme (Thymus vulgaris L., Family Lamiaceae), which is known in Arabic as “zaatar or “zaitra”, is a pleasant-smelling perennial shrub that grows in several regions worldwide [10]. The plant is indigenous to the Mediterranean region and neighboring countries, Northern Africa, and parts of Asia [11]. Thyme is widely used in folk medicine for its expectorant, antitussive, anti-bronchitis, antispasmodic, anthelmintic, carminative and diuretic properties. The aromatic and medicinal properties of the genus Thymus made it one of the most popular plants worldwide. Thymus species have strong antibacterial, antifungal, antiviral, and antioxidant activities [12]. Many pharmacological in vitro studies have revealed the pharmacological activities of both thyme essential oil and plant extracts [13]. Given the various uses of thyme in traditional medicine and the hypothesis that it may have anticancer activity, the present study was undertaken to fractionate Thymus vulgaris in a bioactivity-guided manner, to isolate and identify the bioactive lead(s) that suppress(es) colorectal and breast cancer cell growth, and to study the underlying intracellular signal transduction pathways involved in regulating cell cycle and apoptosis and its/their ability to potentiate the chemo-sensitivity of colorectal and breast cancer cells to DNA-acting drugs.

Methods
Cell lines
Human colorectal cancer cell lines (SW1116 and SW837), human breast cancer cell lines (HTB26, HTB132), and normal human fibroblast cells (CRL1554) were obtained from American type Culture Collection (ATCC; VA, USA). SW1116, SW837, HTB26 and HTB132 cells were cultured in 90% Leibovitz’s L15 medium supplemented with 10% heat-inactivated fetal bovine serum and grown at 37°C in a non-CO2 incubator. CRL1554 cells were cultured in Eagle minimum essential medium, EMEM (90%) supplemented with 10% heat-inactivated fetal bovine serum and grown at 37°C in the presence of 5% CO2 and 95% ambient air.

Chemicals and reagents
Trypsin, Leibovitz’s L-15 and EMEM medium, fetal bovine serum (FBS), and penicillin/ streptomycin solution (100×) were obtained from Mediatech, Inc. (Herndon, VA, USA). An Annexin V-FITC apoptosis detection kit was obtained from BD Hoffmann-La Roche Inc. (Nutley, NJ, USA). A DNA-prep kit was obtained from Beckman & Coulter (FL, USA). All reagents for RT-PCR and real-time qPCR were obtained from Applied Biosystem (Foster City, CA, USA). Nuclear/cytosol fractionation kit was obtained from BioVision, Inc. (Moutain View, CA, USA). Antibodies against PI3K, phospho-Akt1/2/3 (Ser473), Akt, NFkBp65, pIκBx and β-actin were purchased...
from Santa Cruz Biotechnology (Santa Cruz, CA and Cambridge, UK). All other reagents were purchased from Sigma Chemicals (St Louis, MO, USA). Plasticware was purchased from Falcon Lab (Franklin Lakes, NJ, USA).

**General experimental procedure**

Melting points were determined in open capillary tubes using a Mettler 9100 electrothermal melting point apparatus and were uncorrected. IR spectra were recorded using a JASCO FTIR-4100 spectrophotometer. UV spectra were measured in MeOH using a UV-160 IPC UV-visible dual-beam spectrophotometer. The $^1$H and $^{13}$C NMR spectra were obtained on a Bruker Advance II 600-MHz spectrometer operating at 600 and 150 MHz, respectively. Both $^1$H and $^{13}$C NMR spectra were recorded in methanol-$d_4$, and the chemical shift values were expressed in δ (ppm) relative to the internal standard TMS. For the $^{13}$C NMR spectra, spectral editing was determined by DEPT. 2D NMR data were obtained using the standard pulse sequence of the Bruker 600 for COSY, HSQC and HMBC. Low resolution EIMS were obtained using a double-focusing magnetic sector mass spectrometer (GS-MS DFS/Thermo).

**Plant material**

*Thymus vulgaris* was obtained commercially from the local market. Its identity was established as *Thymus vulgaris* by Dr. KT Mathew of Kuwait University. A voucher specimen was deposited at Kuwait University Herbarium and given the number KTM & IYQ (5920).

**Extraction and isolation**

The dried ground plant (1.0 kg) was percolated at room temperature with 96% EtOH (1 L × 3), and the extract was evaporated *in vacuo* to leave 43 g of residue. Part of this crude extract (10 g) was partitioned between water and ethyl acetate. Part of the ethyl acetate fraction (2 g) was chromatographed over a Si gel (190 g, 38 cm × 4 cm), using 15% acetone in chloroform as the eluent, to yield, after crystallization, 5.3 mg (0.01% yield) of pure Nar as pale yellow needles. This process was scaled up to yield Nar quantities enough for the study.

**Time- and dose–dependent anti-proliferative effects of Nar on human breast and colorectal cancer cells and normal human fibroblast**

Cell viability was measured using MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases [14]. Briefly, human colorectal (SW1116, SW837) and breast (HTB26, HTB132) cancer cell lines and normal human fibroblast cells (CRL1554) were seeded (27 × 10^4 cells/well) in 96-well plates and incubated in a non-CO$_2$ or CO$_2$ incubator for 18 h, depending on the cancer cell type and medium used. The cells were then treated with various concentrations of Nar (0.05 - 4 mM) for 6–24 h. Untreated cells, to which only DMSO was added at a final concentration of 0.2%, were used as a control. MTT solution (5 mg/ml, filtered) was added to the incubated cells (20 μl/well); the cells were then incubated for another 4 h, and the medium was discarded. DMSO (200 μl) was added to each well, and absorbance was measured in a micro-plate reader at a λ492 nm. The percentages of cell viability and cytotoxicity were calculated using the following equations: % cell viability = (OD$_{492}$ of treated sample/OD$_{492}$ of untreated sample) × 100; % cytotoxicity = 100 - (OD$_{492}$ of treated sample/OD$_{492}$ of untreated sample) × 100).

**Colony forming assay**

The antiproliferative effects of Nar on human colorectal (SW1116 and SW837) and breast (HTB26 and HTB132) cancer cell lines were confirmed using a colony-formation assay. Briefly, cancer cells (5 × 10^4/well) were plated in a 24-well plate and incubated at 37°C in a non-CO$_2$ incubator for 24 h. Thereafter, the cells were treated with Nar (3 mM) and incubated at 37°C for 24 h. After that, untreated and treated human cancer cells were collected by trypsinization and washed with Hank’s Balanced Salt Solution (HBSS), counted, and plated in 24-well plates at 500 cells/well and incubated at 37°C for 10–14 days. The colonies were washed with cold phosphate-buffered saline (PBS), fixed with 100% methanol, and stained with 0.5% crystal violet. The colonies were counted using an inverted microscope. The data are presented as the number of colonies formed with and without treatment.

**Cell cycle analysis by flow cytometry**

The distribution of cell-cycle phases (G$_0$/G$_1$, S, and G$_2$/M) was determined using flow cytometry by measuring the DNA content of nuclei labeled with propidium iodide as described previously [15]. Briefly, human colorectal (SW1116 and SW837) and breast (HTB26 and HTB132) cancer cell lines were plated (5 × 10^5 cells/ml) in 24-well plates and incubated at 37°C in a non-CO$_2$ incubator for 18 h. The cells were then treated with Nar (3 mM) for 24 h. Untreated and treated human cancer cells were collected by trypsinization, washed with cold phosphate-buffered saline (PBS) and counted. Cells were processed using a DNA-prep kit (Beckman & Coulter) and a DNA-Prep EPICS workstation (Beckman & Coulter). During this process, the cells were treated with a cell-membrane permeabilizing agent (non-ionic detergent) followed by propidium iodide (PI) and RNase. The samples were incubated at room temperature for 15 min before analysis by flow cytometry (FC500, Beckman & Coulter). The percentages of cells in different cell cycle phases were calculated using the Phoenix statistical software.
package, advanced DNA cell-cycle software (Phoenic Flow System, San Diego, CA).

DNA fragmentation assay

Induction of apoptosis was monitored using a DNA fragmentation assay [16]. Briefly, human colorectal (SW1116 and SW837) and breast (HTB 26 and HTB 132) cancer cell lines were plated (5 × 10^5 cells/ml) into 24-well plates and incubated at 37°C in non-CO₂ incubator for 18 h. Cells were treated with Nar (3 mM) and then collected by trypsinization, washed with cold phosphate-buffered saline (PBS) and centrifuged (1000 × g, 5 min). The cells were then resuspended in 200 µl of DNA lysis buffer (10 mM EDTA, 0.5% Triton X-100, 0.5 mg/ml proteinase K and 50 mM Tris–HCl, pH 8). The lysate was immediately incubated for 24 h at 56°C. After adding phenol/chloroform/isoamyl alcohol (25:24:1) to the lysate, DNA was precipitated with 100% ethanol. The suspension was centrifuged, and the isolated DNA was dissolved in 100 µl of TE buffer. The DNA samples were then loaded onto a 1% agarose gel containing 0.1% ethidium bromide and electrophoresed for 2 h at 70 V. The DNA bands were visualized under UV illumination.

Annexin V/PI double staining assay for apoptosis

Induction of apoptosis was determined by Annexin V-FITC apoptosis detection kit (BD Hoffmann-La Roche Inc.) according to the manufacturer's instructions. Briefly, human colorectal (SW1116 and SW837) and breast (HTB26 and HTB132) cancer cell lines were plated (5 × 10^5 cells/ml) in 24-well plate and incubated at 37°C in a non-CO₂ incubator for 18 h. The cells were treated with Nar (3 mM) and collected by centrifugation (1000 × g, 5 min, 4°C). The cells were then washed with ice-cold phosphate-buffered saline (PBS) and re-centrifuged. Nuclear fraction (NFκB) was prepared using cytosol/nuclear fractionation kit (BioVision, Inc.) according to the manufacturer instructions. Whole cell extract (PI3K, phospho-Akt, Akt, pIkBα and β-actin) was prepared as recently described [15]. Briefly, the cell pellets obtained were re-suspended and lysed in buffer containing 20 mM Tris (pH 7.4), 200 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 0.5% NP-40, 0.05% sodium deoxycholic acid, and protease inhibitor cocktail, and the lysates were clarified by centrifugation at 17000 × g for 30 min at 4°C. Protein concentrations of nuclear fractions and whole cell extracts were measured using the Bradford assay (Bio-Rad, Hercules, CA). Untreated and Nar-treated nuclear or whole cell extracts were mixed with equal volumes of 5 × SDS sample buffer, boiled for 5 min, and separated by 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were blocked in 5% nonfat dry milk dissolved in PBST (3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl, 135 mM NaCl, 0.05% Tween-20) overnight at 4°C. Primary antibodies specific to PI3K, phospho-Akt1/2/3 (Ser473), Akt, pIkBα and β-actin (Santa Cruz, CA) were adsorbed to the membranes of whole cell extracts and primary antibody specific to NFκBp65 was adsorbed to the membrane of nuclear fractions. After washing with PBS containing 0.1% Tween 20, the blots were incubated with an alkaline phosphatase-conjugated species-specific IgG secondary antibody for 2 h at room temperature. Bound antibodies were detected using nitroblue tetrazolium and bromochloroindoyl-phosphate. The specificities of the antibodies used in this study were examined by testing their reactivity with unrelated antigens, such as bovine serum albumin (BSA). The signal intensities of the respective bands were quantified by GS-800 calibrated imaging densitometer (Bio-Rad Laboratories, Inc. CA, USA).

Relative quantitative Real-Time PCR assay

mRNA expression of cell cycle- and apoptosis-associated genes in control and Nar-treated cells was determined by real-time polymerase chain reaction (qRT-PCR) using an ABI 7000 SDS system (Applied Biosystems, USA) and the comparative ΔΔCt method [15]. Commercial assays that target specific genes with probes and primers were obtained from Applied Biosystems. The targets and their Applied Biosystems assay numbers for the cell-
cycle regulatory genes were as follows: cdk4 (assay ID: Hs00364847_m1); cdk6 (assay ID: Hs00608037_m1); Cdk7 (assay ID: Hs00361486_m1); p18 (assay ID: Hs00176227_m1); p19 (assay ID: Hs00176481_m1); and p21 (assay ID: Hs00355782_m1). The targets and their Applied Biosystems assay numbers for the pro-apoptotic, anti-apoptotic and caspase genes were as follows: Bax (assay ID: Hs00180269_m1); Bak (assay ID: Hs00832876_m1); AIF (assay ID: Hs00269879-m1); c-IAP-2 (assay ID: Hs00985029_m1); Bcl2 (assay ID: Hs00608023_m1); x-IAP (Assay ID: Hs00236913_m1); casp3 (assay ID: Hs00234387_m1); casp7 (assay ID: Hs00169152_m1); casp8 (assay ID: Hs01018151-m1); and casp9 (assay ID: Hs00154260_m1). GAPDH (assay ID: Hs99999905_m1) was used as an endogenous control to normalize the expression values for each sample. For the comparative Ct method, we performed a two-step RT-PCR to obtain cDNA and real-time quantification using the target gene expression assays and Taqman Universal Master Mix (Applied Biosystems). Colorectal and breast cancer cells (5 x 10^6 cells/ml) were plated in 24-well plates and incubated in a non-CO2 incubator for 18 h. The cells were then treated with Nar (3 mM) for 24 h. mRNA was extracted using the nucleospin RNAII ready-to-use system (MACHEREY-NAGEL), and 200 ng/μl of mRNA was used in the RT reaction. Contaminated DNA was eliminated with DNase-I treatment for 20 min at 25°C, followed by heat inactivation for 10 min at 65°C, prior to cDNA synthesis using the high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s instructions. For each sample, 2.5 μl of cDNA and 12.5 μl of Taqman Universal Master Mix (2×) were used, and the final volume was adjusted to 25 μl with nuclease-free water on an optical 96-well reaction plate (Applied Biosystems). Real-time PCR was performed on an ABI 7000 SDS system using ABI Prism’s SDS collection software version 1.1 (Applied Biosystems). Real-time PCR conditions followed the protocol given by the manufacturer of the Taqman Universal Master Mix: step 1, 95°C for 10 min; step 2, 94°C for 15 s; and step 3, 60°C for 1 min. The samples were analyzed using SDS collection software version 1.1, with the baseline set between 3 and 15 and the threshold set at 0.2. The amount of target normalized to an endogenous reference and relative to a calibrator (untreated) was determined by 2^ΔΔCt, and the log comparative Ct is presented graphically.

Nar enhances the anticancer effects of DNA-acting drugs on colorectal and breast cancer cells

The potential of Nar to sensitize human colorectal (SW1116, SW837) and breast (HTB26, HTB132) cancer cells to DNA-acting drugs was studied as previously described [17]. Briefly, cancer cells were plated (27 x 10^3 cells/well) in 96-well plates at 37°C in a non-CO2 incubator. Eighteen hours after culture initiation, the cells were simultaneously treated for 24 h with Nar (1.0 mM) and various concentrations of the following DNA-damaging drugs: camptothecin (CPT), 5-fluorouracil (5FU), doxorubicin (DOX), cis-platin (CIP), ellipticine (ELP), and etoposide (ETP) at concentrations of 1 x 10^-10 - 1 x 10^-3 M; carboplatin (CAP) at concentrations of 1 x 10^-10 - 1 x 10^-4 M; and cyclophosphamide (CPA) at concentrations of 1 x 10^-11 - 1 x 10^-5 M. The drugs were then removed, the cells were washed with HBSS, and cell growth was monitored using an MTT assay.

Statistical analyses

The results are expressed as the mean ± SEM of at least three independent experiments. Statistical analyses were performed with SPSS-21. The statistical significance of the differences between the control and treated groups were determined by one-way ANOVA. P values < 0.05 were considered significant.

Results

Isolation and identification of Nar

Nar was isolated as yellow needles (EtOAc-ether): mp 240–242°C; UV(MeOH) λmax (log E) 240 (3.75) nm; IR (KBr) νmax 3343, 1639, 1562, 1515, 1419 cm^-1; 1H NMR (CD3OD, 600 MHz) see Table 1; 13C NMR (CD3OD, 150 MHz) see Table 1; EIMS (70 eV) m/z 272 [M]+.

Anti-proliferative effects of Nar on human colorectal and breast cancer cells

To investigate the effect of Nar on human colorectal and breast cancer cells, cell viability was studied using an MTT assay. Treatment of human colorectal (SW1116, SW837) and breast (HTB26, HTB132) cancer cell lines with various concentrations of Nar (0.5-4 mM) for 6–24 h showed time- and dose-dependent anti-proliferative effects (Figure 1A, B). Treatment of SW1116 with Nar for 6 and 12 h produced 42 ± 2.8% (Figure 1Aa) and 52 ± 3.5% (IC50 = 4 mM) (Figure 1Ab) growth inhibition, respectively. Nar did not show any effect on normal human fibroblast cells (CRL1554) at 6 and 12 h, and the difference between the effects of Nar on SW1116 and CRL1554 was significant at 6 h (P ≤ 0.008) and non-significant at 12 h (P ≤ 0.093). In addition, treatment of SW1116 cells with Nar for 15 h markedly inhibited their growth (% cytotoxicity = 0.0 - 98%, P ≤ 0.004, IC50 = 1.73 mM) compared with its effect on CRL1554 cells (% cytotoxicity = 0.0 - 34%) (Figure 1Ac). Moreover, exposure of SW1116 cells to Nar for 18 h showed significantly greater (% cytotoxicity = 0.0 - 99.4%, P ≤ 0.007, IC50 = 0.91 mM) growth inhibition than that exerted on CRL1554 cells (% cytotoxicity = 0.0 - 26%) (Figure 1Ad).
Furthermore, treatment of SW1116 cells with Nar for 24 h produced a higher growth inhibition (% cytotoxicity = 0.0 - 100%, IC₅₀ = 1.0 mM) than that produced on CRL1554 cells (0.0 - 32%). The difference in the growth inhibition was significant (P ≤ 0.001) (Figure 1Ae).

Treatment of SW837 with various concentrations of Nar for 12 h produced a higher growth inhibition (% cytotoxicity = 0.0 - 55%, P ≤ 0.06, IC₅₀ = 4.0 mM) than that produced on CRL1554 cells (% cytotoxicity = 0 - 19%) (Figure 1Ab). In addition, treatment of SW837 cells with Nar for 15 h produced a higher cytotoxicity (% cytotoxicity = 0.0 - 99.5%, P ≤ 0.015, IC₅₀ = 2.0 mM) than that exerted on CRL1554 cells (% cytotoxicity = 0 - 34%) (Figure 1Ac). Moreover, exposure of SW837 cells to Nar for 18 h markedly inhibited their proliferation (% cytotoxicity = 0.0 - 99.9%, P ≤ 0.042, IC₅₀ = 1.36 mM) compared with its effect on CRL1554 cells (% cytotoxicity = 0.0 - 26%) (Figure 1Ad). Further incubation of SW837 cells with Nar for 24 h produced a higher growth inhibition (% cytotoxicity = 0.0 - 100%, P ≤ 0.066, IC₅₀ = 1.55 mM) than that produced on CRL1554 cells (% cytotoxicity = 0.0 - 32%) (Figure 1Ae).

Treatment of the human breast cancer cell line HTB26 with Nar (0.2-4 mM) for 6 h exhibited a slight growth inhibition (% cytotoxicity = 0 - 13%, P ≤ 0.897) with no effect on CRL1554 cells (Figure 1B). A higher growth inhibitory effect was observed on HTB132 cells (% cytotoxicity = 0.0 - 52%, P ≤ 0.035, IC₅₀ = 4.0 mM) (Figure 1Ba.) after exposure to Nar for 6 h. Exposure of HTB26 cells for 12 h produced a substantially higher growth inhibition (% cytotoxicity = 0.0 - 73%, P ≤ 0.041, IC₅₀ = 3.09 mM) compared with CRL1554 cells (% cytotoxicity = 0.0 - 19%). A similar growth inhibition was noted with HTB132 cells treated with Nar for 12 h (% cytotoxicity = 0.0 - 55%, P ≤ 0.038; IC₅₀ = 3.73 mM) (Figure 1Bb). Moreover, exposure of HTB26 to Nar for 15 h markedly inhibited its growth (% cytotoxicity = 0.0 - 100%, P ≤ 0.001, IC₅₀ = 1.36 mM) compared with CRL1554 cells (% cytotoxicity = 0.0 - 34%). Exposure of HTB132 cells to Nar for 15 h produced a higher anti-proliferative effect (% cytotoxicity = 0.0 - 67%, P ≤ 0.007, IC₅₀ = 3.0 mM) than that produced on CRL1554 cells (Figure 1Bc). In addition, exposure of HTB26 (% cytotoxicity = 0.0 - 99.6%, P ≤ 0.011, IC₅₀ = 1.46 mM) and HTB132 (% cytotoxicity = 0.0 - 95%, P ≤ 0.005, IC₅₀ = 0.91 mM) cells to Nar for 18 h produced greater growth inhibition than that observed in CRL1554 cells (% cytotoxicity = 0.0 - 26%) (Figure 1Bd). Furthermore, a similar significant growth inhibition was observed in HTB26 (% cytotoxicity = 0.0 - 99.9%, P ≤ 0.024, IC₅₀ = 0.82 mM) and HTB132 (% cytotoxicity = 0.0 - 95.3%, P ≤ 0.002, IC₅₀ = 0.36) cells compared with CRL1554 cells (% cytotoxicity 0.0 - 32%) after a 24-h exposure to Nar (Figure 1Be).

The anti-proliferative effects of Nar on human colorectal (SW1116, SW837) and breast (HTB26, HTB132) cancer cells were confirmed by the inhibition of colony formation. Treatment of SW1116 cells with Nar markedly inhibited colony formation (mean number of colonies = 17 ± 2, P ≤ 0.0001) compared with untreated SW1116 cells (mean number of colonies = 152 ± 1.84) (Figure 1Ca). Additionally, exposure of SW837 cells to Nar greatly inhibited colony formation (mean number of colonies = 9 ± 0.9, P ≤ 0.0001) compared with untreated SW837 cells (mean number of colonies = 98 ± 2.6) (Figure 1Cb).

Exposure of HTB26 cells to Nar showed a marked inhibition of colony formation (mean number of colonies = 11 ± 1.6, P ≤ 0.0001) compared with untreated HTB26 cells (mean number of colonies = 177 ± 3) (Figure 1Cc). Similar marked inhibition of colony formation was observed with Nar-treated HTB132 cells (mean number of colonies = 22 ± 1.8, P ≤ 0.0001) compared with untreated HTB132 cells (mean number of colonies = 176 ± 3) (Figure 1Cd).

Nar arrests the growth of human colorectal and breast cancer cells

To further demonstrate that the growth inhibition of human colorectal and breast cancer cells leads to alterations of the cell cycle distribution, cells were treated with Nar and stained with PI. The percentage of cells in each stage of the cell cycle was analyzed using flow cytometry. Treatment of the human colorectal cancer cell line SW1116 with Nar for 24 h resulted in accumulation

### Table 1 NMR Data of Nar<sup>a</sup>

| Position | δ<sub>H</sub> (m, J Hz) | δ<sub>C</sub> m<sup>13</sup> |
|----------|------------------|------------------|
| 2        | 5.33 (dd, 13.2, 3.0) | 80.5, d |
| 3        | 3<sub>q</sub>, 3.11 (dd, 13.2, 17.1) | 44.0, t |
|          | 3<sub>q</sub>, 2.69 (dd, 17.1, 3.0) |         |
| 4        | -                | 197.8, s |
| 5        | -                | 165.5, s |
| 6        | 5.90 (d, 1.8)    | 96.2, d |
| 7        | -                | 168.4, s |
| 8        | 5.89 (d, 1.8)    | 97.1, d |
| 9        | -                | 164.9, s |
| 10       | -                | 103.4, s |
| 1<sup>1</sup> | -              | 131.1, s |
| 2<sup>2</sup> | 7.32 (dd, 7.2, 1.8) | 129.1, d |
| 3<sup>3</sup> | 6.83 (dd, 6.6, 2.4) | 116.3, d |
| 4<sup>4</sup> | -                | 159.0, s |
| 5<sup>5</sup> | 6.83 (dd, 7.2, 1.8) | 116.3, d |
| 6<sup>6</sup> | 7.32 (dd, 7.2, 1.8) | 129.1, d |
| OH       | 4.89 (s)         |         |

<sup>a</sup>Spectra recorded in methanol-d₄. <sup>13</sup>C multiplicities were determined by DEPT 135°.
Figure 1 (See legend on next page.)
of cells in S-phase (53.8% ± 2.2 vs. 41.6% ± 1.8 for untreated; UT, \( P \leq 0.005 \)) and G\(_2\)/M-phase (8.2% ± 1.3 vs. 5.6% ± 1.3 for UT, \( P \leq 0.204 \)) with a corresponding decrease in the fraction of cells in G\(_0\)/G\(_1\) phase (37.8% ± 1.7 vs. 52.76% ± 1.2 for UT, \( P \leq 0.001 \)) (Figure 2Aa, b).

Furthermore, Nar growth-arrested SW837 cells in both S-phase (48% ± 1.73 vs. 43.4% ± 1.96 for UT, \( P \leq 0.0123 \)) and G\(_2\)/M-phase (14.7% ± 1.2 vs. 12.8% ± 1.1 for UT, \( P \leq 0.471 \)) with a corresponding decrease of cells in G\(_0\)/G\(_1\)-phase (37.1% ± 1.2 vs. 43.7% ± 2.7 for UT, \( P \leq 0.045 \)) (Figure 2Ac, d).

On the other hand, treatment of the human breast cancer cell line HTB26 with Nar resulted in accumulation of HTB26 cells in both S-phase (49% ± 1.2 vs. 47.1 ± 1.2 for UT, \( P \leq 0.289 \)) and G\(_2\)/M-phase (13.7% ± 1.2 vs. 7.5% ± 1.4 for UT, \( P \leq 0.013 \)), with a corresponding decrease of cells in G\(_0\)/G\(_1\)-phase (37.2% ± 1.7 vs. 45.3% ± 1.9 for UT, \( P \leq 0.019 \)) (Figure 2Ba, b). Additionally,
exposure of HTB132 cells to Nar resulted in accumulation of HTB132 cells in S-phase (49.6% ± 2.3 vs. 45.4% ± 2.3 for UT, P ≤ 0.246) and G₂/M-phase (14.6% ± 1.7 vs. 8.6% ± 1.2 for UT, P ≤ 0.035) with a corresponding decrease of cells in G₀/G₁-phase (35.7% ± 2.3 vs. 45.8% ± 2.3 for UT, P ≤ 0.021) (Figure 2Bc, d).

**Nar induces apoptosis in human colorectal and breast cancer cells**

Agents with the ability to induce apoptosis in tumors have the potential to be used for antitumor therapy. The effect of Nar (3 mM) on the induction of programmed cell death in human breast (HTB26 and HTB132) and colorectal (SW1116 and SW837) cancer cell lines was assessed with DNA fragmentation analysis. The results from DNA gel electrophoresis showed the formation of DNA laddering in both breast and colorectal cancer cell lines treated with Nar (data not shown).

To investigate the type of cell death induced by Nar, cells were stained with annexin V-FITC/PI and analyzed by flow cytometry. Annexin V is a Ca²⁺-dependent phospholipid-binding protein possessing a high affinity for PS, a membrane-bound component localized to the inner surface of the cell membrane. An indicator of early-stage apoptosis is the detection of exposed PS residues that have translocated to the cell surface. The annexin V assay permits the simultaneous detection of early apoptotic events based on annexin V binding to exposed PS and late apoptotic/dead events through the uptake of propidium iodide.

Treatment of SW1116 cells with Nar induced apoptosis, including early apoptosis (6.1% vs. 2.8% for UT), late apoptosis (56.4% vs. 2.5% for UT) and necrosis (29.9% vs. 1% for UT) (Figure 3Aa, b). Similar results were obtained with SW837 cells treated with Nar, which showed early apoptosis (3.7% vs. 3.5% for UT), late apoptosis (3.7% vs. 3.5% for UT) and necrosis (25.6% vs. 1% for UT) (Figure 3Ab, b).

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**Figure 3** Induction of apoptosis in human colorectal and breast cancer cells treated with Nar. Human colorectal cancer cells (A) and human breast cancer cells (B) were plated (2.5 × 10⁵ cells/well) into 24-well plates in non-CO₂ incubator at 37°C for 18 h. The cells were then treated with Nar (3.0 mM) for 48 h. The cells were processed and stained with annexin V-FITC/PI and analyzed by flow cytometry as described in the Materials and Methods section.
apoptosis (65.5% vs. 7.5% for UT) and necrosis (25.6% vs. 0.2% for UT) (Figure 3Ac, d). Moreover, exposure of the human breast cancer cell line HTB26 to Nar induced apoptosis, including early apoptosis (4.6% vs. 3.4% for UT), late apoptosis (95.2% vs. 2.4% for UT) and necrosis (0.1% vs. 0.1% for UT) (Figure 3Ba, b). Similar results were obtained with the breast cancer cell line HTB132, in which Nar induced apoptosis with early apoptosis (2.6% vs. 2.4% for UT), late apoptosis (97.1% vs. 4.4% for UT) and necrosis (0.2% vs. 1.2% for UT) (Figure 3Bc, d).

Nar affects the expression of Akt, pAkt, PI3K, pIkBα and NFkBp65 in colorectal and breast cancer cells
Nuclear factor -κB (NF-κB), a major cell survival signal, participates in multiple steps in cancer cell resistance to chemical and radiation therapies. Studies with animal models and cell culture systems have established links between NF-κB and carcinogenesis, highlighting its significance as a target in cancer treatment and chemoprevention [18]. We therefore tested the possibility that Nar might inhibit NF-κB in colorectal and breast cancer cells. Indeed, Nar inhibited the phosphorylation of IkB, an upstream mediator of NF-κB function, and thus, p65 localization into nuclear was also inhibited in colorectal and breast cancer cells treated with Nar (Figure 4a, b), supporting the involvement of NF-κB inactivation in Nar-induced apoptosis. It is known that PI3K and its downstream substrate Akt also have a role in apoptosis [19]. We examined the expression of these proteins, identifying dose-dependent reductions in PI3K (Figure 4c), phospho-Akt (Figure 4d), Akt (Figure 4e) and the ratio of pAkt/Akt in both colorectal and breast cancer cell lines.

Nar modulates the expression of genes-related to cell cycle and apoptosis in human colorectal and breast cancer cells
Inhibition of the cell cycle and the induction of apoptosis in cancer cells are two major goals in cancer treatment. Therefore, we analyzed the expression of genes controlling both cell cycle and apoptosis in human cancer cells treated with Nar to elucidate the potential molecular anticancer mechanisms of Nar, which remains unclear.

Cell cycle progression in eukaryotic cells is partly controlled by the cyclin-dependent kinase (Cdk) family of protein kinases, their activating partners, the cyclins, and the cyclin-dependent kinase inhibitors (Cdks) [20].

Our data showed that Nar differentially downregulated the expression of Cdk4, Cdk6, and Cdk7 in the human colorectal cancer cell lines SW1116 (Figure 5Aa) and SW837 (Figure 5Ab) and in the human breast cancer cell lines HTB26 (Figure 5Ba) and HTB132 (Figure 5Bb). In contrast, Nar differentially upregulated the expression of p18, p19, and p21 in both human colorectal cancer cell lines, SW1116 (Figure 5Ac) and SW837 (Figure 5Ad), and breast cancer cell lines, HTB26 (Figure 5Bc) and HTB132 (Figure 5Bd).

On the basis of these observations, we propose that alterations in the level of various cell cycle regulatory proteins were responsible for the cell cycle arrest observed in Nar-induced death in human colorectal and breast cancer cells. Cell cycle arrest may, at least partially, account for the induction of apoptosis and the cytotoxic effects of Nar in human colorectal and breast cancer cells.

Pro-apoptotic and anti-apoptotic proteins are central regulators of apoptosis, and the interactions among these proteins set the threshold for cell survival [21]. Nar differentially up-regulated the expression of the pro-apoptotic genes, including caspases-3, 7, 8, 9, Bak, AIF and Bax in SW1116 (Figure 6Aa) and SW837 (Figure 6Ab) as well as HTB26 (Figure 6Ba) and HTB132 (Figure 6Bb). Meanwhile, Nar differentially downregulated anti-apoptotic genes, such as Bcl2, x-IAP and c-IAP-2, in both SW1116 (Figure 6Ac) and SW837 (Figure 6Ad), as well as HTB26 (Figure 6Bc) and HTB132 (Figure 6Bd). Nar promotes apoptosis in human colorectal and breast cancer cells by altering the ratio of pro and anti-apoptotic genes in favor of apoptosis.

Nar enhances the sensitivity of colorectal and breast cancer cells to DNA-acting drugs
The potential of Nar to potentiate the chemo-sensitivity of colorectal and breast cancer cells to DNA-damaging drugs was examined. The results are summarized in Figures 7 and 8 and Table 2. The results reported in this study clearly indicate the potential of Nar to increase the sensitivity of colorectal and breast cancer cells to DNA-damaging drugs. The synergistic and/or additive interaction between the tested DNA-acting drugs and Nar needs to be further investigated and might be dependent on the type of drug tested, the polymorphism of the genes encoding the drug-metabolizing enzymes transporters or drug targets.

Discussion
The need for new drugs has prompted studies evaluating possible anticancer agents in fruits, vegetables, herbs, and spices. Natural products with diverse bioactivities are becoming an important source of novel agents with pharmaceutical potential. The potential for plant extracts to act as anticancer therapeutic agents is due to their ability to inhibit tumor growth, angiogenesis, and metastasis with few side effects [22]. Much of this activity appears to stem from flavonoids, which are principal components of many such extracts that demonstrate the capacity to inactivate carcinogens, inhibit angiogenesis,
Expression of NFκB, pIκB, PI3K, Akt and pAkt in human colorectal and breast cancer cells treated with Nar. Colorectal (SW1116) and breast (HTB26) cancer cells (2.5 × 10⁵ cells/well) were plated into 24-well plates and incubated at 37°C for 18 h. The cells were then treated with Nar (3 mM) for 24 h. Expression levels of NFκB/pIκB, in the nuclear extracts, (Aa, b and Ba, b); PI3K/pAkt/Akt (Ac-e and Bc-e) and β-actin (Af, Bf), in the whole cell extracts, were detected by western blot analyses. The relative protein expression of NFκB/pIκB (C a, b) and PI3K, pAkt, Akt, pAkt/Akt (Cc-f) was normalized to β-actin. *P ≤ 0.05 vs. control (untreated). The data were obtained from at least duplicate experiments.

Figure 4 Expression of NFκB, pIκB, PI3K, Akt and pAkt in human colorectal and breast cancer cells treated with Nar. Colorectal (SW1116) and breast (HTB26) cancer cells (2.5 × 10⁵ cells/well) were plated into 24-well plates and incubated at 37°C for 18 h. The cells were then treated with Nar (3 mM) for 24 h. Expression levels of NFκB/pIκB, in the nuclear extracts, (Aa, b and Ba, b); PI3K/pAkt/Akt (Ac-e and Bc-e) and β-actin (Af, Bf), in the whole cell extracts, were detected by western blot analyses. The relative protein expression of NFκB/pIκB (C a, b) and PI3K, pAkt, Akt, pAkt/Akt (Cc-f) was normalized to β-actin. *P ≤ 0.05 vs. control (untreated). The data were obtained from at least duplicate experiments.
and halt cell proliferation or promote apoptosis [23]. Nar, the aglycone of naringin, is a flavonoid with activity against uterine, blood, stomach, brain, and lung cancer cell lines [24] with no toxic effect on normal cells [25,26]. Its inhibitory effects on tumor growth have spurred interest in its therapeutic application. However, the detailed molecular mechanisms of its anti-proliferative effects and apoptosis induction on human colorectal and breast cancer cells remained to be elucidated. In this study, we endeavored to investigate the effects of Nar on human colorectal and breast cancer cell growth, the intracellular transduction pathways that regulate apoptosis and the potential for Nar to enhance the sensitivity of both colorectal and breast cancer cells to DNA-acting drugs.

The dried powdered Thymus vulgaris was percolated repeatedly in 96% EtOH at room temperature. The combined extracts were evaporated in vacuo until desiccation. A total of 10 g of the dried residue was partitioned successively, between water and ethyl acetate. Flash chromatography of the ethyl acetate fraction over silica gel, using 15% acetone in chloroform as an eluent, yielded the known flavanone Nar [27] in addition to the flavanonol aromadendrin [28], and Nar was isolated as pale yellow needles. Its molecular formula was determined as $C_{15}H_{12}O_5$ on the basis of the ion peak at $m/z$ 272 [M]$^+$ and NMR data. Its IR spectrum showed absorption bands for a hydroxyl group (3343 cm$^{-1}$), a conjugated carbonyl group (1639 cm$^{-1}$) and an aromatic moiety (1562, 1515, and 1419 cm$^{-1}$). The $^{1}$H and $^{13}$C NMR spectra identified the possible identity of this compound as a flavanone. The $^{13}$CNMR spectrum showed fifteen carbon resonances distributed as a triplet, five doublets (two of them are of double intensity) and seven singlets. The double intensity doublets resonated in the aromatic region (at $\delta_C$ 116.3 and 129.1, Table 1), indicating the presence of a para-di-substituted aromatic ring; two resonances of the remaining doublets resonated, again, in the aromatic region of $\delta_C$ 96.2 and 97.1 (Table 1).

The coupling pattern and constants in the $^1$H NMR spectrum confirmed the presence of 1,2,3,5-tetrasubstituted and 1,4-disubstituted aromatic rings (ring A and ring B, respectively). The last doublet resonated in the oxygenated aliphatic region at $\delta_C$ 80.5 ppm (C-2), while the only triplet carbon resonated at $\delta_C$ 44.0 ppm (C-3). This result confirmed the presence of a flavanone ring
The HSQC spectra showed a cross peak between C-2 (δC 80.5) and a proton resonated at δH 5.33 as a double doublet with J = 13.2, 3.0 Hz (H-2). This coupling constant and pattern are only possible when H-2 adapts an axial (β-) orientation. Ring B, however, is attached to C-2 with an α-disposition. Therefore, the absolute stereochemistry around C-2 was assigned as S. The D2O-exchangeable sharp singlet resonating at δH 4.89 indicated the presence of at least one hydroxyl group. Other HSQC, HMBC and COSY spectra facilitated the full unambiguous assignment of the protons and carbons of this compound and revealed its identity as the known flavanone Nar [27].

The anti-proliferative effects of Nar on colorectal (SW1116, SW837) and breast (HTB26, HTB132) cancer cells as well as normal human fibroblast CRL1554 cells were investigated first. Nar was found to be cytotoxic to both colorectal and breast cancer cells in a dose- and time-dependent manner. CRL1554 human fibroblast control cells were slightly affected by Nar. Therefore, Nar appeared to reduce cancer cell growth with minimal collateral damage. These results are in line with those reported in other studies using Nar or Nar derivatives in various cancer cell lines or animal models [29,30]. Our results contradict the recent findings that Nar exhibits no anti-cancer activity against human cervical cancer cells [31]. Very recently, the nano-chemoprevention concept was introduced as a novel approach for improving phytochemical bioavailability and anti-tumor effects. Oral administration of Nar-loaded nanoparticles (NARNPs) to 7,12-dimethylbenz(a)anthracene (DMBA)-treated animals completely prevented tumor formation compared with the free Nar and significantly reduced the degree of histological lesions, in addition to restoration of the status of biochemical and molecular markers during oral carcinogenesis [32]. NARNPs were found to have a more potent antitumor effect than free Nar in completely preventing the formation of squamous cell carcinoma and in restoring the biochemical constituents to normal range [33].

Cell cycle arrest and apoptosis are two important mechanisms involved in anti-cancer drug treatment [34]. Uncontrolled cellular proliferation is a hallmark of all cancer cells, and overcoming it is a major therapeutic goal. Nar, through its anti-proliferative and pro-apoptotic effects, provides a promising approach toward cancer treatment.

**Figure 6** mRNA expression of pro-apoptotic and anti-apoptotic genes in human colorectal and breast cancer cells treated with Nar. The expression of pro-apoptotic and anti-apoptotic genes in human colorectal (A) and breast (B) cancer cells was determined by measuring mRNA levels through real-time RT-PCR and using the comparative ΔΔCt method to calculate expression changes. The amount of the target normalized to an endogenous reference and relative to calibrator, is given by 2–ΔΔCt.
cancer, and the blockade of the cell cycle is regarded as an effective strategy for eliminating cancer cells [35]. Many chemotherapeutic agents have been shown to impart anti-proliferative effects via arresting cell division at certain checkpoints in the cell cycle [36]. The concept of cell cycle-mediated apoptosis has gained increasing attention, as targeting this pathway may provide an opportunity to overcome acquired drug resistance, decrease mutagenesis and reduce toxicity [36].

In the present study, cell cycle arrest was observed when cells were cultured with Nar. Characterization of this effect demonstrated that expression levels of cell cycle regulators were modulated by Nar treatment; arrest appeared to occur at S- and G2/M-phases in both colorectal and breast cancer cells. These results are consistent with those reported in other studies using different types of malignancies [31].

Numerous studies have helped scientists appreciate apoptosis as an ideal way to eliminate precancerous and/or cancer cells [37]. However, most cancer cells block apoptosis, which allows malignant cells to survive despite genetic and morphologic transformation. Therefore, searching for agents that can trigger apoptosis in tumor cells has become an attractive strategy in anti-cancer drugs discovery. Here, Nar markedly induced apoptosis in both colorectal and breast cancer cell lines. The

Figure 7 Nar enhances the chemosensitivity of human colorectal cancer cells to DNA-acting drugs. Human colorectal cancer cells SW1116 were plated (27 x 10^3 cells/well) into a 96-well plate at 37°C in a non-CO2 incubator. At 18 h after starting the culture, the cells were treated for 24 h with Nar (1 mM) and various concentrations of camptothecin, CPT doxorubicin, DOX, 5-fluorouracil, SFU cisplatin, CIP, etoposide, ETP ellipticine, ELP (1 x 10^{-10} - 1 x 10^{-3} M), carboplatin, CAP (1 x 10^{-10} - 3.5 x 10^{-4} M) and cyclophosphamide, CPA (1 x 10^{-11} - 1 x 10^{-5} M). Cell proliferation was monitored using an MTT assay.
results are in line with other several studies that have shown the capability of Nar to stimulate apoptosis in different cancer cell lines [24,31,38,39]. Moreover, emerging evidence from literature has also demonstrated that the anti-proliferative effect of natural products is associated with apoptosis induction [40-42]. Again, the results from this study are in line with these findings. Taken together, dysregulation of the cell cycle mechanism and the induction of cancer cell apoptosis are recognized as an important goal of cancer therapy.

It is known that PI3K and its downstream substrate Akt have a role in apoptosis [43]. The expression of these proteins was examined, identifying dose-dependent reductions of PI3K and phospho-Akt in both colorectal and breast cancer cell lines. Total Akt protein levels remained unchanged throughout the course of the experiment. These results are in agreement with those documented from other studies in other malignancies [42,44].

The PI3K/Akt signaling pathway is influential in the regulation of cell survival due to the activation of anti-
apoptotic downstream effectors [45] and phosphorylation-dependent inhibition of pro-apoptotic signals such as Bad, caspase-9, and the family of Forkhead transcription factors [46]. Akt inhibition results in the upregulation of FasL expression in vascular smooth muscle cells and Hela cells [47]. Downregulation of Akt leads to a decrease in the phosphorylation of the endogenous Forkhead factors and its location to the nucleus. It impairs the induction of FasL promoter [46]. In addition, Akt promotes cell survival via NF-κB activation [48]. The present study sought to determine the effects of PI3K/Akt on Nar-induced apoptosis. The results showed that Nar down-regulated the PI3K/Akt signaling pathway and that the PI3K/Akt pathway inhibition may increase Nar-induced apoptosis. These data are in accord with those reported in other studies in other types of malignancies [29,42,44].

NF-κB participates in multiple steps in cancer cell resistance to chemical and radiation therapies [18] highlighting its significance as a target in cancer treatment and chemoprevention [49]. We therefore explored this transcription factor as a target for the treatment of human colorectal and breast cancer cells by using Nar. Our results indicate that NF-κB is constitutively active in human breast and colorectal cancer cell lines examined and that Nar down-regulated the nuclear pool, or active form, of NF-κB and suppressed IκBα phosphorylation and the expression of the NF-κB-regulated gene products IκBα, Bcl2 and c-IAP-2. This led to the suppression of proliferation and induction of apoptosis. Nar appears to suppress IκB activation (under investigation) which leads to inhibition of IκBα phosphorylation, as reported in the current study abrogating IκBα degradation by ubiquitin-proteasome pathway.

An imbalance between cell proliferation and cell death due to cell cycle disruption will lead to cancer development. Thus, the cell cycle could serve as a target for anticancer agents to inhibit the uncontrolled proliferation of cancer cells and initiate their apoptosis [50-52]. In the present study, expression of cell cycle-regulatory genes resulted in the upregulation of cell cycle-dependent apoptosis-inducing effects. Human colorectal and breast cancer cells treated with Nar exhibited a marked down-regulation in the mRNA of genes related to cell cycle control, including Cdk4, Cdk6 and Cdk7. However, the same treatment resulted in the upregulation of cell cycle-dependent kinase inhibitor genes, p18, p19 and p21. These results are consistent with the findings in other types of malignancies [31,42]. Increased levels of p18, p19, p21 and maintenance of the key cell cycle regulatory proteins that counter proliferation signals, eventually leading to apoptosis, are possible mechanisms of colorectal and breast cancer cell death induced by Nar.

Apoptosis is a cell suicide mechanism that is frequently dysregulated in oncogenesis. Generally, apoptosis occurs via two fundamental pathways: 1) the mitochondrial or

Table 2 Percentage mean cytotoxicities of DNA-acting drugs and their combinations with Nar on human colorectal and breast cancer cells

| Treatment with DNA-acting drugs and their combinations with Nar | Percentage mean cytotoxicity* | Fold increase in therapeutic efficacy |
|---------------------------------------------------------------|-------------------------------|-------------------------------------|
|                                                               | SW837                        | HTB132                              |
| 5 FU (1 × 10⁻¹⁰ - 1 × 10⁻³ M)                                  | 8 ± 1.0                      | 54 ± 0.42                           |
| 5 FU (1 × 10⁻¹⁰ - 1 × 10⁻³ M) + Nar (0.5 mM)                   | 50 ± 1.6                     | 96 ± 0.44                           |
| CIP (1 × 10⁻¹⁰ - 1 × 10⁻³ M)                                   | 3 ± 0.8                      | 49 ± 0.5                            |
| CIP (1 × 10⁻¹⁰ - 1 × 10⁻³ M) + Nar (0.5 mM)                    | 39 ± 2                       | 80 ± 0.6                            |
| CAP (1 × 10⁻¹⁰ - 1 × 10⁻³ M)                                   | 9 ± 2                        | 40 ± 2                              |
| CAP (1 × 10⁻¹⁰ - 1 × 10⁻³ M) + Nar (0.5 mM)                    | 27 ± 1.0                     | 83 ± 1.5                            |
| CPT (1 × 10⁻¹⁰ - 1 × 10⁻² M)                                   | 8 ± 1.1                      | 60 ± 2.2                            |
| CPT (1 × 10⁻¹⁰ - 1 × 10⁻² M) + Nar (0.5 mM)                    | 50 ± 2.6                     | 95 ± 0.3                            |
| DOX (1 × 10⁻¹⁰ - 1 × 10⁻³ M)                                  | 6 ± 1.3                      | 54 ± 1.3                            |
| DOX (1 × 10⁻¹⁰ - 1 × 10⁻³ M) + Nar (0.5 mM)                    | 41 ± 3                       | 84 ± 0.6                            |
| CPA (1 × 10⁻¹¹ - 1 × 10⁻⁷ M)                                  | 15 ± 2.6                     | 44 ± 0.8                            |
| CPA (1 × 10⁻¹¹ - 1 × 10⁻⁴ M) + Nar (0.5 mM)                    | 47 ± 1.1                     | 88 ± 1.1                            |
| ETP (1 × 10⁻¹⁰ - 1 × 10⁻³ M)                                  | 10 ± 2                       | 51 ± 1.6                            |
| ETP (1 × 10⁻¹⁰ - 1 × 10⁻³ M) + Nar (0.5 mM)                    | 57 ± 0.6                     | 83 ± 0.93                           |
| ELP (1 × 10⁻¹¹ - 1 × 10⁻⁷ M)                                  | 23 ± 2.5                     | 51 ± 1.6                            |
| ELP (1 × 10⁻¹¹ - 1 × 10⁻⁷ M) + Nar (0.5 mM)                    | 70 ± 1.8                     | 93 ± 0.6                            |

*The data are based on the mean of absorbance from 3 independent experiments.

**P-values of the combined treatments with DNA-acting drugs and Nar versus DNA-acting drugs = 0.0001.
intrinsic pathway and 2) the death receptor or extrinsic pathway [53]. The intrinsic pathway is triggered by the release of mitochondrial proteins, such as cytochrome c, which bind to Apaf-1 and procaspase-9 in an ATP-dependent manner to form the apoptosome [54]. The apoptosome activates caspase-9, thereby initiating the apoptotic caspase cascade [55]. Conversely, the extrinsic pathway is initiated by the interaction of ligands with their respective death receptors, sequentially leading to cleavage of the initiator, caspase-8. Active caspase-8 cleaves the executioner procaspase-3, resulting in apoptosis [56]. The apical proteases in the intrinsic and extrinsic pathways are caspase-9 and caspase-8, respectively. Activated caspase-8 and caspase-9 further initiate activation of the caspase cascade, leading to biochemical and morphological changes associated with apoptosis [57]. Caspase-3 is a well-known downstream adaptor caspase that is proteolytically activated by caspase-9 or caspase-8 via mitochondrial or cell death receptor signaling pathways [58]. Thus, caspases have been shown to be activated during apoptosis in many cells and play critical roles in both the initiation and execution of apoptosis [59]. To characterize the apoptotic mechanisms induced by Nar, the expression of pro- and anti-apoptotic proteins were assessed. The human colorectal and breast cancer cells exposed to Nar displayed a marked up-regulation in the mRNA expression of pro-apoptotic genes including Caspases-3, 7, 8, and 9, Bak, AIF and Bax. Meanwhile, the mRNA expression of the anti-apoptotic genes including Bcl2, x-IAP and c-IAP-2 was differentially down-regulated in both colorectal and breast cancer cells. These results are in agreement with those findings in other malignancies [29,31,41,42,60].

Numerous studies have demonstrated that the Bcl-2 family significantly regulates apoptosis, either as an activator (Bax) or as an inhibitor (Bcl2) [61]. Bcl2 is a known anti-apoptotic protein that is frequently examined for potential clinical use as a prognostic biomarker in cancer, and its overexpression is associated with resistance to cytotoxic drugs such as cisplatin and 5-fluorouracil. In addition, studies have shown that aberrant expression of this protein facilitates tumor progression [62].

The results presented here demonstrated that Nar-induced apoptosis relates to augmented levels of Bax and down-regulation of Bcl2 inducing mitochondrial dysfunction and leading to apoptosis of colorectal and breast cancer cells. Bax is an essential mitochondrial-mediated apoptosis activator, as its insertion in the mitochondrial membrane results in the release of cytochrome c into the cytosol leading to the activation of caspases and committing the cells to apoptosis [63].

The caspase family, aspartate-specific cysteine proteases, also plays a critical role in regulating apoptosis. Caspase signaling is initiated and propagated by proteolytic autocatalysis and by the cleavage of downstream caspases and substrates such as PARP and lamin A [64]. In particular, caspase-3 is one of the key executioners of apoptosis because it is either partially or totally responsible for the proteolytic cleavage of many key proteins that are important for cell viability [65]. Caspase-9 is an initiator caspase in the apoptotic process, and its function is to activate the effector caspases 6, 7 and 3 [66]. Caspase-independent pathways are involved in mitochondrial dysfunction leading to release of AIF and Endo G from mitochondria and apoptosis [66]. The results from real-time PCR also showed that Nar promoted AIF expression in both colorectal and breast cancer cells, suggesting that Nar-induced apoptosis also involves caspase-independent mechanisms.

The upregulation of pro-apoptotic genes in conjunction with the down-regulation of anti-apoptotic genes in colorectal and breast cancer cells treated with Nar may serve to shift the balance from pro-survival to pro-apoptotic. Nar treatment, thus, may lower the threshold of colorectal and breast cancer cells to pro-death signals, thereby accounting for its anticancer effects. It is well established that agents capable of inducing apoptosis as a mode of cell death are good anticancer candidates [67]. Therefore, Nar is a potential anticancer candidate that is believed to selectively induce apoptosis in cancer cells.

Chemotherapy is one of the main strategies to eliminate residual cancer cells and prevent metastasis after surgery or radiotherapy. The survival period and quality of life of cancer patients have improved with the advent of novel chemotherapeutic agents, such as docetaxel [68]. However, patients continue to suffer various toxic effects including myelosuppression, vomiting and hypersensitivity reactions. Meanwhile, the development of drug resistance continues to threaten patient prognosis [69]. Chemotherapy may also sometimes aggravate cancer progress and lead to patient death [70]. Recently, the concept “to live in harmony with the tumor” has been proposed. This notion has become a new direction in cancer research [71]. In the present study, Nar markedly reduced the apoptotic threshold of both colorectal and breast cancer cells. These results encouraged us to evaluate the potential of Nar to enhance the sensitivity of colorectal and breast cancer cells to DNA-damaging drugs. The results clearly indicated that simultaneous treatment with Nar and DNA-acting drugs exhibited substantially higher cytotoxicity on both colorectal and breast cancer cells compared with a single treatment with a DNA-acting drug. Nar potentiates the chemosensitivity of tested cancer cells in a drug- and cancer-type dependent manner (Figures 6, 7, Table 2). These results are in line with findings in other malignancies [31,72,73].
Thus, Nar has the potential to be a useful adjunct to improve the effectiveness of chemotherapeutic agents in the treatment of human cancers. Nar potentiates the anticancer effects of DNA-acting drugs by activating pro-apoptotic signaling, negating survival signaling, and attenuating their side effects. Testing this strategy with a larger number of cancer cell lines would increase the value of this study. In addition, in vivo studies using animal models are necessary to confirm the validity of this combination strategy for the treatment of colorectal and breast cancers and possibly other types of cancers.

Conclusions
The findings of the present study have important clinical implications. This study demonstrated that Nar induces apoptosis and potentiates the sensitivity of both colorectal and breast cancer cells through multifactorial mechanisms including cell cycle arrest, up-regulation of the expression of pro-apoptotic genes, down-regulation of anti-apoptotic genes and inhibition of pro-survival signaling pathways. Nar therefore displays promise as a pro-apoptotic factor that may benefit colorectal and breast therapy. However, further studies should be conducted in appropriate animal models of cancer, and ultimately, human cancer prevention trials should be performed.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
MA designed the study, analyzed data, and wrote manuscript; MA and EQ performed all the biological studies; KO and EQ conducted isolation and identification of Nar, KO assisted in writing and editing the manuscript; RA and MA performed the flow cytometry analysis. All authors read and approved the final manuscript.

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References
1. World Health Organization. The global burden of disease: 2004 update. Geneva: World Health Organization; 2008.
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61:69–90.
3. El-Basmy A, Al-Mohannadi A, Al-Awadi A. Some epidemiological measures of cancer in Kuwait: national cancer registry data from 2000–2009. Asian Pac J Cancer Prev. 2012;13:113–8.
4. Carvalho M, Jeronimo C, Valentea P, Andrade PB, Silva BM. Green tea: a promising anticancer agent for renal cell carcinoma. Food Chem. 2010;122:49–54.
5. Tan AC, Konczak I, Sze DM, Ramzan I. Molecular pathways for cancer chemoprevention by dietary phytochemicals. Nutr Cancer. 2012;63:495–505.
6. Wahl K, Brown I, Rotondo D, Heys SD. Plant phenolics in the prevention and treatment of cancer. Adv Exp Med Biol. 2011;698:36–51.
7. Clere N, Faure S, Martinez MC, Andriantsitohaina R. Anticancer properties of flavonoids: role in various stages of carcinogenesis. Cardiovasc Hematol Agents Med Chem. 2011;9:62–77.
8. Yoshida H, Takamura N, Shuto T, Ogata K, Tokunaga J, Kawa K, et al. The citrus flavonoids hesperetin and naringenin block the lipolytic actions of TNFα in mouse adipocytes. Biochem Biophys Res Commun. 2010;2010:728–32.
9. Arul D, Subramanian P. Inhibitory effect of naringenin (citrus flavone) on N-(1H-indol-3-yl)ethanamine induced hepatocarcinogenesis in rats. Biochem Biophys Res Commun. 2013;434:203–9.
10. Hendawse Y, Ezzy El-din A, Aszt EE, Omar EA. Productivity and oil quality of Thymus vulgaris under organic fertilization condition. Ocean Journal of Applied Sciences. 2010;3:203–16.
11. El-Nekeety AA, Mohamed SR, Hatouth AS, Hassan NS, Aly SE, Abdel-Wahhab MS. Antioxidant properties of Thymus vulgaris oil against aflatoxin-induced oxidative stress in male rats. Toxicol. 2011;57:984–91.
12. Stahl-Biskup E, Saez F, Thyme. London Taylor and Francis; 2002.
13. Amirghofran A, Karimi MH. Cytotoxicity of Thymus vulgaris, Achillea millefolium and Thuja orientalis on growing cell lines. MJUR. 2001;15:149–54.
14. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55–63.
15. Abaza MSI, Bahrani A, Al-Attia R, Valproic acid, an antiepileptic drug with histone deacetylase inhibition activity, and proteasome inhibitors exert synergistic antiproliferative, proapoptotic and chemosensitizing effects in human colorectal cancer cells: underlying molecular mechanisms. Int J Mol Med. 2014;34:513–32.
16. Fan XM, Wong BC, Wang WP, Zhou XM, Cho CH, Yuen ST, et al. Inhibition of proteasome function induced apoptosis in gastric cancer. Int J Cancer. 2001;93:481–8.
17. Abaza MSI. Augmentation of the anticancer effects of proteasome inhibitors by combination with sodium butyrate in human colorectal cancer cells. Exp Ther Med. 2010;1:675–93.
18. Chen W, Li Z, Bai L, Lin Y. NFκB in lung cancer, a carcinogenesis mediator and a prevention and therapy target. Front Biosci. 2011;16:1172–85.
19. Kulla G, Klippel A, Weber MJ. Antiproliferative signaling by the insulin-like growth factor receptor, phosphatidylinositol 3-kinase, and Akt. Mol Cell Biol. 1997;17:1595–606.
20. Malumbres M, Barbacid M. Mammalian cyclin-dependent kinases. Trends Biochem Sci. 2003;30:430–41.
21. Cragg GM, Newman DJ, Snader KM. Natural products in drug discovery and development. J Nat Prod. 1997;60:552–60.
22. Kandaswamy C, Lee MT. The antitumor activities of flavonoids. In Vivo. 2005;19:895–909.
23. Jin CY, Park C, Hwang HI, Kim GY, Choi BT, Kim WJ, et al. Naringenin up-regulates the expression of death receptor 5 and enhances TRAIL-induced apoptosis in human lung cancer A549 cells. Mol Nutr Food Res. 2010;55:300–9.
24. Kanno S, Tomizawa A, Ohtake T, Koiwai K, Ujibe M, Ishikawa M. Naringenin-induced apoptosis via activation of NF-κB and necrosis involving the loss of ATP in human promyelocytic leukemia HL-60 cells. Toxicol Lett. 2006;166:131–9.
25. Wang BD, Yang ZY, Wang Q, Cai TK, Crewdson P. Synthesis, characterization, cytotoxic activities, and DNA-binding properties of the La (III) complex with naringenin Schiff-base. Bioorg Med Chem. 2006;14:1880–8.
26. Jain R, Mitta M. Naringenin, a flavanone from the stem of Nyctanthes Arbortis Linn. International Journal of Biology, Pharmacy and Allied Sciences. 2012;1:964–72.
27. Lee I, Bae J, Kim T, Kwon O, Kim TH. Polyphenolic constituents from the aerial parts of Thymus quinquecostatus var. japonica collected on Ulleung Island. J Korean Soc Appl Biol Chem. 2011;54:811–6.
28. Arul D, Subramanian P. Naringenin (citrus flavone) induces growth inhibition, cell cycle arrest and apoptosis in human hepatocellular carcinoma cells. Pathol Oncol Res. 2013;19:763–70.
29. Frydooonfar HR, McGath DR, Spigelman AD. The variable effect on proliferation of a colon cancer cell line by the citrus fruit flavonoid naringenin. Colorectal Dis. 2003;5:149–52.
31. Kim JH, Kang JW, Kim MS, Bak Y, Park YS, Jung KY, et al. The apoptotic effects of the flavonoids N101-2 in human cervical cancer cells. Toxicol in Vitro. 2012;26:67–73.

32. Sulfiikkalan N, Krishnakumar N, Manoharan S, Nirmal RM. Chemopreventive efficacy of naringenin-loaded nanoparticles in 7,12-dimethylbenz(a)anthracene-induced experimental oral carcinoma. Pathol Oncol Res. 2013;19:287–96.

33. Krishnakumar N, Sulfiikkalan N, Manoharan S, Nirmal RM. Screening of chemopreventive efficacy of naringenin-loaded nanoparticles in DMBA-induced hamster buccal pouch carcinogenesis by FT-IR spectroscopy. Mol Cell Biochem. 2013;382:27–36.

34. Dickson MA, Schwartz GK. Development of cell cycle inhibitors for cancer therapy. Curr Oncol. 2009;16:36–43.

35. Adhami VM, Aziz MH, Reagan-Shaw SR, Nih M, Mukhtar H, Ahmad N. Sanguinarine causes cell cycle blockade and apoptosis of human prostate carcinoma cells via modulation of cyclin kinase-cyclin-cyclin-dependent kinase machinery. Mol Cancer Ther. 2004;3:933–40.

36. Kögel D, Fulda S, Mittelbronn M. Therapeutic exploitation of apoptosis and autophagy for glioblastoma. Anticancer Agents Med Chem. 2010;10:438–49.

37. Conkery DJ. Therapy-induced apoptosis in primary tumors. Adv Exp Med Biol. 2007;608:31–51.

38. Tan M, Zhu J, Pan Y, Chen Z, Liang H, Liu H, et al. Synthesis, cytotoxic activity, and DNA binding properties of copper (II) complexes with hesperetin, naringenin, and apigenin. Bioorganic Chem Appl. 2009;2009:947872–80.

39. Sabarinathan D, Mahalakshmi P, Vanisree AJ. Naringenin promote apoptosis in cerebrally implanted G6 glioma cells. Mol Cell Biochem. 2010;345:215–22.

40. Zhong Z, Chen X, Tan W, Xu Z, Zhou K, Wu T, et al. Germacrone inhibits the proliferation of breast cancer cell by inducing cell cycle arrest and promoting apoptosis. Eur J Pharmacol. 2011;667:50–5.

41. Park HS, Kim GY, Nam TJ, Deuk Kim N, Hyun Choi Y. Antiproliferative activity of fucoidan was associated with the induction of apoptosis and autophagy in AGS human gastric cells. J Food Sci. 2011;76:77–83.

42. Bak Y, Kim H, Kang JMW, Lee DH, Kim MS, Jung KY, et al. A synthetic naringenin derivative, 5-Hydroxy-7,4-diacetyloxyflavanone-N-phenylhydrazone (N101-43), induces apoptosis through up-regulation of Fas/FasL expression and inhibition of PI(3)K/Akt signaling pathways in non-small-cell lung cancer cells. J Agric Food Chem. 2011;59:10286–97.

43. Franke TF, Yang SL, Chan TO, Datta K, Kadauskas A, Morrison DK, et al. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell. 1995;81:277–36.

44. Park JH, Jin CY, Lee BK, Kim GY, Lee BK, Kim GY, et al. Naringenin induces apoptosis through downregulation of Akt and caspase-3 activation in PDGF-activated phosphatidylinositol 3-kinase. Cell. 1995;81:727–33.

45. Ciechomska I, Pyrzynska B, Razmierczak P, Kaminska B. Inhibition of Akt signaling pathway and modulation of Akt substrates: Implications for cancer therapy. Cell Death Differ. 2005;12:233.

46. Uriarte SM, Joshi-Barve S, Song Z, Sahoo R, Gobejishvili L. Akt inhibition up-regulates FasL, downregulates c-FLIPs and induces caspase-8-dependent cell death in Jurkat T lymphocytes. Cell Death Differ. 2003;10:233–42.

47. Martin IC, Smith N, Cooper H. Deaths following chemotherapy—lesson to be learnt. Br J Oral Maxillofac Surg. 2009;47:e51.

48. Tateishi Y, Matsumoto A, Kanie T, Hara E, Nakayama K, Nakayama KI. Development of mice without Cip/Kip CDK inhibitors. Biochem Biophys Res Commun. 2012;427:285–92.

49. Nakagawa Y, Sedulkina AS, Okamoto N, Nagasawa S, Suzuki N, Ohta T, et al. NF-κB signaling mediates acquired resistance after PARP inhibition. Oncotarget: Advance Publications. 2015.

50. Chu IM, Hengst L, Slingerland JM. The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. Nat Rev Cancer. 2000;8:253–67.

51. Tanse S, Ikenishi A, Okayama H, Iwamoto N, Nakayama KI, Takeuchi T. CDK inhibitors, p21(Cip) and p27 (Kip1), participate in cell cycle exit of mammalian cardiomyocytes. Biochem Biophys Res Commun. 2014;443:1105–9.

52. Tateishi Y, Matsumoto A, Kanie T, Hara E, Nakayama K, Nakayama KI. Development of mice without Cip/Kip CDK inhibitors. Biochem Biophys Res Commun. 2012;427:285–92.