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Combination of naringenin and epicatechin sensitizes colon carcinoma cells to anoikis via regulation of the epithelial–mesenchymal transition

Muzaffer DÜKEL*

Department of Molecular Biology and Genetics, Faculty of Science and Letters, Mehmet Akif Ersoy University, Burdur, Turkey

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Abstract: Anoikis is a programmed cell death mechanism that supports tissue integrity, and resistance to anoikis is a vital hallmark for metastatic cancer cells. Flavonoids are considered potent candidates for anticancer drugs as they affect several anticancer pathways, including apoptosis. Despite several studies on the anticancer effects of flavonoids on several cancers, still so little is known about how flavonoids cooperate with each other to regulate detachment-induced cell death. In this study, we examined the effects of combined naringenin + epicatechin in both attached and suspended colon carcinoma cells (HCT-116 and T84). We performed MTT assays to test the cytotoxic effect of these flavonoids on colon cancer cells. To test cell death type in flavonoid-treated cells, we used Annexin V and Hoechst 33342. In addition, the levels of apoptosis or epithelial–mesenchymal transition (EMT)-related protein and mRNA were analyzed via western blot and qRT-PCR, respectively, in all experimental groups. Cell viability data showed that the combination of these flavonoids presents a stronger cytotoxic effect than each flavonoid alone, and this combination significantly induced anoikis after cell detachment. Our results have also demonstrated that combined treatment of these flavonoids could suppress metastatic characteristics such as colony formation, cell invasion, and migration. In addition, we observed that naringenin/epicatechin combination significantly decreased cell survival under detached conditions by regulating cell survival proteins in colon cancer cells. Moreover, immunoblot data has indicated that combination of naringenin and epicatechin decreased regulator proteins of EMT in colon carcinoma cells. In conclusion, our findings showed that naringenin/epicatechin combination sensitized colon carcinoma cells to anoikis.

Key words: Anoikis, naringenin, epicatechin, colon cancer, epithelial–mesenchymal transition

1. Introduction

The major cause of cancer-associated deaths is metastasis, and cancer cells acquire this ability via a multistep process known as the metastatic cascade (Lambert et al., 2017). Colon cancer exhibits slow-growing nature and 20% of individuals at the time of diagnosis have already developed metastatic sites on commonly liver and lung (Markowitz et al., 2002). Numerous studies showed that tumor metastasis requires epithelial–mesanchymal transition (EMT)-mediated anoikis resistance (Guadamillas et al., 2011; Tiwari et al., 2012). Anoikis (Greek for "homelessness") is a type of programmed cell death that arranges tissue homeostasis via eliminating misplaced cells and metastatic cells that develop anoikis resistance during carcinogenesis (Frisch and Ruoslahti, 1997; Frisch and Screaton, 2001). During carcinogenesis, tumor cells promote anoikis resistance mainly through altered expression of cell adhesion molecules (CAM), such as Claudin-1 (Singh et al., 2012), E-cadherin (Kumar et al., 2011), which are vital for cell-extracellular matrix (ECM) interactions. Both naringenin and epicatechin are found in a variety of herbs and fruits as natural plant flavonoids that present a wide range of pharmacological effects on several diseases, including cancer (Abdulkhaleq et al., 2017; Salehi et al., 2019). An earlier study demonstrated that epicatechin suppresses activation of transcription factor NF-kB, which is essential for EMT, in lymphoma cell lines (Mackenzie and Oteiza, 2006). In addition, another report indicated that epicatechin inhibits pancreatic carcinoma tumorigenesis via modulating activity of NF-kB transcription (Siddique et al., 2012). Naringenin has been reported as a promising natural flavonoid for antimetastatic effects in a variety of cancers, including colon (Leonardi et al., 2010), lung (Chang et al., 2017), prostate (Gumushan Aktas and Akgun, 2018), and skin (Choi et al., 2020). Moreover, other investigators showed that Naringenin prevented abilities of tumor cell invasion and migration via regulation of EMT in pancreatic (Lou et al., 2012) and prostate cancer cells (Han et al., 2018). In our previous work, we showed that treatment with naringenin and epicatechin decreased

* Correspondence: mdukel@mehmetakif.edu.tr

both mRNA and protein levels of the upregulated CAM, which play vital roles during metastatic cascade, such as Claudin-1, E-cadherin, and EPCAM, in colon cancer cells (Dükel et al., 2021). Despite the fact that naringenin and epicatechin have been shown to have anticancer properties in several studies, the combined effect of these flavonoids on the metastatic characteristics of colon cancer cells has remained unknown. Here, we investigated whether combined treatment of naringenin and epicatechin sensitizes colon carcinoma cell lines to anoikis. Our findings show that the molecular mechanism of flavonoid sensitization of colon cancer cells to anoikis involves inhibition of cell survival pathway as well as EMT.

2. Materials and methods

2.1. Cell culture and flavonoid treatment

Colon cancer cell lines LoVo, HT29, T84, and RKO were obtained from ATCC (Manassas, VA). The colon cancer cell lines Caco-2, Colo 201, DLD-1, SW480, SW620, and HCT-116 and normal colon cell line CCD-18Co were a kind gift from Hülya Ayar Kayalı (İzmir, Turkey). Caco-2, CCD-18Co, DLD-1, and RKO DMEM (Sigma), and Colo 201, HCT-116, HT29, SW480, SW620, and LoVo were cultured in RPMI medium (Gibco) supplemented with 1% penicillin/streptomycin (Corning), 10% FBS (FCS; Life Technologies), respectively. T84 cells were cultured in DMEM F-12. All lines were maintained under sterile conditions and tested for mycoplasma by short tandem repeat. Cells were incubated at 37 °C with 5% CO2. Both naringenin and epicatechin were obtained from Sigma with catalog numbers N5893 and E4018, respectively. Both flavonoids were solved in DMSO and applied to growth media. Both cells were treated with flavonoids at concentrations ranging from 10 to 400 μM. Naringenin and epicatechin IC_{50} (the effective dose of drug that inhibits 50% of growth) values were determined as previously described (Dükel et al., 2021). For combination assays, flavonoids were mixed at a 1:1.25 ratio, and combination index (CI) values were determined using the Chau-Talalay equation (Chou, 2006).

2.2. Cell viability assay

To evaluate cell viability of adherent cell up on to flavonoid treatment, we used both MTT and resazurin. Both assays were performed via commercial kits (MTT, Abcam, ab146345; Resazurin, Abcam, ab129732) as per manufacturers' instructions. Briefly, 5×10^3 cells were seeded in a volume of 100 μL in an individual well of 96-well plates and then incubated with flavonoids or 0.1 %DMSO as control groups for up to 72 h. After flavonoid treatment, medium was replaced, and either MTT or resazurin solvent was added for 3 h. Cell viability was measured by using a microplate reader. IC50 values were calculated for both assays and then data were expressed as mean ± SD.

2.3. Western blotting

SDS-PAGE and immunoblotting procedures were performed as previously described (Dukel et al., 2016). In brief, cells were lysed using lysis buffer containing 50 mM Tris-HCI, pH 8.0, 150 mM NaCI, 1% Triton X-100, 5 mM EDTA, and a cocktail of protease inhibitors (Sigma-Aldrich, p8340). After centrifugation, supernatants were collected and protein concentrations were determined using BCA assay. Following transfer, membranes were probed with antibodies directed against anti-ERK1/2, anti-p-ERK, anti-GAPDH, anti-β-Actin, anti-Zeb1, anti-MMP-2, anti-MMP-9 (sc-514302, sc-7383, sc-25778, sc-47778, sc-81428, sc-13595, sc-21733 Santa Cruz Biotechnology), anticlaudin-1, anti-Stat3, anti-Snail, anti-BIM, anti-BAX, anti-Mcl-1, anti-Bcl-2, antisurvivin (ab15098, ab68153, ab216347, ab7888, ab243140, ab243136, ab117115, ab182132, ABCAM), anticleaved caspase-3, anti-p-Stat3(Tyr705), anti-Flip, anticleaved PARP, anti-AKT, anti-p-AKT(Ser473), anti-N-cadherin, anti-E-cadherin, anti-Vimentin, (9664, 9145, 8510, 5625, 9272, 4060, 13116, 14472, 5741 Cell Signaling Technology). After treatment with secondary antibodies, immunoblot signals were developed with an enhanced chemiluminescence method.

2.4. RNA isolation and qRT-PCR

Total RNA was extracted from colon cancer cells using RNeasy Kits (Qiagen, Hidden, Germany) according to the manufacturer's instructions. After purification, first-strand cDNA was synthesized using 500 ng of RNA with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Carlsbad, CA, USA) by following the manufacturers' protocol. For q-PCR, SYBR Green master mix (Applied Biosystems, Cat No: 4309155) was used to measure gene expression levels. For each sample, a total volume of 10 μL of reaction was carried out in an Applied Biosystems StepOnePlus thermal cycler and data analysis was performed with the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001). Primer sequences for targeted genes and temperatures for PCR amplification are shown in Table S1.

2.5. Anoikis assay

To determine detachment-induced cell death, we cultured cells in poly-HEMA coated plates. Culture plates were coated with poly-HEMA at 20 mg/mL in 95% ethanol. Adherent HCT-116 and T84 cells were trypsinized and seeded into 24-well polystrene and 24-well ultralow attachment plates at a density of 3×10^5. Under suspended conditions, cells were treated with either nontoxic concentrations of naringenin (25 mM) and epicatechin (25 mM) alone, or in combination (4:8 mM) for 24 h. Indicated concentrations were selected to observe whether

these flavonoids affect cell viability in suspension without inducing cell death. For each cell line and group, untreated cells were left as a control. Resazurin assay was performed to determine cell viability after anoikis application.

2.6. Soft agar colony formation test

To test effect of these flavonoids on colony formation ability of colon carcinoma cells, we performed soft agar colony formation test. The lower and upper layers of medium were adjusted to 1.2% and 1.4% nobble agar, respectively. Cells were seeded at 1×10^3/mL density into 6-well plates with either nontoxic concentrations of naringenin (25 mM) and epicatechin (25 mM) alone, or in combination (4:8 mM) for 24 h. After the first week, colonies from 3 independent repeats were imaged using a dissecting microscope. Culture media was changed once a week and colony growth was checked up to 4 weeks. Colony formation was determined by using inverted microscope (Olympus, Tokyo, Japan) at a magnification of 40×. Numbers of colonies were calculated using ImageJ program.

2.7. Cell migration and invasion assays

Both HCT-116 and T84 cells were treated with naringenin alone, epicatechin alone, or combined at nontoxic and toxic concentrations for 24 h. Controls remained untreated for each of the cell lines. For cell migration, we used wound-healing assay, in which cells were grown to 80% confluence in 24-well plates (3×10^5 cells/well), and then the wound was generated by scratching the surface of the plates with a pipette tip. To evaluate cell migration, we used an inverted microscope (Olympus, Tokyo, Japan) at a magnification of 200× and the numbers of migrated cells were counted from four random fields.

For the invasion assay, we used BD BioCoat Tumor Invasion Assay System (BD Biosciences, Bedford, MA) according to the manufacturer's protocol. In brief, firstly, the upper compartment of transwell was coated with Matrigel (0.5 mg/mL) for 4 h at room temperature before starting the assay. The upper chamber of the system was then seeded with HCT-116 and T84 (5 x 104) cells in serum-free medium, and flavonoid-treated media was added to both the upper and lower chambers. After 24 h of incubation, invading cells were stained with 4 μg/mL calcein AM for 1 h, and invaded cells were determined at excitation/emission wavelengths of 530/590 nm in a plate reader. Membranes were then visualized under a fluorescent microscope (Olympus BX61, Tokyo, Japan) at 40× magnification.

2.8. Apoptosis analysis

We analyzed type of cell death following flavonoid treatment using two different assays. Firstly, HCT-116 and T84 cells were treated with flavonoids with both nontoxic and toxic concentrations of naringenin and epicatechin alone, and in combination for 24 h. Flavonoid-treated cells were then stained with 2 μg/mL of Hoechst 33342 and 1 μg/mL of propidium iodide (PI) for 1 h at 37 °C in the dark. To determine the type of cell death, the cells were visualized and imaged under a fluorescent microscope (Olympus BX61, Tokyo, Japan) at 60× magnification. Hoechst 33341-stained cells were considered apoptotic cells, while PI-stained cells were considered necrotic cells. Apoptotic cells were also detected by Annexin V-FITC Apoptosis Detection Kit (6592S, Cell Signaling) following the manufacturer's protocol. Briefly, HCT-116 and T84 cells were treated with flavonoids for 24 h. Following flavonoid treatment, the cells were harvested and resuspended with 500 μL of 1X Annexin V Binding Buffer. The cells were then costained via adding 2 μL of Annexin V- FITC and 8 μL of PI to the binding buffer. The level of apoptosis was examined via flow cytometer (BD Biosciences, San Jose, CA, USA) and data was analyzed using FlowJo software.

2.9. Live/dead viability assay

To quantify the ratio of live/dead cells under suspension condition, both cell lines were treated with a nontoxic combination of naringenin and epicatechin alone at a concentration of 4:8 mM and 0.1% DMS as a control for 24 h. After 24 h of incubation, the cells were stained with 1 μM calcein AM and 1 μM PI for 30 min in the dark at room temperature. The cells were then visualized with a fluorescent microscope (Olympus BX61, Tokyo, Japan) at 40× magnification.

2.10. MMP-2/MMP-9 activity assay

To test MMP-2/MMP-9, studied cell lines were treated with indicated concentration of naringenin and epicatechin either alone or in combination, then MMP-2/MMP-9 activity was detected with InnoZyme™ Gelatinase (MMP-2/MMP-9) Activity Assay kit according to manufacturer's protocol. Briefly, 90 μL of each Gelatinase (MMP-2/MMP-9) standard and sample was added to each well. After 10 μL of Substrate Working Solution application to each well, the samples were incubated 6 h at 37 °C. Fluorescence signals were measured using a fluorescence plate reader (Waltham, MA, United States) at an excitation wavelength of 320 nm and an emission wavelength of 405 nm.

2.11. ROS production analysis

Cellular ROS level was measured in both attached and suspended living cells using Total ROS assay kit reagent as described by the manufacturer (Life Technologies, CA, USA). HCT-116 and T84 cells were seeded in 6-well plates at a cell density of 1.5×10^6 cells/well. The cells were cultured under both attached and suspension conditions. They were then treated with naringenin and epicatechin in combination at 4:8 mM concentration for 24 h. For both treated and untreated cells, 2 μL of ROS assay stain stock solution was added, and the cells were incubated for 1 h

at 37 °C. ROS production was determined using a flow cytometer, and the data was analyzed using the FlowJo software.

2.12. Statistical analysis

The results of cell viability for flavonoid-treated cells, anchorage-dependent cell survival and gene transcript abundance (determined by qRT-PCR) measurements were expressed as calculated means with standard deviation (SD). For each experiment, the results are presented as mean ± SD, and a two-tailed Student's t-test was used to compare measurements taken from 3 independent experiments. Multiple comparisons for statistically significant differences between multiple groups were conducted using SPSS (Chicago, IL) and GraphPad Prism 5 was used for creating graphs. In all cases, a p-value of 0.05 was considered significant.

3. Results

3.1. Effect of combined naringenin and epicatechin on cell survival and clonogenicity of colon cancer cells

In our previous work, we examined cytotoxic effect of naringenin and epicatechin in colon cancer cells SW620, Caco-2, and DLD-1, and normal colon cell line CCD-18Co (Dükel et al., 2021). Here, we aimed to examine whether these flavonoids present any cytotoxic effect on several colon cancer cells either each flavonoid alone or in combination. IC50 data and CI values are listed in Table. Consistent with our previous work, we observed similar IC50 concentrations for SW620, Caco-2, and DLD-1, and normal colon cell line CCD-18Co. In addition, our findings show that those flavonoids exert cytotoxic effect on both adenocarcinoma (HT-29, LoVo, SW480, Colo 201) and carcinoma cell lines (HCT-116, RKO, T84) at ranged concentration. Interestingly, the combination of those flavonoids showed synergetic effect in only 4 colon carcinoma cell lines Caco-2, HCT-116, RKO, and T84 while the combination exhibited either antagonistic or additivity for colon cell line and adenocarcinoma cell lines. Hence, we aimed to investigate the effects of these flavonoids on metastatic characters of colon carcinoma cells, and we selected HCT-116 and T84 cell lines that have the best CI scores, and both cell lines exert metastatic characters (Demers et al., 2009). To elaborate the effects of these flavonoids on cell viability of HCT-116 and T84 cells, we conducted cell viability and soft agar colony formation assays. HCT-116 and T84 cells were treated with these flavonoids alone or in combination at different concentrations ranging between 10 μM and 400 μM for 8 to 72 h. Cell viability data indicates that both flavonoids suppress cell viability of HCT-116 and T84 cells, consistent with our previous findings. As shown in Table, naringenin and epicatechin suppressed cell growth on HCT-116 cells at IC_{50} concentrations of 45 ± 1.9 μM and 55 ± 2.1 μM, on T84 cells at IC_{50} concentrations of 51 ± 4.2 μM and 57 ± 3.8 μM, respectively. Similar to our previous data, we measured the best cytotoxic effect of these flavonoids for 24 h time point for both cell lines. Thus, we treated cells with the indicated flavonoids for 24 h for further experiments (Figure 1A). In addition, analysis of synergy based on the isobologram method showed that the combination index (CI) was 0.37 for the combination of naringenin (20 μM) and epicatechin (25 μM) in HCT-116. Similar to HCT-116 cells, for T84 cells, the CI was 0.29 for the combination of naringenin (20 μM) and epicatechin (25 μM). Moreover, as shown

Table. IC50 and CI at ED50 values of naringenin and epicatechin in ten colon cancer cell lines and one colon cell line, alone and in combination. Combination index (CI) = synergism, between 0.9 and 1.1 = additivity, and > 1.1 = antagonism values at 50% effective dose (ED50) for naringenin and epicatechin in colon cancer and normal colon cell lines. Response to combination treatment is as follows: CI at ED50 < 0.90.

Cell lines	Naringenin IC_{50} (μM)	Epicatechin IC_{50} (μM)	CI at ED50	Naringenin + epicatechin response
Caco-2	2.6 ± 1.5	6.5 ± 1.7	0.39 ± 0.04	Synergism
CCD-18Co	20.6 ± 1.8	8.4 ± 3.2	1.38 ± 0.02	Antagonism
Colo 201	40.7 ± 1.3	50.4 ± 2.0	0.98 ± 0.01	Additivity
DLD-1	6.3 ± 2.3	7.9 ± 0.3	1.5 ± 0.01	Antagonism
HCT-116	45 ± 1.9 μM	55 ± 2.1 μM	0.29 ± 0.03	Synergism
HT-29	6.1 ± 3.3	7.3 ± 1.5	0.98 ± 0.06	Additivity
LoVo	10.5 ± 1.8	16.2 ± 3.0	0.99 ± 0.03	Additivity
RKO	39.2 ± 1.7	46.3 ± 2.8	0.43 ± 0.04	Synergism
SW480	12.3 ± 1.4	12.9 ± 2.3	0.97 ± 0.04	Additivity
SW620	12 ± 3.1	17 ± 1.4	0.99 ± 0.05	Additivity
T84	51 ± 4.2 μM	57 ± 3.8 μM	0.37 ± 0.02	Synergism

in Figure 1B, combination of naringenin and epicatechin had a stronger inhibitory effect on cell viability than either flavonoid alone in both HCT-116 and T84 cells. Since the combination of naringenin and epicatechin with the ratio of 20:25 (μM) had a synergistic effect on both cell lines, we used this dose ratio for the toxic combination in further experiments. To determine nontoxic combination of these flavonoids for further assays, we also treated studied cell lines with naringenin and epicatechin in combination at a concentration range of 1–50 μM, at 1: 1.25 ratios. We observed that lower than 10 μM treatment for combination of naringenin and epicatechin was nontoxic to studied cell lines. We used 4 μM of naringenin and 8 μM of epicatechin for further experiments as nontoxic concentrations for combined treatment. In addition, colony formation assays indicated that the combination of naringenin (20 μM) and epicatechin (25 μM) suppressed colony formation of HCT-116 and T84 cells more efficiently compared with each flavonoid alone, as well as nontoxic concentration of studied flavonoids (Figure 1C). Moreover, we observed that each flavonoid could suppress colony formation in T84 cells in single treatment. These results suggest that combination of these flavonoids could strongly inhibit cell growth in metastatic colon carcinoma cell lines.

3.2. Naringenin in combination with epicatechin induces cell death more efficiently than either flavonoid alone in colon carcinoma cells

We used Annexin V and Hoechst 33342/(PI) costaining to investigate the type of cell death caused by flavonoids. Both T84 and HTC-116 cell lines were treated with naringenin and epicatechin at 25 μM concentration alone, and combination of naringenin (20 μM) and epicatechin (25 μM), as well as nontoxic concentration 4:8 μM. Annexin V results indicated that combination of these flavonoids significantly elevated early and late apoptotic cells in T84 (40.27% and 15.6%, respectively) and HCT-116 (46.6% and 6.38%, respectively) compared with each flavonoid alone or the control group (Figure 2A). Moreover, our data indicate that nontoxic level of combination of those flavonoids had no significant effect on apoptotic cell death on both cell lines under attached conditions. The number of apoptotic cells was further tested using Hoechst 33342/(PI) costaining. We randomly selected three fields for each group, and the apoptotic and necrotic cells were counted. IF data showed that, consistent with Annexin V results, combination of both flavonoids increased apoptotic cells compared with either flavonoid alone or control group in studied cell lines (Figure 2B). Immunoblot findings confirmed that combination of naringenin and epicatechin induced cell death via upregulation of proapoptotic proteins such as Caspase 3 and BAX at combined concentration of naringenin (20 μM) and epicatechin (25 μM) (Figure 2C).

3.3. Naringenin and epicatechin in combination inhibits the invasion and migration of colon carcinoma cells

The antimigration and antiinvasion abilities of both studied flavonoids alone and in combination were evaluated via Wound Healing and Transwell assays, respectively. As shown in Figure 3A, following flavonoid treatment in HCT-116, we observed that either flavonoid slightly inhibits cell migration at 25 μM concentration of each flavonoid alone for 24 h. However, toxic combined treatment of these flavonoids strongly suppressed cell migration at concentrations of naringenin (20 μM) and epicatechin (25 μM) for 24 h compared with control and each flavonoid alone, but flavonoid treatment slightly suppressed cell migration in T84 cells (data not shown). In addition, nontoxic combination of naringenin and epicatechin at concentrations of 4 μM and 8 μM, respectively, inhibited cell migration in HCT-116 cells, but not T84 cells (data not shown). Moreover, we next measured MMP-9 and MMP-2 levels, which are the migration-associated proteins, in each group. Immunoblot bands demonstrated that both MMP-9 and MMP-2 protein levels were sharply reduced following combination treatment of these flavonoids. On the other hand, each flavonoid at 25 μM concentration or nontoxic combination moderately decreased MMP-9 and MMP-2 in HCT-116 cells (Figure 3B). Next, we examined the invasion of HCT-116 and T84 cells after treatment with flavonoids, either alone or in combination. Figure 3C indicated that these flavonoids notably inhibited cell invasion either alone or in combination in HCT-116 cells. Similar to migration assay, flavonoid treatment has not shown a strong inhibition effect on T84 cell invasion (data not shown). The antiinvasive activities of naringenin and epicatechin for colon carcinoma cell line HCT-116 were statistically significant ($p < 0.05$). These results suggest that naringenin and epicatechin may play an important role in repressing metastatic characteristics in colon cancer cells.

The combination of naringenin and epicatechin sensitizes metastatic colon cancer cells to anoikis

As resistance to anoikis is a vital characteristic for malignant cells to survive during metastasis, overcoming anoikis resistance could be a promising approach in cancer treatment (Chanvorachote et al., 2013). In addition, our findings indicated that nontoxic combination of Naringenin and Epicatechin could suppress metastatic characteristics while this treatment has not caused statistically important cell death in colon cancer cells. To better understand the effect of combination of naringenin and epicatechin on colon cancer cell metastatic characteristics, we examined whether these flavonoids sensitize colon cancer cells to anoikis. Cells under attached conditions were treated with flavonoids at nontoxic concentrations either alone or in combination (final concentration of 25 μM for

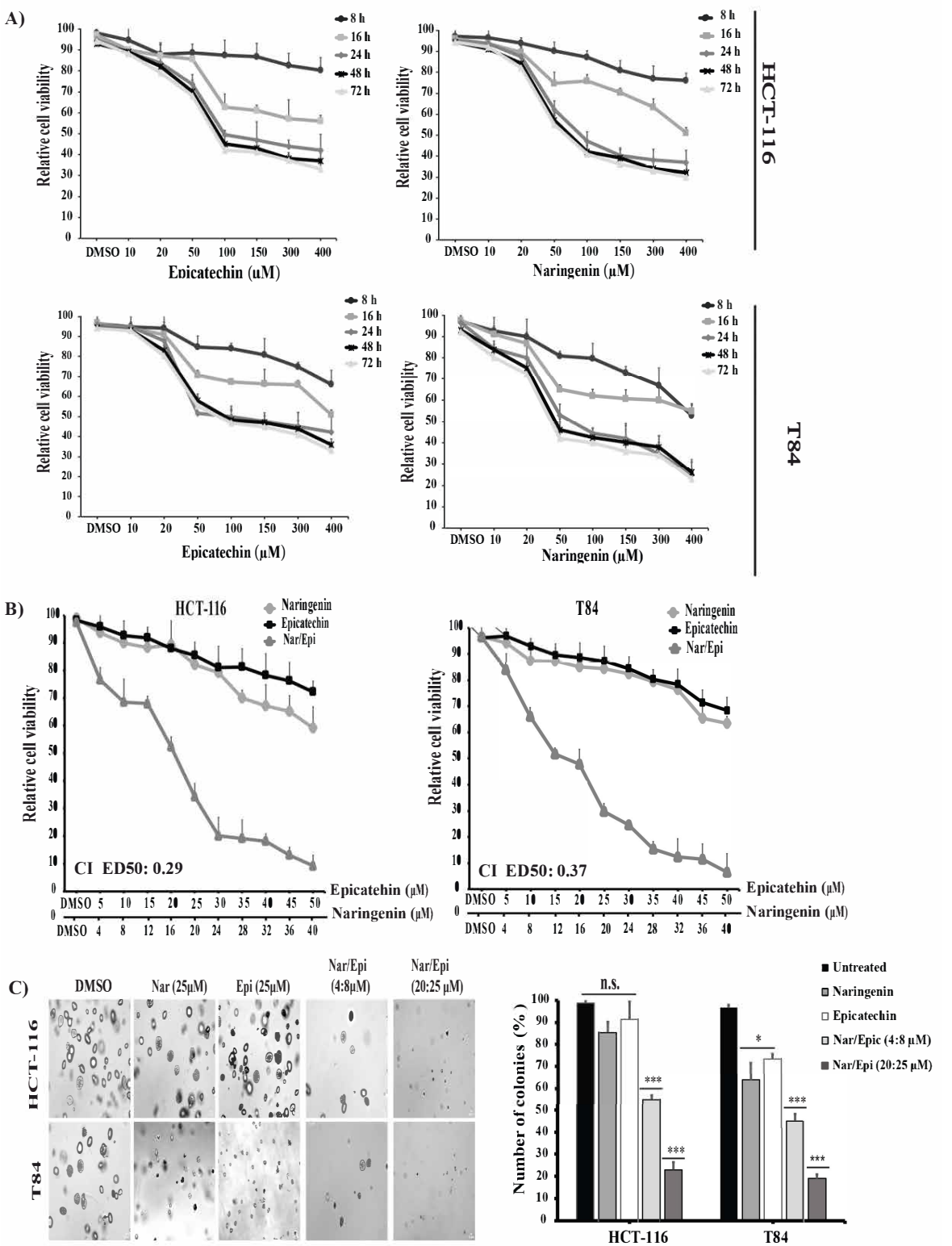

Figure 1. Naringenin, and epicatechin, and their combination inhibit cell growth in HCT-116 and T84 cells. **A, B)** Cells were treated with flavonoids either alone or in combination. The effect of naringenin and epicatechin on HCT-116 and T84 cells was assessed by MTT assay. For each group, untreated cells were used as a control. **C)** The effect of naringenin and epicatechin combination on ability of colony formation in studied cell lines, and histograms show the number of colonies. For all experiments, averages and SD are shown (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

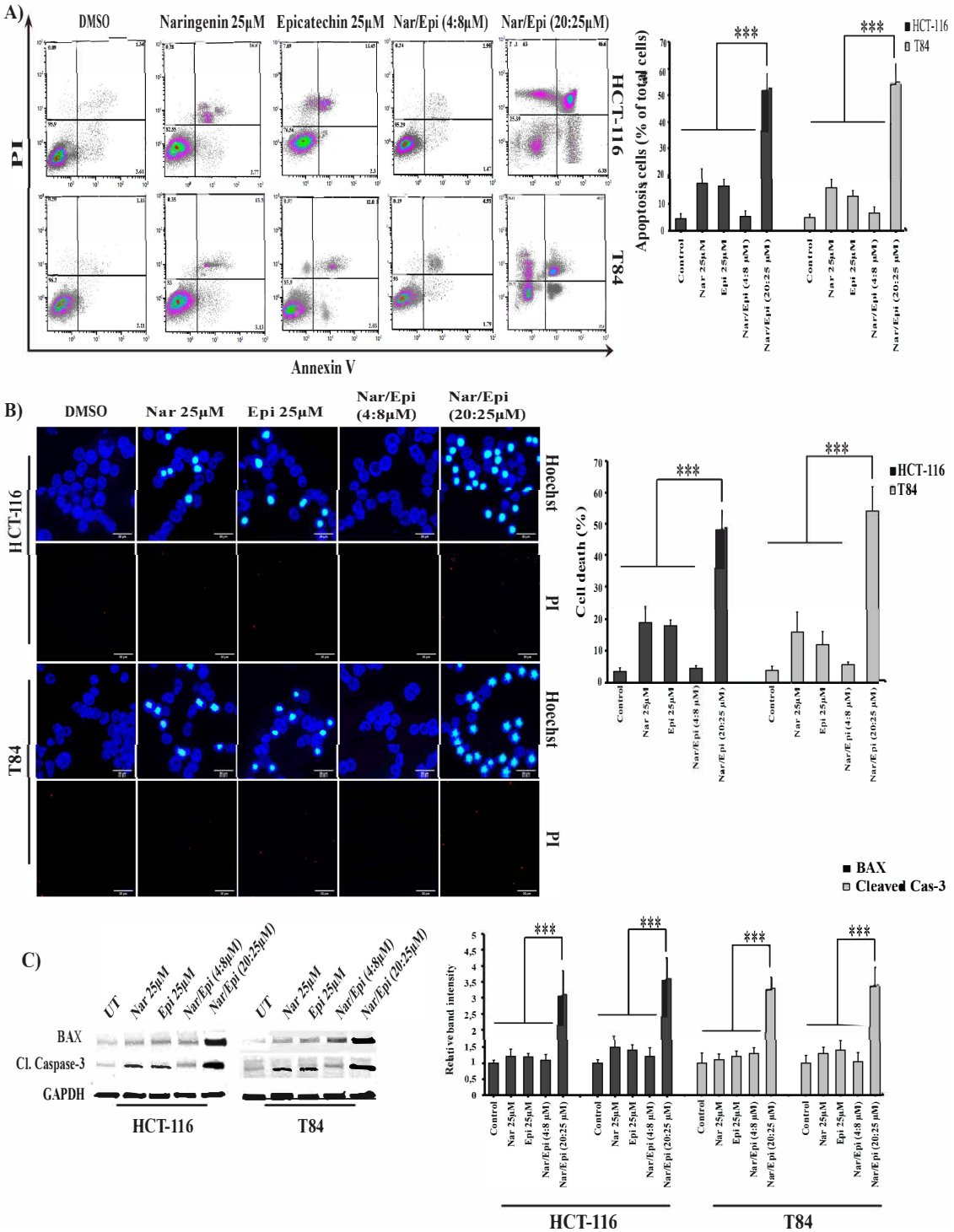

Figure 2. Naringenin and epicatechin synergistically induce apoptosis of colon cancer cells. **A)** HCT-116 and T84 cells were treated with naringenin and epicatechin at 25 µM value, or their nontoxic and toxic combinations with the ratios of 4:8 µM and 20:25 µM, respectively, for 24h and then the percentage of apoptotic cells was measured using Annexin V assay and the percentage of apoptotic cells was statistically compared and *p < 0.05, **p < 0.01, ***p < 0.001 represents significant differences compared with the control. Experiments were performed in three independent replicates, and values are expressed as the mean ± standard error of the mean (s.e.m). **B)** Apoptotic and necrotic cells were determined using Hoechst 33342/PI staining after flavonoid treatment. The white arrow points to apoptotic cells and yellow arrow points to necrotic cells. **C)** Cells were treated with indicated flavonoids, then proteins were isolated. Nitrocellulose membranes were immunoblotted with anticleaved cas3, anti-BAX, or GAPDH as a loading control.

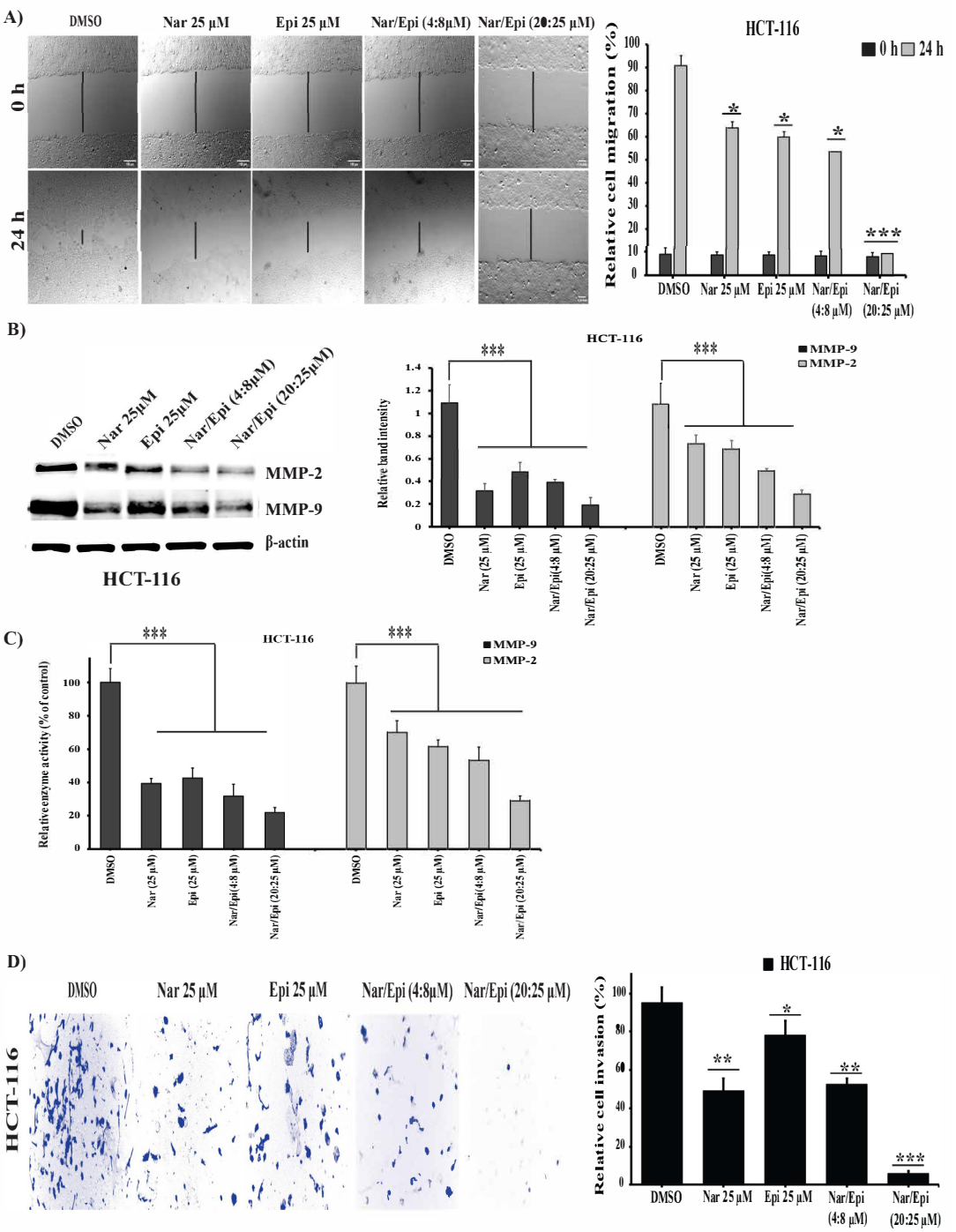

Figure 3. Combination of naringenin and epicatechin attenuates the migration and invasion ability in colon carcinoma cell lines. **A)** Cells were treated with flavonoids at indicated concentrations, and wound healing assay was performed to observe the changes in colon cancer cell motility. Quantitative graphs of wound healing results for HCT-116 are also presented. **B)** Protein levels of MMP-9 and MMP-2 were analyzed by western blot and β-actin was used as internal control. Densitometry analysis was performed with Image J software for quantification, and the results are presented below each band. **C)** Activations of both MMP-9 and MMP-2 were detected as using kit. **D)** After 24 h of flavonoid treatment, the effects on the invasion ability of HCT-116 cells were evaluated by transwell assay. Both the migration and invasion of flavonoid-treated and control groups were tested and compared in both cell lines. Total number of migration and invasion cells through the Matrigel was counted under a microscope in the whole fields. The results are expressed as mean ± SD (representative data from three independent experiments). (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.0013.4$. The combination of naringenin and epicatechin sensitizes metastatic colon cancer cells to anoikis

each flavonoid alone and 4:8 μM for naringenin and epicatechin combination, respectively) for 24 h. For assessment of anoikis resistance, both cell lines were simultaneously treated with indicated concentrations of flavonoids and cultured under suspension for 24 h. After incubation, the MTT assay was performed to measure cell viability. As shown in Figure 4, HCT-116 and T84 cells present anoikis resistance and approximately 90% of those cells remain viable upon cell detachment for 24 h in absence of flavonoids. Interestingly, both naringenin and epicatechin slightly sensitized HCT-116 and T84 cells to anoikis in comparison to the untreated control cells. Furthermore, in the combination treatment, flavonoids more strongly sensitized both HCT-116 and T84 cells to anoikis than either flavonoid alone (Figures 4A and 4B). Moreover, in order to confirm cell death type, we performed Hoechst33342/PI costaining and Annexin V assays in cells treated with nontoxic levels of flavonoids under suspension condition. Under attached conditions, these flavonoids at 25 μM of concentrations (nontoxic for studied cell lines) had only a modest effect on cell death in HCT and T84 cells. On the other hand, nontoxic flavonoid treatment significantly induced cell death in detached HCT-116 and T84 cells (Figures 4C and 4D). To visualize flavonoid-induced cell death under suspension conditions, studied cell lines were stained with calcein-AM/PI while both cell lines were treated with a nontoxic combination of naringenin and epicatechin for 24 h. Consistent with anoikis assay data, IF pictures show that combined flavonoid treatment increased cell death in studied cell lines upon detachment as live cells stained by calcein-AM (green) and dead cells were stained by PI (Figure 4E). Our previous data indicated that naringenin- and epicatechin-induced ROS (reactive oxygen species) production suppresses cell growth in colon cancer cells (Dükel et al. 2021). Here, consistently, we found that both naringenin and epicatechin caused an increase in level of ROS but not with nontoxic concentration for HCT-116 cells. However, either each flavonoid alone or in combination did not increase ROS production under suspension compared to attached cells (Figure 4F).

3.5. Flavonoid sensitization to anoikis involves inhibition of cancer cell survival signaling

Several studies have reported that anoikis is a caspase-dependent process and cancer cells obtain anoikis resistance via activating prosurvival pathways such as FLIP overexpression (Simpson et al., 2008; Kim et al., 2012). An earlier study also documented that activated ERK and AKT proteins enhance cancer cell survival under detachment conditions via inactivating proapoptotic proteins including BIM (Nagaprashantha et al., 2011). To determine whether anoikis sensitization by naringenin and epicatechin involved the alteration of cell survival signaling, cells were treated under suspension and attached conditions with nontoxic concentrations of studied flavonoids in single or combination for 24 h. To assess the role of cell survival signaling in flavonoid-induced anoikis, we tested levels of AKT, p-AKT, Erk, p-Erk, cleaved caspase-3, cleaved PARP, c-FLIP, Bcl-2, BIM, survivin, and BAX proteins. HCT-116 and T84 cells were treated in both attached and suspension conditions with 25 μM for each flavonoid alone and 4:8 μM for combination of naringenin and epicatechin. We used these concentrations as 80% of HCT-116 and T84 cells could survive upon treatment of the indicated concentrations. Cells were harvested after incubation in both suspended and attached conditions, and the levels of pro- and antiapoptotic proteins were determined using immunoblotting. Bands obtained by immunoblotting show that studied flavonoids in combination increased the cleavage of Caspase-3 and PARP, BAX and BIM compared to each flavonoid alone and control groups under suspension. In attached conditions, flavonoid treatments moderately induced apoptotic proteins for both HCT-116 and T84 cells. On the other hand, the antiapoptotic Bcl-2, FLIP, and survivin protein levels were significantly reduced by naringenin and epicatechin combined treatment, except for Mcl-1 in suspended HCT-116 and T84 cells (Figure 5A). In addition, we observed an important reduction in p-Akt and p-Erk levels in only suspended conditions, while both Akt and Erk protein levels were stable upon flavonoid treatment in both suspended and attached cells (Figure 5B). Thus, taken together, these results demonstrate that alteration in cell survival signaling plays an important role in naringenin- and epicatechin-induced anoikis (Figure 5).

3.6. The combination of naringenin and epicatechin suppresses the EMT in colon cancer cells

Alterations in regulation of the EMT pathway render malignant cells to anoikis resistance, and targeting EMT can restore anoikis sensitivity. Our previous work indicates that both naringenin and epicatechin regulates expression of EMT effector genes, including E-cadherin, Claudin-1, and Vimentin, in dose-dependent manner (Dükel et al., 2021). In addition, others indicated that activation of ERK and PI3-K/Akt pathways plays a key role in acquiring anoikis resistance and these pathways can be stimulated via regulation of EMT genes; for example, E-cadherin and Vimentin "could potentially sensitize colon cancer cells to anoikis through EMT regulation (Paoli et al., 2013; Cao et al., 2016). These findings suggest that flavonoids could potentially sensitize colon cancer cells to anoikis through EMT regulation. To test this hypothesis, we first treated HCT-116 and T84 cells with flavonoids at nontoxic concentration either alone or in combination for 24 h both attached and in suspension. Next, we investigated levels of EMT-related proteins in response to flavonoid treatment

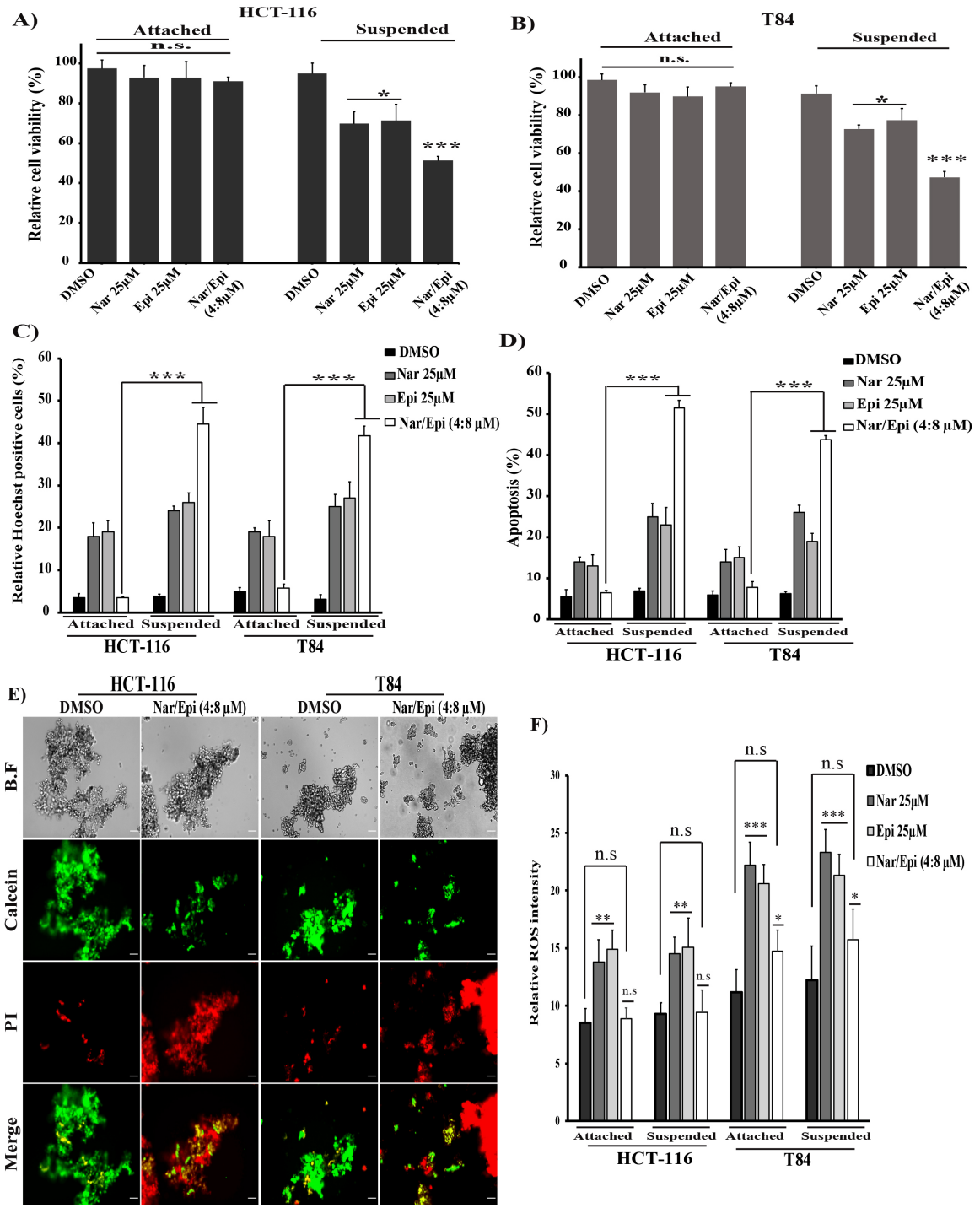

Figure 4. Anoikis-sensitizing effect of combination of naringenin and epicatechin in colon cancer cells **A, B)** HCT-116 and T84 cells were cultured under suspension condition and treated with nontoxic concentrations of flavonoids alone and in combination. Resazurin assay was then performed to test cell viability in detached and suspended cells. **C, D)** Mode of cell death in both attached and suspension cells was confirmed by Hoechst33342/PI co-staining and Annexin V assays. Results are means ± s.e.m of three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 versus control. **E)** Both HCT116 and T84 cells were cultured under suspension and simultaneously treated with combination of naringenin + epicatechin at 4:8 µM concentration for 24 h. The cells were then stained with calcein-AM/PI and IF pictures were captured using fluorescence microscopy (green: live cells; red: dead cells) at 100× magnification. Experiments were performed independently three times. **F)** Both cell lines were cultured either attached or under suspension, then cells were treated with indicated concentration of studied flavonoids. ROS levels were examined by commercially available kits upon flavonoid treatment.

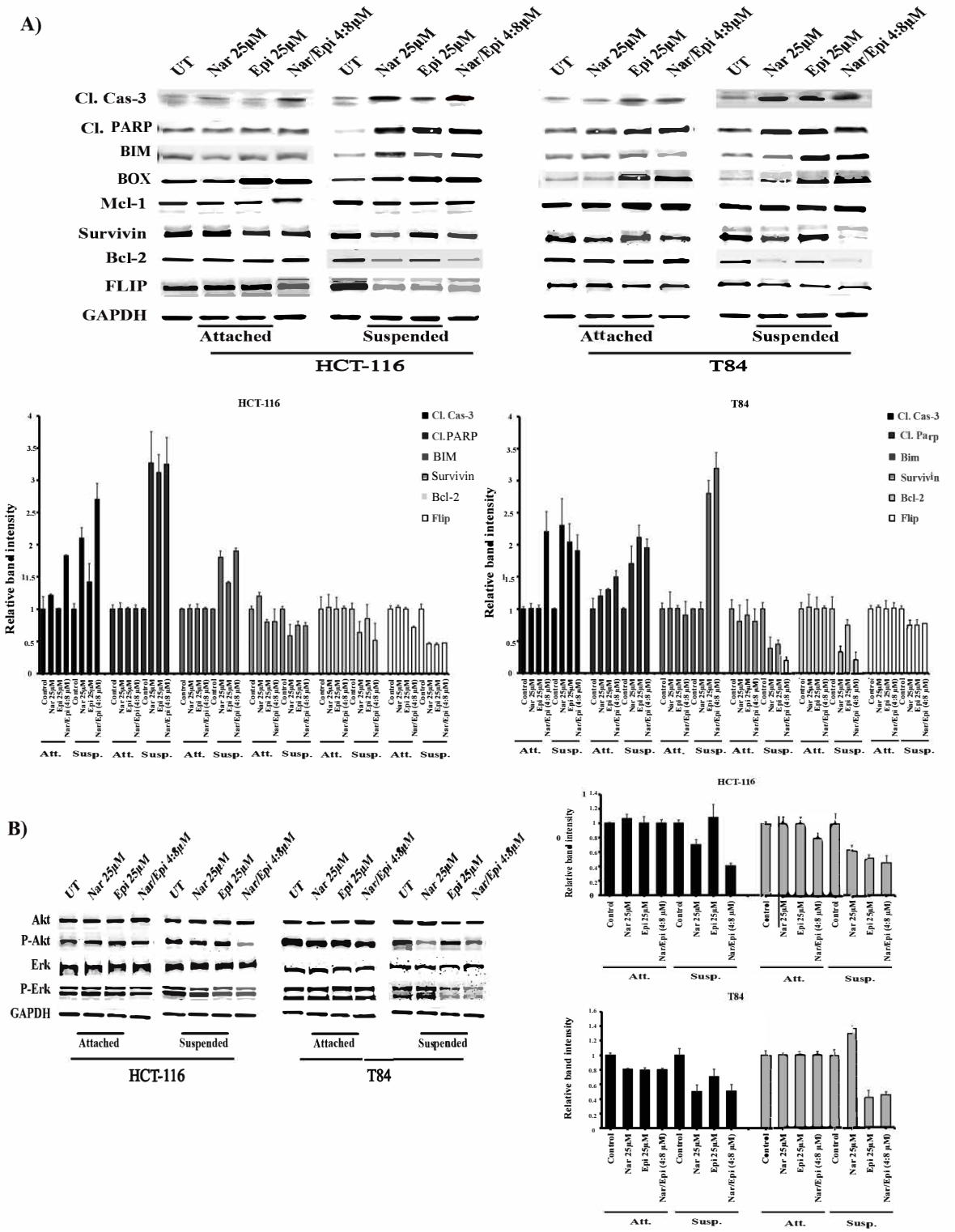

Figure 5. Combination of naringenin and epicatechin strongly suppresses survival-related proteins in HCT-116 cells. **A, B)** HCT-116 and T84 cells were treated with flavonoids at a nontoxic level, then the cells were harvested. Equal protein extracts from flavonoid-treated and control HCT-116 and T84 cells were loaded onto SDSPAGE gels and then transferred to nitrocellulose for western blot analysis. Membranes were immunoblotted with anti-AKT, p-AKT, Erk, p-Erk, cleaved PARP, c-FLIP, Bcl-2, BIM, survivin, and BAX or and GAPDH (bottom) as a loading control. The relative immunoblot signal intensity measured by ImageJ and mean data from three independent experiments were normalized to the untreated control. Values are presented as mean under immunoblot bands.

during either attached or suspension growth via western blot. We observed a sharper decrease in N-cadherin and Vimentin in studied cell lines following combined flavonoid treatment in suspended cells compared to attached cells. Similarly, Claudin-1, a cell adhesion molecule that is changed during EMT for many cancers, showed a sharp decrease under suspension conditions in flavonoid-treated HCT-116 and T84 cells. Furthermore, in only suspended cells, an approximately 2-fold decrease for EMT transcription factor Zeb1 protein was measured in combination of naringenin- and epicatechin-treated HCT-116 and T84 cells, but no detectable change in attached cells. On the other hand, immunoblot bands demonstrated that another EMT transcription factor, Snail was stable in either suspended or attached cells after flavonoid treatment. Moreover, at nontoxic level, naringenin and epicatechin combination induced expression of epithelial marker E-cadherin in both detached and suspended HCT-116 and T84 cells. Furthermore, our analyses indicated that p-STAT3 was decreased to response suspension growth in both cell lines treated with flavonoids while there was no alteration in STAT3, which known as an inducer of EMT (Figures 6A and 6B). Our western blot results indicated that these flavonoids suppress EMT in both attached and suspension cells, while they present a stronger effect under suspension growth. When these cell lines were examined for EMT-related genes, mRNA abundance upon flavonoid treatment, either under attachment or suspension, qRT-PCR data was consistent with immunoblot bands. Specifically, we observed around a 3-fold increase in E-cadherin mRNA under suspension in HCT-116 and T84 cells upon flavonoid treatment. In addition, for N-cadherin, Vimentin, Claudin-1, and Zeb-1, we scored an approximately 2-fold decrease in both cell lines. In sum, our results show that combination of naringenin and epicatechin strongly suppresses expression of EMT-related genes in colon cancer cells under suspension conditions.

4. Discussion

Colon cancer is the third most commonly diagnosed cancer but the second leading cause of cancer death in both sexes because of metastasis, which has been linked to late diagnosis (Bouvier et al., 2015; Bray et al., 2018). Anoikis is a type of programmed cell death induced following cell detachment from the ECM or neighboring cells. Metastatic cancer cells must overcome this cell death to survive within the circulatory system, and to invade distant tissues or organs (Kim et al., 2012). Several clinical findings indicated that level of circulating tumor cells within the circulatory system of colon cancer patients is correlated with more aggressive stage and poorer survival outcomes (Cohen et al., 2008; Molnar et al., 2001; Sastre et al., 2008). Hence, targeting anoikis may be a promising therapeutic approach for colon cancer patients to decrease metastasis and improve patient outcomes.

A large number of studies have documented that naringenin and epicatecin elicit a pronounced cell growth–inhibitory effect on many cancer cells, including colon, lung, breast, and gastric (Patel et al., 2014; Abdulkhaleq et al., 2017). Here, we have shown for the first time that combination of naringenin and epicatechin was effective in inhibiting growth of colon carcinoma cell lines and also decreased growth of colon cancer cells in soft agar. It has also been shown that these flavonoids are promising natural products for cancer treatment and they could exert their antitumor effect by targeting multiple carcinogenesis-related processes, including cell migration and invasion, angiogenesis, and apoptosis (Kanno et al., 2005; Lou et al., 2012; Song et al., 2016; Abdulkhaleq et al., 2017; Choi et al., 2020). Our data also demonstrated that combination of naringenin and epicatechin inhibited cell migration and invasion in metastatic HCT-116 and T84 cell lines.

In this study, we also observed that combination of studied flavonoids sensitized colon cancer cells to anoikis at nontoxic concentrations in detached conditions and this treatment caused no toxicity to attached colon cancer cells. Anoikis occurs in various normal cell types through both intrinsic and extrinsic pathways. However, cancer cells acquire resistance to anoikis via altered expression of proapoptotic and antiapoptotic genes (Kim et al., 2012). We further investigated how these flavonoids could alter anoikis resistance using suspended cells. Immunoblot data revealed that flavonoid treatment decreased antiapoptotic protein levels, such as Bcl-2, FLIP, and survivin under suspension conditions, but not Mcl-1. Consistent with that, earlier studies indicated that Bcl-2 (Singh et al., 2012), FLIP (Mawji et al., 2007) and survivin (Hori et al., 2013) proteins play central roles in cell survival and anoikis resistance in cancer cells. In addition, Maamer-Azzabi and colleagues observed that Mcl-1 was overexpressed in response to suspended growth, but degradation of Mcl-1 did not change anoikis resistance of SW480 colon adenocarcinoma cell line (Maamer-Azzabi et al., 2013). Here, our immunoblot data showed that suspension growth did not alter protein level of Mcl-1 in HCT-116 and T84 colon carcinoma cells. These data may suggest an additional mechanism(s) that controls expression of Mcl-1 in anchorage-independent growth in colon cancer cells. On the other hand, it has been demonstrated that proapoptotic proteins are downregulated in cancer cell response to loss of ECM connection (Sethi et al., 1999; Jinka et al., 2012). Importantly, similar to our current findings, Kim et al. observed that Epicatechin could change expression of apoptotic genes resulting in significant increase in apoptosis (Kim et al., 2012). Moreover, a recent report showed that naringenin could suppress cell proliferation

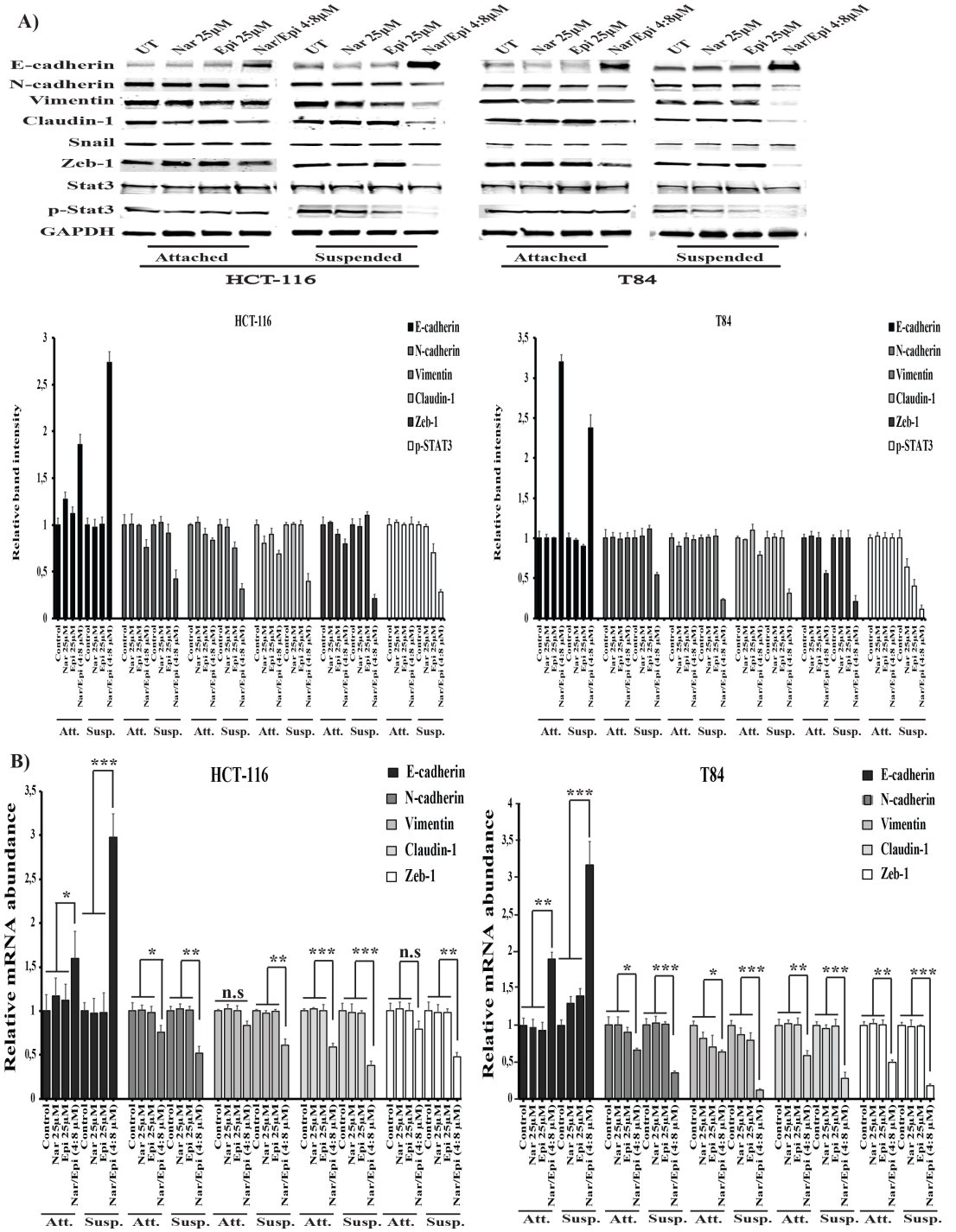

Figure 6. Flavonoid combination suppresses epithelial to mesenchymal transition (EMT) in human colon carcinoma cells. **A)** HCT-116 and T84 cells were treated with flavonoids at nontoxic level for 24 h and then changes on epithelial to mesenchymal transition (EMT)-related protein levels of these flavonoids were determined by immunoblotting. After protein isolation, equal protein extracts from both HCT-116 and T84 cells were loaded onto SDSPAGE gels, and then membranes were immunoblotted with anti-Claudin1, E-cadherin, N-cadherin, Vimentin, Zeb1, Snail, STAT-3, and p-STAT3 or GAPDH (bottom) as a loading control. The relative immunoblot signal intensity measured by ImageJ and mean data from three independent experiments were normalized to the untreated control. Band intensity are presented as means of the independent triplicate experiments. **B)** Total RNA was isolated and then relative transcripts' abundance of EMT-related genes levels were measured by qRT-PCR. qRT-PCR assays were conducted at least in triplicate, and the graph shows the means of all assays conducted. Error bars, SD; ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

via inhibiting the PI3K/AKT/mTOR cell survival pathway and that induces apoptosis in 12SW620 and HCT-116 cell lines (Cheng et al., 2020). Here, analysis of survival signaling revealed that activation of AKT and ERK was inhibited via combination of naringenin and epicatechin, and that increased proapoptotic proteins, including BIM and BAX, resulting in anoikis for detached HCT-116 and T84 cells.

Furthermore, others have shown that both naringenin and epicatechin suppress EMT in cancer cells (Mackenzie and Oteiza, 2006; Han et al., 2018). In our previous work, we also observed that these flavonoids could alter expression of EMT marker genes, such as E-cadherin, Vimentin, and Claudin-1 (Dükel et al., 2021). To that end, it is known that EMT is a crucial event that enhances migratory capacity, invasiveness, and resistance to anoikis during metastatic cancer progression, and its specific targeting marks a new avenue of cancer therapy (Kim et al., 2012; Heerboth et al., 2015). In the present study, we also focused on EMT genes upon flavonoid treatment because alteration of EMT genes in colon cancer has been demonstrated to be controlled by ERK/AKT signaling, which resulted in anoikis resistance (Ferraro et al., 2013). Moreover, Zeb1 and Snail are vital transcriptional factors that are known to be required for anoikis resistance and metastasis by regulating EMT genes such as E-cadherin (Smit et al., 2009; Smit and Peeper, 2011). Earlier reports have also shown that Zeb1-regulated EMT is crucial for colon cancer tumorigenesis and metastasis (Peña et al., 2005; Spaderna et al., 2006, 2008). Furthermore, it has been observed that STAT3 activation is involved in inducing anoikis resistance in pancreatic (Fofaria and Srivastava, 2015) and cervical (Hu et al., 2013) cancers. Consistently, we observed that combined treatment of naringenin and epicatechin resulted in decreased N-cadherin, Vimentin, Claudin-1, Zeb1, and p-STAT3 protein levels and that sensitizes colon carcinoma cells to anoikis. We also found out that this combination caused a sharp increase in both protein and mRNA of E-cadherin in detached colon cancer cells. Consistent with our work, Singh et al. have demonstrated that Claudin-1 plays a central role in resistance to anoikis in SW620 cells and downregulation of Claudin-1 sensitizes SW620 cells to anoikis in AKT-dependent manner (Singh et al., 2012).

In summary, we document that combination of naringenin and epicatechin can sensitize colon cancer cells to anoikis and results in alterations in gene expression patterns of EMT that drive decreased motility and invasiveness. We also document that these flavonoids in combination suppress cell survival under detachment conditions through inhibition of AKT and ERK activation.

5. Conclusion

Naringenin and epicatechin caused cytotoxicity in colon carcinoma cells either alone or in combination. In addition, naringenin/epicatechin combination inhibits cell growth more strongly than either flavonoid alone. We also observed that a toxic concentration of these flavonoids, either alone or combined, induced cell death. Furthermore, our findings suggest that a combination of these flavonoids may inhibit metastatic characteristics such as colony formation, cell invasion, and migration. Importantly, based on our results, these flavonoids in combination sensitized colon carcinoma cells to anoikis via following mechanisms: 1) inducing detachment-mediated cell death, 2) downregulation of cell survival factors, and 3) suppressing EMT.

Conflict of interest

The author declares that there are no competing interests.

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References

Abdulkhaleq LA, Assi MA, Noor MHM, Abdullah R, Saad MZ et al. (2017). Therapeutic uses of epicatechin in diabetes and cancer. Veterinary World 10: 869–872. https://doi.org/10.14202/vetworld.2017.869-872

Bouvier AM, Launoy G, Bouvier V, Rollot F, Manfredi S et al. (2015). Incidence and patterns of late recurrences in colon cancer patients. International Journal of Cancer 137: 2133–2138. https://doi.org/10.1002/ijc.29578

Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA et al. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians 68: 394–424. https://doi.org/10.3322/caac.21492

Cao Z, Livas T, Kyprianou N (2016). Anoikis and EMT: Lethal "Liaisons" during Cancer Progression. Critical Reviews in Oncogenesis 21: 155–168. https://doi.org/10.1615/CritRevOncog.2016016955

Chang HL, Chang YM, Lai SC, Chen KM, Wang KC et al. (2017). Naringenin inhibits migration of lung cancer cells via the inhibition of matrix metalloproteinases-2 and -9. Experimental and Therapeutic Medicine 13: 739–744. https://doi.org/10.3892/etm.2016.3994

Chanvorachote P, Chunhacha P, Pongrakhananon V (2013). Anoikis: a potential target to prevent lung cancer metastasis? Lung Cancer Management 2: 169–171. https://doi.org/10.2217/lmt.13.13

Cheng H, Jiang X, Zhang Q, Ma J, Cheng R et al. (2020). Naringin inhibits colorectal cancer cell growth by repressing the PI3K/AKT/mTOR signaling pathway. Experimental and Therapeutic Medicine 19: 3798–3804. https://doi.org/10.3892/etm.2020.8649

Choi J, Lee DH, Jang H, Park SY, Seol JW (2020). Naringenin exerts anticancer effects by inducing tumor cell death and inhibiting angiogenesis in malignant melanoma. International Journal of Medical Sciences 17: 3049–3057. https://doi.org/10.7150/ijms.44804

Chou TC (2006). Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacological Reviews 58: 621–681. https://doi.org/10.1124/pr.58.3.10

Cohen SJ, Punt CJA, Iannotti N, Saidman BH, Sabbath KD et al. (2008). Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology 26: 3213–3221. https://doi.org/10.1200/JCO.2007.15.8923

Demers MJ, Thibodeau S, Noël D, Fujita N, Tsuruo T et al. (2009). Intestinal epithelial cancer cell anoikis resistance: EGFR-mediated sustained activation of Src overrides Fak-dependent signaling to MEK/Erk and/or PI3-K/Akt-1. Journal of Cellular Biochemistry 107: 639–654. https://doi.org/https://doi.org/10.1002/jcb.22131

Dukel M, Streitfeld WS, Tang TCC, Backman LRF, Ai L et al. (2016). The Breast Cancer Tumor Suppressor TRIM29 Is Expressed via ATM-dependent Signaling in Response to Hypoxia. The Journal of Biological Chemistry 291: 21541–21552. https://doi.org/10.1074/jbc.M116.730960

Dükel M, Tavsan Z, Kayali HA (2021). Flavonoids regulate cell death-related cellular signaling via ROS in human colon cancer cells. Process Biochemistry 101: 11–25. https://doi.org/https://doi.org/10.1016/j.procbio.2020.10.002

Ferraro A, Mourtzoukou D, Kosmidou V, Avlonitis S, Kontogeorgos G et al. (2013). EZH2 is regulated by ERK/AKT and targets integrin alpha2 gene to control Epithelial-Mesenchymal Transition and anoikis in colon cancer cells. The International Journal of Biochemistry & Cell Biology 45: 243–254. https://doi.org/10.1016/j.biocel.2012.10.009

Fofaria NM, Srivastava SK (2015). STAT3 induces anoikis resistance, promotes cell invasion and metastatic potential in pancreatic cancer cells. Carcinogenesis 36: 142–150. https://doi.org/10.1093/carcin/bgu233

Frisch SM, Ruoslahti E (1997). Integrins and anoikis. Current Opinion in Cell Biology 9: 701–706. https://doi.org/10.1016/s0955-0674(97)80124-x

Frisch SM, Screaton RA (2001). Anoikis mechanisms. Current Opinion in Cell Biology 13: 555–562. https://doi.org/10.1016/S0955-0674(00)00251-9

Guadamillas MC, Cerezo A, Pozo MA (2011). Overcoming anoikis – pathways to anchorage- independent growth in cancer. Journal of Cell Science 124 (19): 3189-3197. https://doi.org/10.1242/jcs.072165

Gumushan HA, Akgun T (2018). Naringenin inhibits prostate cancer metastasis by blocking voltage-gated sodium channels. Biomedicine & Pharmacotherapy 106: 770–775. https://doi.org/https://doi.org/10.1016/j.biopha.2018.07.008

Han KY, Chen PN, Hong MC, Hseu YC, Chen KM et al. (2018). Naringenin Attenuated Prostate Cancer Invasion via Reversal of Epithelial-to-Mesenchymal Transition and Inhibited uPA Activity. Anticancer Research 38: 6753–6758. https://doi.org/10.21873/anticanres.13045

Heerboth S, Housman G, Leary M, Longacre M, Byler S et al. (2015). EMT and tumor metastasis. Clinical and Translational Medicine 4: 6. https://doi.org/10.1186/s40169-015-0048-3

Hori M, Miki T, Okamoto M, Yazama F, Konishi H et al. (2013). The detergent-soluble cytoplasmic pool of survivin suppresses anoikis and its expression is associated with metastatic disease of human colon cancer. PloS one 8: e55710. https://doi.org/10.1371/journal.pone.0055710

Hu Y, Chen H, Duan C, Liu D, Qian L et al. (2013). Deficiency of Erbin induces resistance of cervical cancer cells to anoikis in a STAT3-dependent manner. Oncogenesis 2: e52. https://doi.org/10.1038/oncsis.2013.18

Jinka R, Kapoor R, Sistla PG, Raj TA, Pande G (2012). Alterations in Cell-Extracellular Matrix Interactions during Progression of Cancers. International Journal of Cell Biology 2012: 219196. https://doi.org/10.1155/2012/219196

Kanno SI, Tomizawa A, Hiura T, Osanai Y, Shouji A et al. (2005). Inhibitory effects of naringenin on tumor growth in human cancer cell lines and sarcoma S-180-implanted mice. Biological & Pharmaceutical Bulletin 28: 527–530. https://doi.org/10.1248/bpb.28.527

Kim D, Mollah ML, Kim K (2012). Induction of apoptosis of SW480 human colon cancer cells by (-)-epicatechin isolated from Bulnesia sarmienti. Anticancer Research 32: 5353–5361.

Kim YN, Koo KH, Sung JY, Yun UJ, Kim H (2012). Anoikis resistance: An essential prerequisite for tumor metastasis. International Journal of Cell Biology 2012: 306879. https://doi.org/10.1155/2012/306879

Kumar S, Park SH, Cieply B, Schupp J, Killiam E et al. (2011). A pathway for the control of anoikis sensitivity by E-cadherin and epithelial-to-mesenchymal transition. Molecular and Cellular Biology 31: 4036–4051. https://doi.org/10.1128/MCB.01342-10

Lambert AW, Pattabiraman DR, Weinberg RA (2017). Emerging Biological Principles of Metastasis. Cell 168: 670–691. https://doi.org/10.1016/j.cell.2016.11.037

Leonardi T, Vanamala J, Taddeo SS, Davidson LA, Murphy ME et al. (2010). Apigenin and naringenin suppress colon carcinogenesis through the aberrant crypt stage in azoxymethane-treated rats. Experimental Biology and Medicine (Maywood, N.J.) 235: 710–717. https://doi.org/10.1258/ebm.2010.009359

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2^(-ΔΔCT) method. Methods (San Diego, Calif.) 25: 402–408. https://doi.org/10.1006/meth.2001.1262

Lou C, Zhang F, Yang M, Zhao J, Zeng W et al. (2012). Naringenin decreases invasiveness and metastasis by inhibiting TGF-β-induced epithelial to mesenchymal transition in pancreatic cancer cells. PloS one 7: e50956. https://doi.org/10.1371/journal.pone.0050956

Maamer-Azzabi A, Ndozangue-Touriguine O, Bréard J (2013). Metastatic SW620 colon cancer cells are primed for death when detached and can be sensitized to anoikis by the BH3-mimetic ABT-737. Cell Death & Disease 4: e801. https://doi.org/10.1038/cddis.2013.328

Mackenzie GG, Oteiza PI (2006). Modulation of transcription factor NF-kappaB in Hodgkin's lymphoma cell lines: effect of (-)-epicatechin. Free Radical Research 40: 1086–1094. https://doi.org/10.1080/10715760600788396

Markowitz SD, Dawson DM, Willis J, Willson JKV (2002). Focus on colon cancer. Cancer Cell 1: 233–236. https://doi.org/10.1016/s1535-6108(02)00053-3

Mawji IA, Simpson CD, Gronda M, Williams MA, Hurren R et al. (2007). A chemical screen identifies anisomycin as an anoikis sensitizer that functions by decreasing FLIP protein synthesis. Cancer Research 67: 8307–8315. https://doi.org/10.1158/0008-5472.CAN-07-1687

Molnar B, Ladanyi A, Tanko L, Sréter L, Tulassay Z (2001). Circulating tumor cell clusters in the peripheral blood of colorectal cancer patients. Clinical Cancer Research : an Official Journal of the American Association for Cancer Research 7: 4080–4085.

Nagaprashantha LD, Vatsyayan R, Lelsani PCR, Awasthi S, Singhal SS (2011). The sensors and regulators of cell-matrix surveillance in anoikis resistance of tumors. International Journal of Cancer 128: 743–752. https://doi.org/10.1002/ijc.25725

Paoli P, Giannoni E, Chiarugi P (2013). Anoikis molecular pathways and its role in cancer progression. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1833: 3481–3498. https://doi.org/10.1016/j.bbamcr.2013.06.026

Patel K, Singh GK, Patel DK (2014). A review on pharmacological and analytical aspects of naringenin. Chinese Journal of Integrative Medicine. 24 (7): 551-560. https://doi.org/10.1007/s11655-014-1960-x

Peña C, García JM, Silva J, García V, Rodríguez R et al. (2005). E-cadherin and vitamin D receptor regulation by SNAIL and ZEB1 in colon cancer: clinicopathological correlations. Human Molecular Genetics 14: 3361–3370. https://doi.org/10.1093/hmg/ddi366

Salehi B, Fokou PVT, Sharifi-Rad M, Zucca P, Pezzani R et al. (2019). The Therapeutic Potential of Naringenin: A Review of Clinical Trials. Pharmaceuticals (Basel, Switzerland) 12: 11. https://doi.org/10.3390/ph12010011

Sastre J, Maestro ML, Puente J, Veganzones S, Alfonso R et al. (2008). Circulating tumor cells in colorectal cancer: correlation with clinical and pathological variables. Annals of Oncology 19: 935–938. https://doi.org/10.1093/annonc/mdm583

Sethi T, Rintoul RC, Moore SM, MacKinnon AC, Salter D et al. (1999). Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance in vivo. Nature Medicine 5: 662–668. https://doi.org/10.1038/9511

Siddique HR, Liao DJ, Mishra SK, Schuster T, Wang L et al. (2012). Epicatechin-rich cocoa polyphenol inhibits Kras-activated pancreatic ductal carcinoma cell growth in vitro and in a mouse model. International Journal of Cancer 131: 1720–1731. https://doi.org/10.1002/ijc.27409

Simpson CD, Anyiwe K, Schimmer AD (2008). Anoikis resistance and tumor metastasis. Cancer Letters 272: 177–185. https://doi.org/10.1016/j.canlet.2008.05.029

Singh AB, Sharma A, Dhawan P (2012). Claudin-1 expression confers resistance to anoikis in colon cancer cells in a Src-dependent manner. Carcinogenesis 33: 2538–2547. https://doi.org/10.1093/carcin/bgs275

Smit MA, Geiger TR, Song JY, Gitelman I, Peeper DS (2009). A Twist-Snail axis critical for TrkB-induced epithelial-mesenchymal transition-like transformation, anoikis resistance, and metastasis. Molecular and Cellular Biology 29: 3722–3737. https://doi.org/10.1128/MCB.01164-08

Smit MA, Peeper DS (2011). Zeb1 is required for TrkB-induced epithelial-mesenchymal transition, anoikis resistance and metastasis. Oncogene 30: 3735–3744. https://doi.org/10.1038/onc.2011.96

Song HM, Park GH, Eo HJ, Jeong JB (2016). Naringenin-Mediated ATF3 Expression Contributes to Apoptosis in Human Colon Cancer. Biomolecules & Therapeutics 24: 140–146. https://doi.org/10.4062/biomolther.2015.109

Spaderna S, Schmalhofer O, Hlubek F, Berx G, Eger A et al. (2006). A transient, EMT-linked loss of basement membranes indicates metastasis and poor survival in colorectal cancer. Gastroenterology 131: 830–840. https://doi.org/10.1053/j.gastro.2006.06.016

Spaderna S, Schmalhofer O, Wahlbuhl M, Dimmler A, Bauer K et al. (2008). The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer. Cancer Research 68: 537–544. https://doi.org/10.1158/0008-5472.CAN-07-5682

Tiwari N, Gheldof A, Tatari M, Christofori G (2012). EMT as the ultimate survival mechanism of cancer cells. Seminars in Cancer Biology 22: 194–207. https://doi.org/10.1016/j.semcancer.2012.02.013

Supplementary

Table S1. Primer sequences used for qRT-PCR amplifications of the EMT genes and housekeeping genes.

Genes	Forward primer	Reverse primer	Annealing temp. (°C)	PCR fragment size (bp)
E-cadherin (CDH1)	5'- CCCAGGAGCCAGACACATTT -3'	5'- TGCTGTTCTTCACGTGCTCA -3'	60	150
N-cadherin (CDH2)	5'-TGACAATGACCCCACAGCTC-3'	5'- TCCTGCTCACCACCACTACT-3	60	120
Vimentin (VIM)	5'-CCTGTGAAGTGGATGCCCTT-3'	5'-AGGCGGCCAATAGTGTCTTG-3'	60	115
Claudin-1 (CLDN1)	5'-TGCTTCTCTCTGCCTTCTGG-3'	5'-TGGAAGGTGCAGGTTTTGGA-3'	60	103
ZEB1	5'-CAGGGAGGAGCAGTGAAAGA-3'	5'-ACATCCTGCTTCATCTGCCT-3'	60	110
GAPDH	5'-CCACAGTCCATGCCATCACT-3'	5'-GGCAGGGATGATGTTCTGGA-3'	60	101
TBP	5'-CGCTGGCCCATAGTGATCTT-3'	5'-CCAGCACACTCTTCTCAGCA-3'	60	101