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Clove Extract Inhibits Tumor Growth and Promotes Cell Cycle Arrest and Apoptosis

Haizhou Liu[*,†,‡], **John C. Schmitz**[†,‡], **Jianteng Wei**[*], **Shousong Cao**[§], **Jan H. Beumer**[‡,¶], **Sandra Strychor**[‡], **Linyou Cheng**[*], **Ming Liu**[*], **Cuicui Wang**[*], **Ning Wu**[*], **Xiangzhong Zhao**[*], **Yuyan Zhang**[*], **Joshua Liao**[#], **Edward Chu**[†,‡], and **Xiukun Lin**[*]

[*]Department of Pharmacology, Capital Medical University, Beijing, China

[†]Department of Medicine, University of Pittsburgh, Pittsburgh, PA, USA

[‡]Cancer Therapeutics Program, University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA, USA

[§]Roswell Park Cancer Institute, Buffalo, NY, USA

[¶]Departmentof Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, PA, USA

[#]Hormel Institute, University of Minnesota, Austin, MN, USA

Abstract

Cloves (*Syzygium aromaticum*) have been used as a traditional Chinese medicinal herb for thousands of years. Cloves possess antiseptic, antibacterial, antifungal, and antiviral properties, but their potential anticancer activity remains unknown. In this study, we investigated the in vitro and in vivo antitumor effects and biological mechanisms of ethyl acetate extract of cloves (EAEC) and the potential bioactive components responsible for its antitumor activity. The effects of EAEC on cell growth, cell cycle distribution, and apoptosis were investigated using human cancer cell lines. The molecular changes associated with the effects of EAEC were analyzed by Western blot and (qRT)-PCR analysis. The in vivo effect of EAEC and its bioactive component was investigated using the HT-29 tumor xenograft model. We identified oleanolic acid (OA) as one of the components of EAEC responsible for its antitumor activity. Both EAEC and OA display cytotoxicity against several human cancer cell lines. Interestingly, EAEC was superior to OA and the chemotherapeutic agent 5-fluorouracil at suppressing growth of colon tumor xenografts. EAEC promoted G_0/G_1 cell cycle arrest and induced apoptosis in a dose-dependent manner. Treatment with EAEC and OA selectively increased protein expression of $p21^{WAF1/Cip1}$ and γ-H2AX and downregulated expression of cell cycle-regulated proteins. Moreover, many of these changes were at the mRNA level, suggesting transcriptional regulation by EAEC treatment. Our results demonstrate that clove extract may represent a novel therapeutic herb for the treatment of colorectal cancer, and OA appears to be one of the bioactive components.

Address correspondence to Dr. Xiukun Lin, Department of Pharmacology, Capital Medical University, Beijing 100069, China. Tel: +86-532-82898916; Fax: +86-532-82898916; linxiukun@yahoo.com. Dr. Joshua Liao, Hormel Institute, University of Minnesota, Austin, MN, 55912, USA. Tel: +1-507-437-9665; djliao@hi.umn.edu.

Keywords

Syzygium aromaticum; Traditional Chinese medicine; Colorectal cancer; Oleanolic acid (OA)

INTRODUCTION

Cancer remains a major public health problem in the world with the global burden of cancer continuing to increase (1). When cancer is diagnosed at an advanced stage, chemotherapy remains the most effective means to improve the patient's quality of life and prolong survival (2). Despite these improvements, current treatments have had little impact on 5-year overall survival in patients with advanced disease, and drug resistance remains a significant obstacle for successful treatment. Natural products have played an important role as an effective source of antitumor agents. It is estimated that up to 30–40% of the anticancer drugs used globally are derived from plant sources (3). The exploration of medicinal plants continues to hold significant promise for the prevention and treatment of cancer (4).

The dried buds of cloves, *Syzygium aromaticum* L, have been widely used as a spice and as traditional Chinese and Indian medicine. Cloves contain a wide range of bioactive compounds, which include eugenol, β-caryophyllene, humulene, chavicol, methyl salicylate, α-ylangene, and eugenone; the flavonoids eugenin, rhamnetin, kaempferol, and eugenitin; triterpenoids like oleanolic acid, stigmasterol, and campesterol; and several sesquiterpenes (5).

In our screening program for identifying potential antitumor agents from traditional Chinese herbs, we found that the extract of cloves displayed potent cytotoxic activity against several human cancer cell lines. In order to investigate the potential biological activities, we performed a series of experiments to identify the bioactive compounds and evaluated the in vivo antitumor activity of clove extract. In addition, we conducted a series of experiments to identify the potential biological mechanisms of the clove extract.

MATERIALS AND METHODS

Cell Culture

Human cancer cells, including ovarian cancer cells (SKOV-3), cervical epithelial cells (HeLa), liver cancer cells (BEL-7402), colon cancer cells (HT-29), breast cancer cells (MCF-7), pancreatic cells (PANC-1), normal colon epithelial cells (CCD 841 CoN), and normal lung fibroblasts (IMR-90) were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in RPMI-1640 media (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in a humidified incubator with 5% CO_2.

Extraction, Isolation, and Characterization of the Individual Compounds With Cytotoxic Activity From Cloves

Air-dried, powdered cloves (10.0 kg, purchased from Qingdao Company of Traditional Chinese Medicine, China) were extracted using 95% ethanol (40 L) at room temperature for

72 h. After filtration, the solution was concentrated to generate the ethanol extract (EEC), which was suspended in distilled water and further extracted with ethyl acetate as described previously (6). The ethyl acetate extract of cloves (EAEC) was fractionated using silica gel column chromatography (CC, 200 mesh and 400 mesh) and Sephadex LH-20 CC. The fraction with anti-proliferative activity was subsequently characterized by the Department of Chemistry and Molecular Engineering at Qingdao University using ^{1}H- and ^{13}C-NMR. EAEC was analyzed for pesticide residue content by the Pacific Agricultural Laboratory (Portland, OR, USA). A comprehensive residue screen (172 pesticides) was performed, and no pesticides were detected. Similarly, EAEC was analyzed for heavy metal content by Avomeen Analytical Services (Ann Arbor, MI, USA). While no lead, mercury, or cadmium was detected, arsenic levels were above the suggested USP parenteral limit but below the oral USP limit (Table 1).

HPLC-UV Quantitative Analysis of Oleanolic Acid and Eugenol

Analytical standards of oleanolic acid (OA), ursolic acid (UA), eugenol (Sigma, St. Louis, MO, USA), and EAEC were analyzed by HPLC based on a previously described assay (7). Briefly, extracts were resuspended in 60% methanol and injected onto a C18 column (RP-18, Agilent Zorbax Eclipse XDB-C18 3.0×150 mm, 3.5 μm) with a C18 guard column (RP18, Applied Biosciences). The mobile phase was composed of methanol with 0.2% ammonium acetate (solvent A) and water with 0.2% ammonium acetate, pH 6.6 (solvent B), with the following gradient: 0–5 min, 60% solvent A; 5–35 min, 60–90% solvent A linearly; 35–45 min, 90% solvent A, at a flow rate of 0.5 ml/min. The detection wavelength was set at 210 nm. The quantities of the components of EAEC were calculated by comparing their peak areas to those of reference standards prepared in 60% (v/v) methanol.

Cell Proliferation Assay

The effect of EEC, EAEC, OA, and eugenol on cell survival was determined using the MTT assay as previously described (8). In brief, cells (3×10^3 cells/well) were seeded in 96-well plates and allowed to attach overnight. Extracts and individual components were dissolved in DMSO and added to wells at various concentrations [stock concentrations: EEC (30 mg/ml), EAEC (20 mg/ml), oleanolic acid (26 mM), eugenol (650 mM)]. After incubation for an additional 48 h, 10 μl of the MTT solution (Sigma) was added to each well at a concentration of 5 mg/ml and incubated for an additional 4 h. The medium was aspirated, and the formazan crystals were solubilized by the addition of 100 μl of DMSO to each well. OD value was determined at 570 nm using a spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). Cell viability was calculated using the following formula: cell viability (%) = OD_{570nm} in cells treated with extracts/OD_{570nm} in control cells × 100%. The IC_{50} value is defined as the concentration of drug required to inhibit cell growth by 50% when compared to control cells. All assays were performed in triplicate with at least three to five independent experiments.

Clonogenic Assay

HT-29 cells were plated in six-well plates at a density of 300 cells/well. On the following day, cells were treated with EAEC or OA for 48 h. After an additional 12 days, colonies

were fixed with trypan blue solution (75% methanol/25% acetic acid/0.25% trypan blue), washed, and air-dried before counting colonies >50 cells.

Antitumor Activity In Vivo

All procedures and experiments involving animals in this study were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The study protocol (#2011-X-072) was approved by the Animal Ethics Committee at Capital Medical University, Beijing, China. HT-29 tumor cells (3×10^6 cells per mouse) were inoculated subcutaneously in nude mice (female, 20.0~22.0 g) at the axillary region as described previously (9). Tumor xenografts were allowed to grow to an average size of 100–200 mm^3 and were randomly assigned to four different treatment groups (five mice per group): (A) vehicle control (50% DMSO in physiological saline); (B) 5-FU 100 mg/kg; (C) EAEC 50 mg/kg; (D) OA 50 mg/kg. The mice were administered drug via intraperitoneal (IP) injections once daily for 5 days. Tumor size was measured on two axes with the aid of Vernier calipers, and tumor volume (mm^3) was calculated using the formula: $1/2(L \times W^2)$, where L is the longest, and W is the shortest axis (10). Percentage of initial tumor size (%) = $V_{tumor}/V_{initialtumor} \times 100$, where V_{tumor} and $V_{initialtumor}$ are defined as the mean tumor volumes in the extract-treated group determined at various times and initial time, respectively. Percentage of body weight (%) = $W_{body}/W_{initial\ body} \times 100$, where W_{body} and $W_{initial\ body}$ are defined as the mean body weight of mice in the drug treatment group determined at various times and initial time, respectively. Mice were euthanized at the end of the study and/or when tumor size exceeded 2,000 mm^3.

Cell Cycle Analysis

The effect of EAEC on cell cycle distribution was determined by flow cytometry with propidium iodide (PI) staining (11). HT-29 cells were incubated with vehicle control (0.45% DMSO) or EAEC (50, 70, or 90 μg/ml). After 24h, cells were harvested, stained with PI, and analyzed on a BD Accuri C6 flow cytometer (BD Cytometers, Inc., Ann Arbor, MI, USA).

Detection of Apoptosis

HT-29 cells were incubated with vehicle control (0.45% DMSO) or EAEC (50, 70, or 90 μg/ml) for 24, 48, and 72 h, respectively. At each time point, cells were harvested, stained with FITC Annexin V Apoptosis Detection Kit (BD Biosciences), and analyzed on an Accuri C6 Flow Cytometer.

Western Blot Analysis

HT-29 cells were treated with vehicle control (0.45% DMSO), EAEC (50, 70, or 90 μg/ml) or OA (110 or 153 μM) for 24 h. Equivalent amounts of protein (40 μg) from each cell lysate were resolved on SDS-PAGE and performed as previously reported (12). Gels were electroblotted onto nitrocellulose membranes (0.45 μm; Bio-Rad), which were then incubated in blocking solution (1 × PBS, 0.1% Tween-20, 5% nonfat dry milk powder) for 1 h at room temperature. Membranes were incubated at 4°C overnight with the following primary antibodies at the indicated dilutions: anti-E2F1, 1:1,000 (Cell Signaling, Danvers, MA, USA); anti-TS (thymidylate synthase), 1:2,000 (Cell Signaling); anti-survivin, 1:1,000

(Cell Signaling); anti-p21$^{waf1/cip1}$, 1:1,000 (Cell Signaling); anti-Rb, 1:5,000 (Cell Signaling); anti-γ-H2AX, 1:1,000 (Cell Signaling); anti-H2AX, 1:1,000 (R&D Systems, Minneapolis, MN, USA); anti-GAPDH, 1:30,000 (Santa Cruz). After additional TBST washes, membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) for 1 h at room temperature and detected by the enhanced chemiluminescence method (SuperSignal West Pico substrate; Pierce, Rockford, IL, USA). Quantitation of signal intensities was performed by densitometry on a Xerox scanner using NIH IMAGEJ software.

Real-Time qRT-PCR Analysis

HT-29 cells were treated with vehicle control (0.45% DMSO) or EAEC (50, 70, or 90 μg/ml) for 24 h. Cells were harvested, and total RNA was extracted in Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. qRT-PCR analysis was performed as described previously (12). The first-strand cDNA was synthesized using 1.0 μg total RNA and the iScript^{TM} Reverse Transcription Supermix for real-time polymerase chain reaction (RT-PCR) (Bio-Rad). PCR was performed in triplicate using the SsoFast^{TM} Probes Supermix (Bio-Rad) in a final reaction volume of 10 μl with gene-specific primer/probe sets and a standard thermal cycling procedure (40 cycles) on a Bio-Rad CFX96^{TM} Real-Time PCR System. RNA levels of p21, E2F1, TS, and 18S were assessed using the TaqMan Gene Expression real-time PCR assays (Applied Biosystems assay IDs: Hs00355782_m1, Hs00153451_m1, Hs00426586_m1, Hs03928990_g1). Results were expressed as the threshold cycle (Ct). Relative quantification of target transcripts was determined by the comparative Ct method (ΔΔCt) according to the manufacturer's protocol. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative changes in gene expression. Control PCR experiments in the absence of reverse transcription were performed to confirm that the total RNA was not contaminated with genomic DNA.

Statistical Analysis

Data are presented as mean ± SD unless otherwise indicated. The Student's *t* test was used to determine statistical significance between two groups. Analysis was done with Prism version 5 (GraphPad Software, Inc.). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Clove Extract Inhibits Cancer Cell Growth

In our initial series of experiments, we analyzed the growth inhibitory effects of dried clove powder (resuspended in water) in human colon cancer HT-29 cells. The IC_{50} concentration using the MTT assay was approximately 2.2 mg/ml (data not shown). To enhance the anti-proliferative effects of the clove extract, we subjected the clove powder to ethanol extraction in an attempt to concentrate the bioactive compounds. As seen in Figure 1A, the ethanol extract of clove (EEC) displayed an approximate 10-fold enhancement in cell growth inhibition against a panel of human cancer cell lines, including breast (MCF-7), ovarian (SKOV-3), cervical (HeLa), liver (BEL-7402), pancreatic (PANC-1), and colon (HT-29) cells. The IC_{50} values ranged from 196 to 455 μg/ml (Table 2). This EEC extract was further

processed with extraction by ethyl acetate. As seen in Figure 1B, the resultant EAEC demonstrated enhanced growth inhibitory effects with twofold lower IC_{50} values against all cell lines tested when compared with EEC. The IC_{50} values ranged from 108 to 217 μg/ml (Table 2) with PANC-1 and HT-29 cells being the most sensitive. Given their sensitivity to EAEC-mediated growth suppression, HT-29 cells were utilized in subsequent experiments. In addition to the MTT assay, clonogenic assays were performed on HT-29 cells resulting in inhibition of clonal growth with slightly lower IC_{50} values (66 ± 8 μg/ml) (Fig. 1D). In order to determine the growth effect of EAEC on normal cells, nontumorigenic colon 841 and lung IMR90 cells were treated with EAEC. We obtained EAEC IC_{50} values similar to those observed in cancer cell lines (data not shown).

Identification of Active Components From Cloves

We next attempted to identify the individual component(s) of clove extract that was mediating its anti-proliferative activity. We subjected the EAEC (40.0 g) to silica gel column chromatography (CC, 200 mesh) with increasing concentrations of ethyl acetate in petroleum ether as the mobile phase. Five fractions were isolated, and fraction III (4.1 g) was identified as having the most potent effect on HT-29 cell growth by MTT assay. Fraction III was then separated using Sephadex LH-20 CC with four subfractions being isolated. Subfraction III (886.5 mg) was found to have the greatest antiproliferative effects, and this fraction was further purified using silica CC (400 mesh). The pure compound was a white amorphous powder having a melting point of 309–311°C and a purple color in the Liebermann–Burchard reaction. This compound was then subjected to NMR analysis: ^{1}H-NMR (500 MHz, $CDCl_3$): δ5.27 (1H, m, H-12), 3.25 (1H, dd, *J* = 15.0, 5.9 Hz), 2.81 (1H, dd, *J* = 12.3, 5.5 Hz), 1.13 (3H, s), 1.06 (3H, s), 0.98 (3H, s), 0.92 (3H, s), 0.90 (3H, s), 0.77 (3H, s), 0.74 (3H, s); ^{13} C-NMR (125 MHz, $CDCl_3$): δ38.4 (C-1), 27.1 (C-2), 79.0 (C-3), 38.7 (C-4), 55.2 (C-5), 18.3 (C-6), 32.9 (C-7), 39.3 (C-8), 47.6 (C-9), 37.1 (C-10), 22.9 (C-11), 122.6 (C-12), 143.6 (C-13), 41.6 (C-14), 27.7 (C-15), 23.5 (C-16), 46.5 (C-17), 40.9 (C-18), 45.9 (C-19), 30.6 (C-20), 33.8 (C-21), 32.4 (C-22), 28.1 (C-23), 15.3 (C-24), 15.5 (C-25), 17.1 (C-26), 25.9 (C-27), 183.4 (C-28), 33.4 (C-29), 23.4 (C-30). Based on comparisons with published data (13), the purified compound was identified as (3β)-3-hydroxyolean-12-en-28-oic acid (oleanolic acid, OA). The cytotoxic activity of OA was analyzed in various human cancer cell lines. As seen in Figure 1C, OA inhibited the growth of MCF-7, SKOV-3, HeLa, BEL-7402, PANC-1, and HT-29 cancer cells with similar IC_{50} values ranging from 88 to 159 μM (Table 2). These values are comparable to those previously reported (14,15). In addition, clonogenic assays were performed on HT-29 cells, which resulted in OA IC_{50} values that were slightly lower than with the MTT assay (63 ± 2.2 μM vs. 88 μM) (Fig. 1E).

To independently confirm the OA identification by NMR, HPLC-UV analysis was performed. The fingerprint analysis of EAEC is presented in Figure 2A. Based on the OA standard curve, OA comprised about 34% of EAEC (dry w/w). From this percentage, we calculated the concentration of OA in EAEC (IC_{50} value, 108 μg/ml; HT-29 cells) as approximately 80 μM. Since the IC_{50} of OA in HT-29 cells is 88 μM (Table 2), this suggests that the majority of the in vitro activity of EAEC stems from the OA component. As clove oil is approximately 80% eugenol, we determined the eugenol content in EAEC to be 14%

of EAEC (dry w/w). Thus, the eugenol concentration in EAEC (at its IC_{50} dose) is 92 μM. Previous studies have shown that eugenol has antiproliferative activity in multiple cancer cell lines (16). To determine whether eugenol may be contributing to EAEC cytotoxicity, human colon cancer HT-29 cells were incubated with various concentrations of pure eugenol. An IC_{50} value of 2.4 mM was obtained, which was significantly higher than OA and EAEC. Thus, these results suggest that OA is one of the bioactive components in EAEC responsible for its in vitro cytotoxicity.

Antitumor Activity of EAEC and OA in Nude Mice Bearing HT-29 Xenografts

To determine whether clove extract exhibited in vivo antitumor activity, EAEC and OA were evaluated in athymic nude mice containing HT-29 xenografts. As seen in Figure 3A, tumor growth was significantly reduced in the EAEC-treated versus control groups. Treatment with EAEC resulted in significantly greater tumor growth suppression than after an equivalent dose of OA. For comparison, we tested the antitumor activity of the fluoropyrimidine 5-fluorouracil (5-FU) in this same model system, given that 5-FU remains the backbone for chemotherapy regimens treating colorectal cancer. 5-FU was found to have antitumor activity that appeared to be somewhat better than OA but slightly less than EAEC administration. Animal weight loss is commonly used as a surrogate marker of toxicity, and as seen in Figure 3B, treatment with EAEC was well tolerated with relatively minimal weight loss when compared to control mice.

Effect of EAEC on Cell Cycle Distribution

To begin to determine the potential mechanisms by which EAEC was exerting its biological activity, we analyzed the dose-dependent effects of EAEC on cell cycle distribution in human colon cancer HT-29 cells by flow cytometry. As seen in Figure 4A, treatment of HT-29 cells with EAEC at concentrations of 50, 70, and 90 μg/ml gave rise to an increased fraction of cells in the G_0/G_1 interphase with 65.8 ± 7.1%, 70.2 ± 3.8%, and 78.4 ± 3.3%, respectively, compared with control cells (56.6 ± 7.7%). In addition, a dose-dependent decrease in the fraction of cells in the S and G_2/M phases was observed with EAEC treatment (Fig. 4B).

Effect of EAEC on Apoptosis

Annexin V-FITC and PI staining were used to evaluate the apoptotic effects of EAEC. Treatment of HT-29 cells with EAEC resulted in a dose-dependent increase in the number of early and late apoptotic cells (Fig. 5A and 5C). The relative percentage of cells in early apoptosis in response to 50, 70, and 90 μg/ml of EAEC was 4.6 ± 2.7%, 11.2 ± 4.9%, and 36.4 ± 12.2%, respectively, while the relative percentage of cells in late apoptosis was 1.4 ± 1.0%, 2.4 ± 1.3%, and 6.0 ± 3.7%, respectively. The fraction of EAEC-treated cells undergoing apoptosis also increased in a time-dependent manner (Fig. 5B and 5D). While small increases in apoptosis were observed after 24 h treatment of 90 μg/ml EAEC, longer exposures to EAEC (48 and 72 h) resulted in significant increases in the percentage of total apoptosis (early plus late), 27.8 ± 10.1% and 42.5 ± 15.2%, respectively.

EAEC Affects the Expression of G_0/G_1-Related and Apoptosis-Related Regulators

To further investigate the underlying mechanisms involved in EAEC-induced effects on G_0/G_1 arrest and apoptosis in HT-29 cells, we determined the expression of several key cellular proteins involved in cell cycle progression and apoptosis. As seen in Figure 6A, 24-h treatment with EAEC significantly altered the expression of several proteins in a dose-dependent manner (quantified in Fig. 6B). The cyclin-dependent kinase inhibitor, $p21^{WAF1/Cip1}$, was significantly upregulated in addition to a reduction in the protein levels of Rb and E2F1. The E2F1-regulated protein, thymidylate synthase, was similarly reduced in response to E2F1 loss. The expression of survivin was suppressed, which coincided with the observed induction of apoptosis. Furthermore, EAEC treatment led to an increase in the expression of γ-H2AX, a marker of DNA damage. These effects on protein expression were observed as early as 12 h after EAEC treatment (Fig. 6C and 6D). In order to determine whether treatment with the bioactive component, OA, was associated with similar effects on protein expression, additional experiments were performed with the purified OA compound. Using a concentration of OA of 110 μM, we observed weaker effects on the expression of cellular proteins compared with EAEC at 70 μg/ml and 90 μg/ml, respectively. However, at a higher OA concentration (153 μM), we observed a dramatic change in protein expression (Fig. 6A). Eugenol, another component of EAEC that we identified, produced similar effects on several proteins, albeit requiring much higher concentrations (Fig. 7). To begin to determine the molecular level at which these changes in protein expression were controlled, mRNA levels were analyzed after EAEC treatment. As seen in Figure 6E, (qRT)-PCR analysis revealed that EAEC treatment resulted in a significant increase in the mRNA level of p21 as well as a decrease in the mRNA levels of E2F1 and TS, which quantitatively accounts for the reduction in expression of the corresponding proteins. These results suggest that the inhibitory effects of EAEC treatment on the expression of cell cycle and apoptosis-related proteins are controlled at the transcriptional level.

DISCUSSION

In the present study, we demonstrated that the ethyl acetate extract of cloves has in vitro antiproliferative and in vivo antitumor activity. We have also shown that the triterpenoidoleanolic acid (OA) appears to be one of the active compounds contained within EAEC. OA is a pentacyclic triterpene present in many herbs, fruits, and vegetables, such as Panar ginseng C.A. Meyer (*Araliaceae*), Eugenia jaumbolana Lam. (*Myrtaceae*), olive leaves (*Oleaeuropaea*), mistletoe sprouts (*Viscum album*), cloves (*Syzyiumaromaticum*), and grapes (*Vitisvinifera*) that exhibits a wide range of pharmacological and biochemical effects including anti-inflammatory, antihyperlipidemic, hypoglycemic effects (17,18). Previous studies from our laboratory have provided evidence that OA arrests the cell cycle and induces apoptosis in Panc-28 cells, possibly via ROS-mediated mitochondrial and lysosomal pathways (19). However, Juan et al. demonstrated that OA was unable to increase ROS production in HT-29 cells (14).

Our results have shown that the in vitro antiproliferative activity for EAEC was likely mediated by OA. However, our in vivo data demonstrated that treatment with EAEC resulted in greater tumor growth suppression than OA itself. This suggests that either

additional components of EAEC may play a role in its in vivo activity or that absorption and metabolism influence its antitumor ability. Interestingly, even though our in vitro results revealed that normal cell lines were sensitive to the antiproliferative effects of EAEC, neither EAEC nor OA administration caused significant weight loss in the mice. However, it has been shown that administration of higher doses of OA can result in body weight loss and liver toxicity (20). One of the main principles of Chinese herbal medicine is that the formulations are usually made up of multiple components, each of which are working in concert to yield a medicine with optimal biological and clinical effects. Along these lines, it is unusual for a single component to be isolated from a Chinese herbal medicine with optimal biological and clinical activity. As such, several studies have shown that combining OA or the closely related ursolic acid with 5-fluorouracil-based therapy results in synergistic cancer growth inhibition (21, 22). Our study provides further evidence that an extract is more effective in vivo than a single isolated component.

A clearer understanding as to how EAEC regulates the cellular death pathway would provide important insights into the potential mechanisms and provide a rational basis for the development of anticancer herbs with improved potency and selectivity. Inhibition of cell cycle progression in cancer cells is considered one of the most effective strategies for the control of tumor growth (23). Our data clearly showed that the significant increase of G_0/G_1 phase cells was accompanied by a decrease of cells in the S and G_2/M phases, respectively. It has been reported that overexpression of p21 inhibits the kinase activity of cyclin-CDK4 complexes (24). D-type cyclins synthesized in response to mitogenic signals form active complexes with CDK4 or CDK6, leading to phosphorylation and inactivation of pRb. This, in turn, dissolves complexes of pRb with members of the E2F family of transcription factors and associated chromatin-modifying enzymes, allowing transcription of genes required for S phase (25). Progression of cells from the G_1 phase of the cell cycle to the S phase is associated with E2F1 activation of a number of genes whose expression is required for the G_1 to S transition (26). In the present study, we have demonstrated that EAEC treatment increased the protein level of the CDK inhibitor $p21^{WAF1/Cip1}$. Pratheeshkumar et al. observed a similar response upon OA treatment of B16F-10 melanoma cells (27). Moreover, EAEC not only downregulated the transcriptional repressor E2F1 but also downregulated TS, an E2F1 target gene. These observations suggest that the stimulatory effects of EAEC on p21 may lead to suppression of E2F1 signaling, which plays a role in the induction of G_0/G_1 arrest in human colon cancer cells. This phenomenon has been observed with other plant components. Both resveratrol and silibinin have been reported to increase p21 expression and cause G_1 arrest, which led to an induction of apoptosis (28,29). In this study, EAEC treatment resulted in induction of apoptosis. Consistent with these observations, treatment with EAEC also resulted in decreased expression of survivin, a member of the inhibitor of apoptosis (IAP) family, as well as promoted the phosphorylation of H2AX (γ-H2AX), a measure of DNA damage. The main component of EAEC, OA, has been shown to decrease survivin to a similar extent in human lung cancer cells (15). Taken together, our findings suggest that there are potentially multiple mechanisms by which EAEC is able to exert its growth inhibitory effects on cancer cells.

In summary, our studies have shown that the ethyl acetate extract of cloves displayed antitumor activity both in vitro and in vivo. Clove extract may represent a novel therapeutic

herb for cancer treatment, and OA is one of the components responsible for part of its antitumor activity. Further studies are needed in order to characterize other potential antitumor components contained within cloves, to address whether there are synergistic effects among these components, and to determine the role of metabolism as it relates to the in vivo activity of EAEC and OA.

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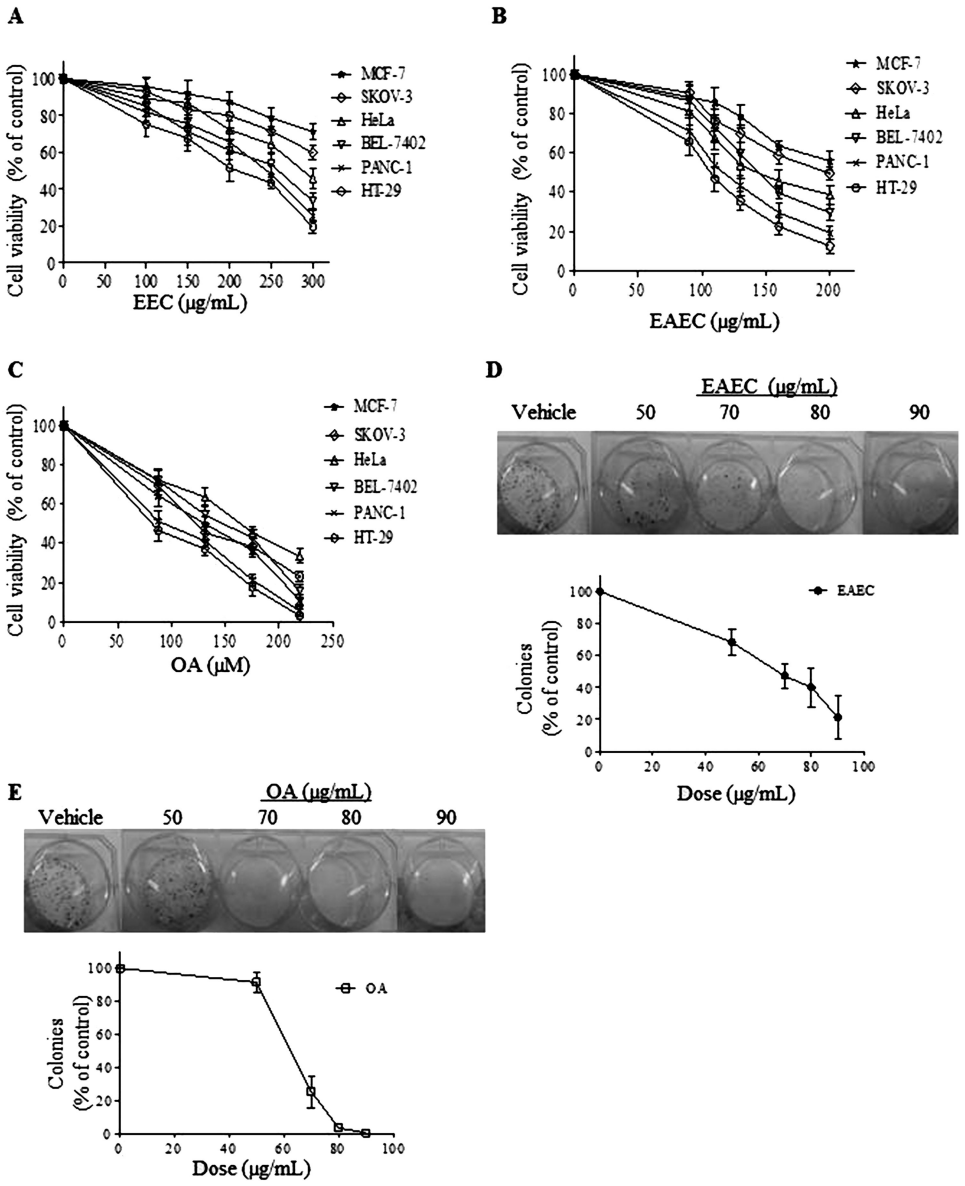

Figure 1.

Effect of EEC, EAEC, and OA on proliferation of MCF-7 (—★—), SKOV-3 (—◇—), HeLa (—△—), BEL-7402 (—▽—), PANC-1 (—×—), and HT-29 cells (—○—) and effect of EAEC and OA on colony formation of HT-29 cells. Cells were treated with vehicle control (DMSO), EEC (A), EAEC (B), or OA (C) for 48 h. HT-29 cells were treated with vehicle control, EAEC (D), or OA (E) for 48 h and allowed to grow for an additional 12 days. Photographs of cell culture dishes from a representative experiment are shown. Colony percentages represent the mean ± SD from three individual experiments performed in duplicate with EAEC or OA. The number of colonies after treatment with vehicle control was normalized to 100%. All values represent the mean ± SD from three independent experiments.

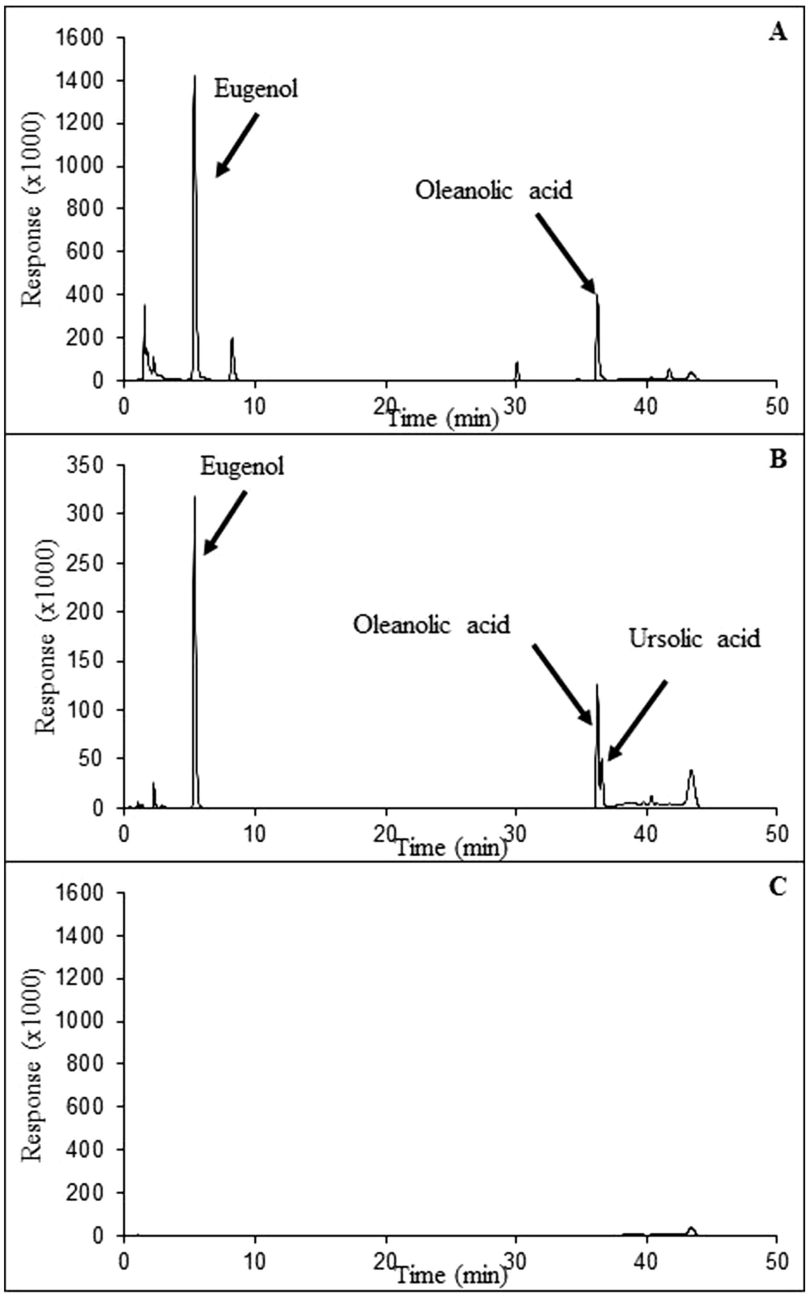

Figure 2.

HPLC-UV analysis of EAEC. EAEC (A) and a mixture of analytical standards (B) were resuspended in 60% methanol, injected onto an Agilent Zorbax Eclipse XDB-C18 column, and detected at 210 nm. Retention times for eugenol, OA, and UA were 5.39, 36.3, and 36.7 min, respectively. (C) Mobile phase.

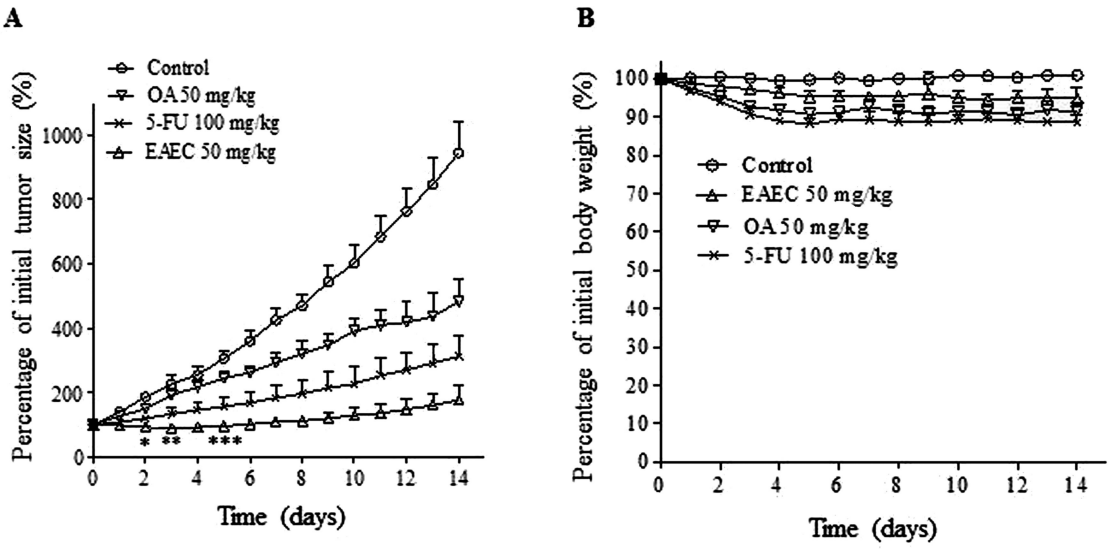

Figure 3.

(A) Antitumor activity of EAEC, OA, and 5-FU in nude mice bearing HT-29 tumors. EAEC (50 mg/kg, IP), OA (50 mg/kg, IP), and 5-FU (100 mg/kg, IP) were administered once daily for 5 days. Significant differences in tumor growth ($p < 0.05$) were achieved on the following days: *EAEC versus control; **EAEC versus OA; ***EAEC versus 5-FU. (B) Effect of EAEC, OA, and 5-FU on body weight. Data in the graphs represent the mean ± SD (n = 5).

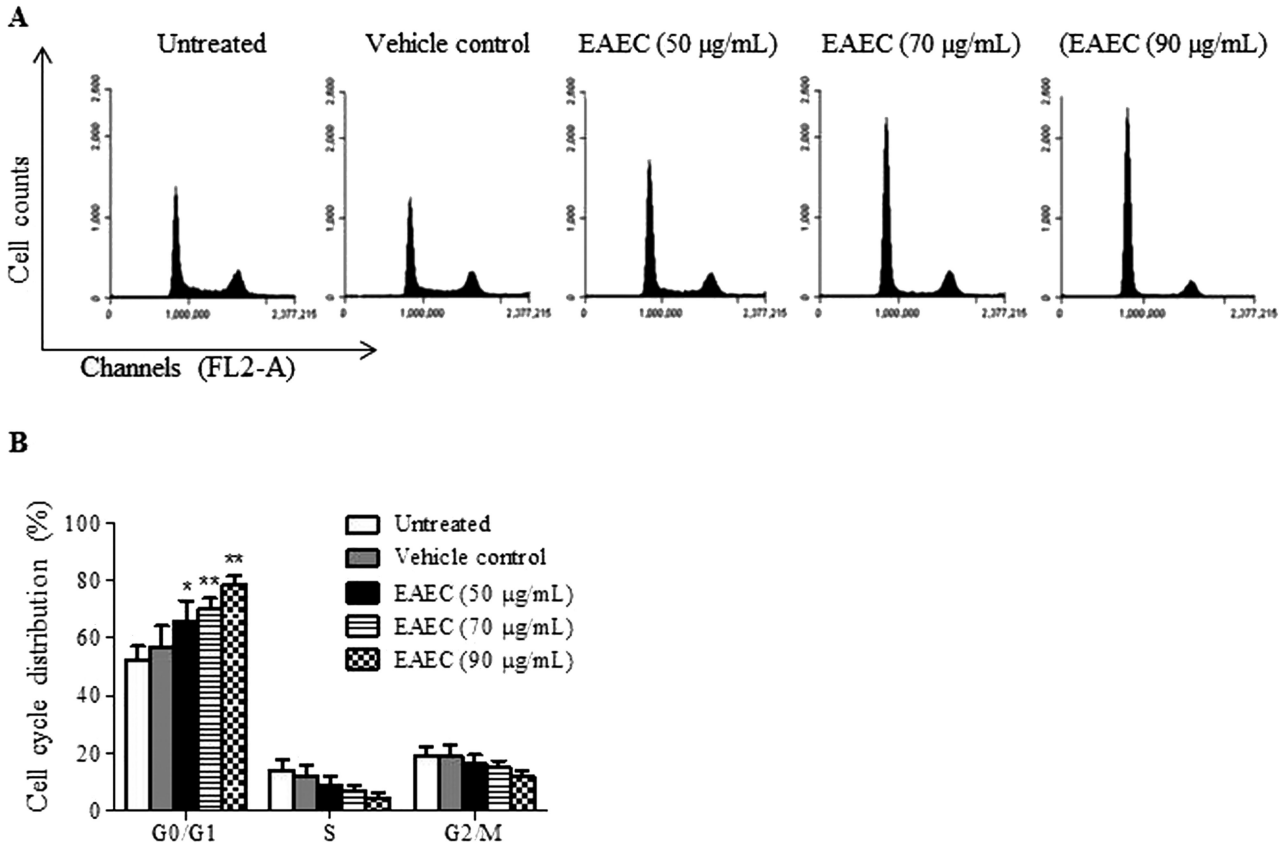

Figure 4.

Effect of EAEC on cell cycle distribution. (A) HT-29 cells were treated with vehicle control or 50, 70, or 90 μg/ml EAEC for 24 h, stained with propidium iodide, and analyzed by flow cytometry. (B) Quantitative analysis of cell cycle distribution. Distribution percentages represent mean ± SD from three independent experiments. Significant difference from vehicle control, $*p < 0.05$; $**p < 0.01$.

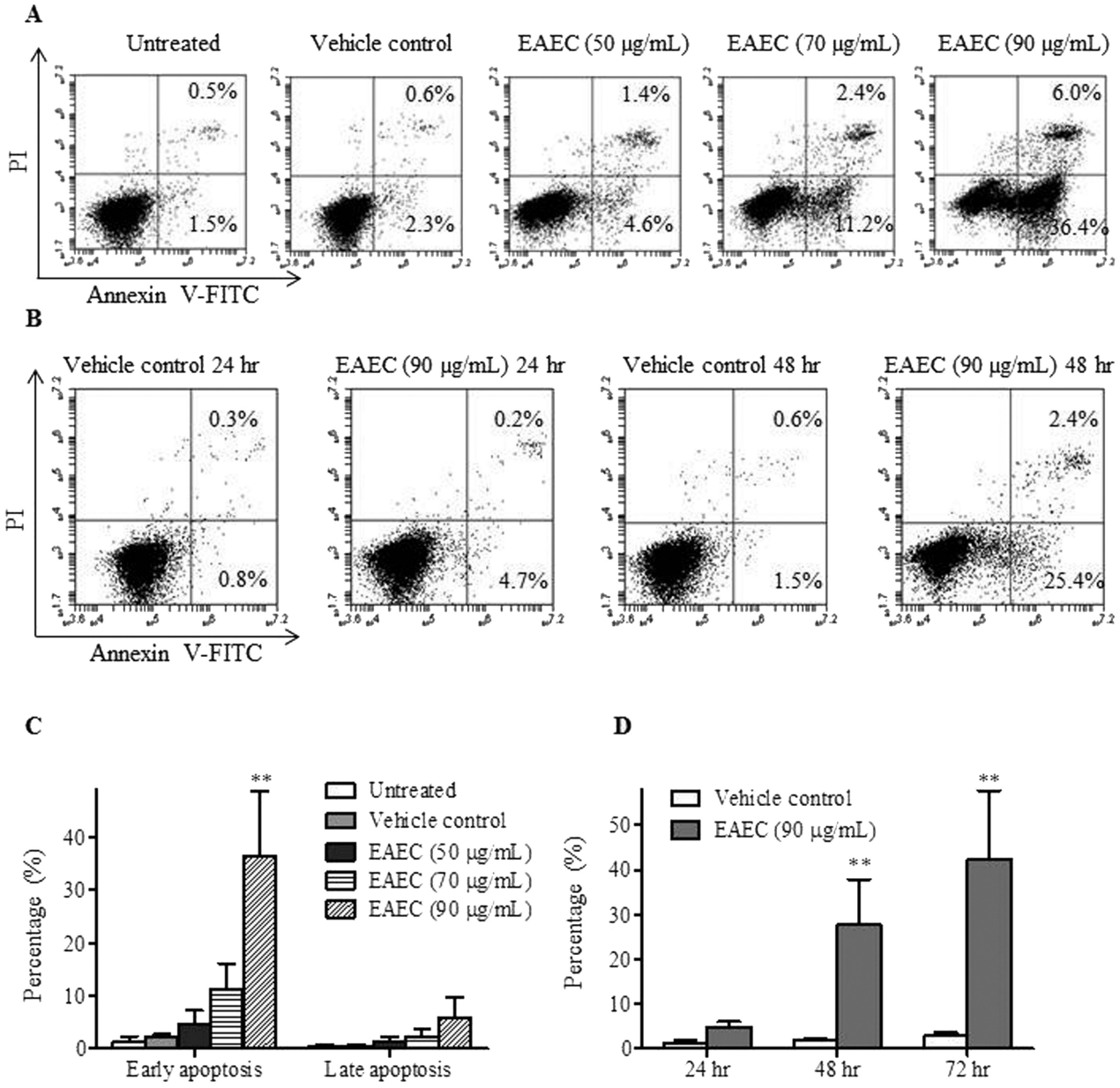

Figure 5.

Effect of EAEC on apoptosis. (A) HT-29 cells were exposed to EAEC for 72 h followed by Annexin V-FITC/PI staining as described in the Materials and Methods section. Top left quadrant, necrotic cells (Annexin V-FITC./PI^+); top right quadrant, cells in late stage of apoptosis (Annexin V-$FITC^+/PI^+$); bottom right quadrant, cells in early stage of apoptosis (Annexin V-$FITC^+/PI^-$); bottom left quadrant, viable cells (Annexin V-$FITC^-/PI^-$). (B) Cells were treated with vehicle control or 90 μg/ml EAEC for 24 h and 48 h, respectively. (C) Quantitative analysis of dose-dependent alteration of EAEC on early and late apoptosis after 72 h. (D) Quantitative analysis of time-dependent alteration of EAEC on total apoptosis (early apoptosis plus late apoptosis). The bar graphs were obtained from three

independent experiments relative to vehicle controls, which were defined as 100%. Significant difference from vehicle control, *$p < 0.05$; **$p < 0.01$.

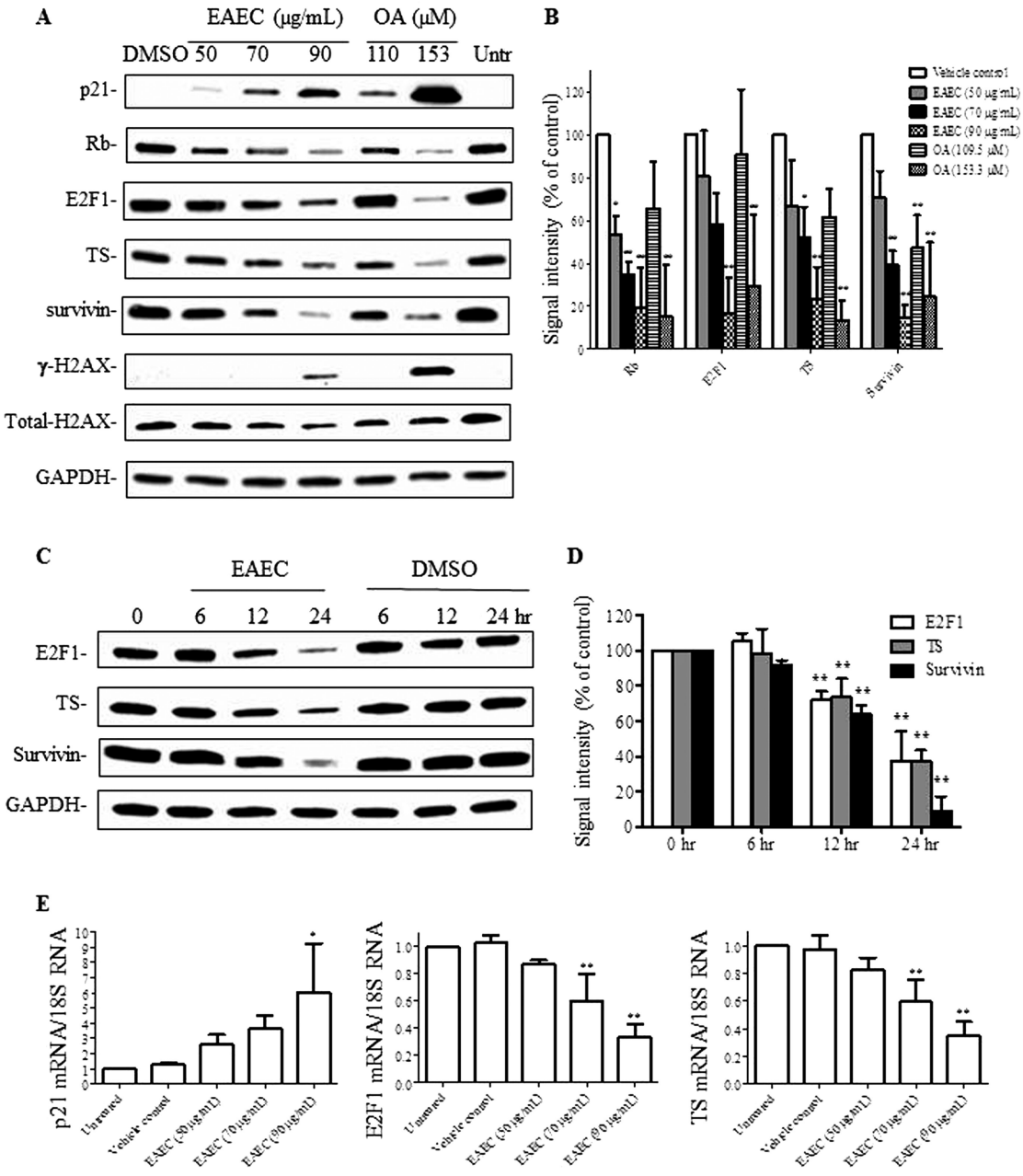

Figure 6.

Dose- and time-dependent effects of EAEC and OA treatment on the expression of protein and mRNA levels. (A) HT-29 cells were treated with either vehicle control (0.45% DMSO), EAEC (50, 70, or 90 μg/ml), or OA (110 or 153 μM) for 24 h, followed by processing for Western blot analysis. (B) Quantitation of signal intensities from Western blots was performed by densitometry on a Xerox scanner using NIH IMAGEJ software for dose-dependent experiments. (C) Cells were treated with vehicle control or EAEC. After various time points, cells were harvested and processed for Western blot analysis. Blots are representative of three independent experiments. (D) Quantitation of signal intensities from Western blots for time-dependent experiments. (E) HT-29 cells were treated with vehicle

control or EAEC for 24 h, after which time total RNA was extracted from cells, and mRNA levels were quantified by (qRT)-PCR analysis as described in the Materials and Methods section. Significant difference from vehicle control, *$p < 0.05$; **$p < 0.01$.

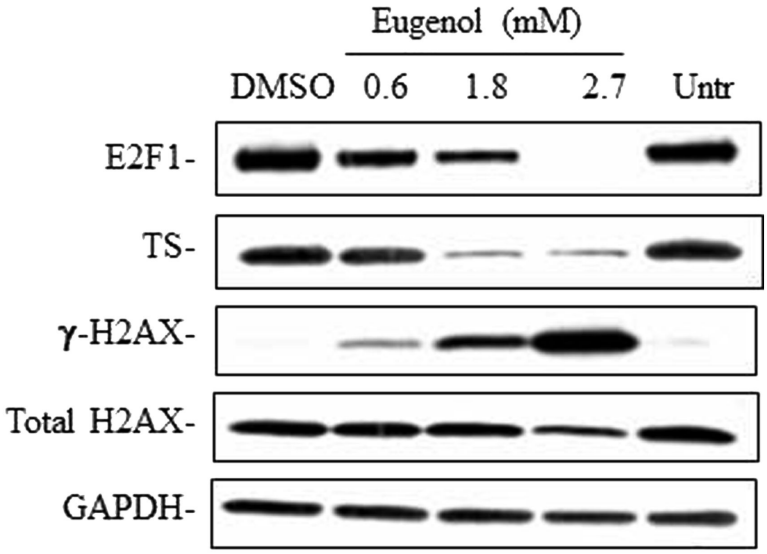

Figure 7.

Dose-dependent effects of eugenol on protein expression in HT-29 cells. Cells were treated with either vehicle control (0.45% DMSO) or eugenol (0.6, 1.8, 2.7 mM) for 24 h, followed by processing for Western blot analysis.

Table 1

Elemental Analysis of EAEC

Element	Detection Level (μg/g)	Results (μg/g)	USP Oral Limit* (μg/g)	USP Parenteral Limit* (μg/g)
Total arsenic	0.1	0.6	1.5	0.15
Total chromium	0.5	0.85	15	1.5
Total copper	1.0	1.9	50	5
Total iron	2.0	95	1500	150
Total manganese	1.0	1.8	700	70
Total zinc	1.0	3	1500	150
Total sodium	10.0	630	n.d.	n.d.
Total calcium	5.0	83	n.d.	n.d.
Total magnesium	3.0	24	n.d.	n.d.
Total potassium	2.0	910	n.d.	n.d.
Total aluminum	1.0	None detected		
Total antimony	0.3	None detected		
Total barium	1.0	None detected		
Total beryllium	0.2	None detected		
Total cadmium	0.1	None detected		
Total cobalt	0.5	None detected		
Total lead	1.0	None detected		
Total mercury	0.1	None detected		
Total molybdenum	0.1	None detected		
Total nickel	1.0	None detected		
Total selenium	0.2	None detected		
Total silver	0.1	None detected		
Total thallium	0.5	None detected		
Total tin	5.0	None detected		
Total vanadium	1.0	None detected		

EAEC was analyzed by Avomeen Analytical Services (ID: 010914UP2516).

* http://www.usp.org/sites/default/files/usp_pdf/EN/USPNF/2008-04-10InorganicImpuritiesStim.pdf n.d., not determined.

Table 2

IC_{50} Values for Clove Extracts and OA

	IC_{50} (μg/ml)		IC_{50} (μM)
Cell Line	**EEC**	**EAEC**	**OA**
MCF-7	455.0 ± 65.7	216.7 ± 14.6	121.9 ± 10.5
SKOV-3	383.2 ± 24.1	190.6 ± 10.3	127.6 ± 10.3
HeLa	297.1 ± 23.1	151.9 ± 16.4	159.1 ± 14.2
BEL-7402	239.8 ± 24.6	146.9 ± 9.3	137.2 ± 11.4
PANC-1	229.1 ± 17.5	118.9 ± 9.9	96.3 ± 8.0
HT-29	196.1 ± 21.6	108.0 ± 8.6	88.4 ± 8.7

IC_{50} values were determined by MTT assay.

EEC, ethanol extract of cloves; EAEC, ethyl acetate extract of cloves; OA, oleanolic acid.