Growth Inhibition of Human Colon Cancer Cells by Plant Compounds

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OBJECTIVE: Evidence is accumulating that compounds of plant origin (phytochemicals) exert anti-cancer effects. The purpose of this study was to determine if resveratrol, cinnamaldehyde, and piperine (from red grapes, cinnamon, black pepper respectively) have anti-proliferative effects on colon cancer.

DESIGN: Quantitative effects of each phytochemical on concentration responses and time courses of proliferation of cultured human colon cancer cells (DLD-1) were assessed.

SETTING: Research was performed at Saint Louis University.

MAIN OUTCOMES MEASURES: Responses were measured by spectrophotometry of surviving cells stained by a dye method.

RESULTS: Phytochemicals displayed anti-proliferative effects on DLD-1 cells in concentration- and kinetic-dependent manners. Cinnamaldehyde offered statistically significant effects at 24 hours [200 μM], 48 hours [100 - 200 μM], and 72 hours [200 μM]. Piperine displayed a trend towards anti-proliferation at 24 hours and statistically significant inhibition at 48 and 72 hours [100 - 200 μM]. Resveratrol displayed significant anti-proliferative effects at 24 hours [50-200 μM], 48 hours [10-200 μM], and 72 hours [10-200 μM].

CONCLUSION: Cinnamaldehyde, piperine, and resveratrol offer significant *in vitro* anti-proliferative effects on cultured human colon cancer cells. While each phytochemical exhibited significant anti-proliferative effects, resveratrol results were most impressive in that lower concentrations administered at regular intervals were significantly effective. These results taken together with everyday dietary availability of concentrations used in this study strongly suggest that regular intake of low doses of these phytochemicals offer preventive effects against colon cancer.

ABREVIATIONS: DLD-1 = human colon cancer cells; DMSO = dimethylsulfoxide.

INDEX TERMS: alternative medicine; cinnamaldehyde; phytochemicals; piperine; resveratrol.

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Each year, approximately 1.5 million people are diagnosed with cancer in the United States and more than 500,000 die annually. Colon cancer is the second leading cause of cancer death in the US. Only lung cancer kills more people. Of the 140,000 people diagnosed with colon cancer each year in the US, 40% die[1].

There are two major problems in the treatment of cancer with chemotherapeutic agents and radiation, development of tumor resistance to therapy with time and nonspecific toxicity towards normal host cells.[2,3] Selective destruction of tumor cells without causing damage to normal cells is therefore an important area of research in cancer chemotherapy. Recently, it has been identified that many plants have anti-proliferative effects on cancer cells.[3] While the healing power of plants is not frequently used in the modern world, herbal medicine is the most widely used alternative medicine in America.[4]

Of grave importance in our society is the report that an environmental factor strongly linked with colon cancer is consumption of a high fat, high calorie, low fiber diet[5] thereby indicating the importance of diet in cancer development. Foods that possess anti-cancer activity may have a cancer preventive effect. Many plants are sources of phytochemicals with reported anticancer potential, thereby making them important compounds in cancer biology and research. Among these phytochemicals are phenolic agents (e.g., flavonoids, catechols, phenylpropenoids, quinones, lignans, stilbenes, gallic acid derivatives) which are found in abundance in our daily diet. Epidemiological and animal studies have demonstrated that plant-derived constituents play an important role in the prevention of disease through interference with targets implicated in carcinogenesis and tumor biology.[6]

A number of food components have been identified that inhibit initiation and/or progression of cancer. In the current study, three plant-derived compounds found in normal dietary sources, resveratrol, piperine, and cinnamaldehyde, were tested for anti-cancer activity, specifically for growth effect on proliferation of human colon cancer cells. Recent studies indicate that the polyphenol, resveratrol, found in red wine,[7] red grapes,[7] and peanuts,[8] has anti-carcinogenic effects, such as inhibition of cancer cell growth by an apoptosis mechanism.[9,10,11] It has been shown that resveratrol also has a wide variety of health uses, such as cardiovascular disease prevention as well as anti-bacterial, anti-viral (such as anti-HIV), neuroprotective and anti-inflammatory activities.[7,9]

Piperine is an alkaloid found in black pepper (*Piper nigrum* and *Piper longum*) with a long history of medicinal uses due to anti-pyretic, anti-hypertensive, and anti-inflammatory properties.[12,13,14] Piperine is the compound in black pepper that gives it its pungent smell and taste[12] and is probably best known for its ability to increase absorbency of various drugs in human serum. For example, it dramatically increases absorbency of curcumin (a component of the spice turmeric).[15] Since few studies have been reported assessing direct effects of piperine, the focus of this study was determination of an anti-proliferative effect of piperine on colon cancer cells.

Cinnamon gets its taste from cinnamaldehyde, a naturally occurring product in the bark of cinnamon trees and other species of the genus *Cinnamomum*.[16] Cinnamon has a history of medicinal uses dating back 5,000 years in Ancient China.[16] Recently, cinnamon has been used to treat ulcers and other digestive issues[17] as well as diabetes,[18] and as an anti-microbial agent.[19] A cytotoxic effect has been ascribed to trans-cinnamaldehyde on human cancer cell lines as recently reported.[20]

Concentration and kinetic responses of these compounds (resveratrol, piperine, cinnamaldehyde) were assayed in microwell format for proliferation inhibitory effects on colon cancer as determined by analysis of their effects on DLD-1 cells, a human colorectal adenocarcinoma cell line.[21] The purpose of this study was to determine if resveratrol, piperine or cinnamaldehyde inhibited growth of human colon cancer cells *in vitro* using the DLD-1 cell line.

MATERIALS AND METHODS

Cells and reagents

Colon cancer cells (DLD-1) are a cancer cell line of epithelial origin harvested from a human colorectal adenocarcinoma[21] and were purchased from the American Type Culture Collection (Manassas VA). Cells were cultured (37°C, 5% CO_2) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, glutamine, sodium pyruvate, and HEPES buffer. Dulbecco's modified Eagle's medium and culture supplements were purchased from Hyclone (Logan UT). Resveratrol, piperine and cinnamaldehyde (Sigma-Aldrich, Inc, St. Louis MO) were prepared and stored at -20°C in dimethylsulfoxide (DMSO) at 100 mM stock solutions.

Cell culture

DMEM was removed from cells growing in tissue culture flasks and cells were rinsed with magnesium and calcium free phosphate buffered saline. After the cells reached a confluency of 60%-80%, they were split for subsequent expansion. To do this, cells were treated with trypsin (0.25%), incubated (37°C, 5% CO_2, three to five minutes), treated with serum containing medium to inactivate trypsin, and manually counted using a hemacytometer. Cell numbers were adjusted to 50,000/ml with culture media and cells (100 µL) were added to wells of 96-well microplates.

Cell proliferation assay

The protocol is the primary one-dose anticancer assay described by the National Cancer Institute for *in vitro* anticancer drug discovery screens with modifications included as necessary for the compounds and cell line utilized.[24] Piperine, resveratrol, or cinnamaldehyde were added to appropriate wells for performance of concentration response curves that included 1, 12.5, 25, 50, 100, and 200 µM final concentrations. The test compounds were solubilized in DMSO such that the maximum final concentration of DMSO was 0.2%. All dilutions were made immediately before addition to cells, all treatments were performed in quadruplicate, and each experiment was performed three times. In addition to treatment wells, control wells were assessed which included cell blanks (media only), positive growth controls (untreated cells plus media), and vehicle controls (untreated cells plus media plus DMSO at the highest concentration utilized).

At the designated time points (24 hours, 48 hours, 72 hours), cells were fixed with glutaraldehyde (20 µL of 11% glutaraldehyde, 15 minutes rotation at 300 rpm) that stopped cell multiplication. Cells were rinsed three times with deionized water and left to air dry for 24 hours. Cells were stained using a crystal violet method[22,23] after fixation and air-drying. Briefly, crystal violet (0.1% crystal violet in 100 mM 2-(N-morpholino) ethane sulfonic acid [MES] buffer at pH 6.0) was added to each well and the plate agitated by rotation (300 rpm, 15 minutes). Plates were rinsed thoroughly three times with deionized water and air-dried overnight. When dry, acetic acid (100 µL of 10%) was added to each well and the plates agitated by rotation (300 rpm, 15 minutes) to solubilize the dyed cells. Number of cells present was quantitated using a microplate reader and a 590 nm filter. Results were reported as percent of positive growth compared to control (cells with no additives) as described earlier.[24]

Statistical analysis

Data are reported as the mean ± standard error of the mean. Comparisons of sample means were performed using ANOVA followed by Dunnett multiple comparisons test when indicated. Significance was noted at $p<0.05$ unless otherwise stated.

RESULTS

Cinnamaldehyde has shown promise in earlier studies for inhibition of proliferation effects of cancer cells.[25] Cinnamaldehyde exerted a statistically significant ($p<0.01$) anti-proliferation effect at 24 hours with 200 µM, at 48 hours with 100 µM and 200 µM, and at 72 hours with 200 µM concentrations (Figure 1). These results indicate that the highest concentrations tested elicited anti-proliferative effects.

Piperine has previously been shown to be effective against sarcoma[26] and melanoma[27] cancer cells *in vivo*. Piperine had an anti-proliferation effect at 24 hours, however, only the 100 µM concentration reached statistical significance ($p<0.05$) (Figure 2). At the 48 and 72 hour time points, 100 and 200 µM piperine exerted a statistically significant ($p<0.01$) anti-proliferation effect (Figure 2). The results suggest that 100 and 200 µM concentrations administered for at least two days elicit significant anti-proliferation effects.

The current literature has many reports on resveratrol effects. There have been indications that resveratrol increases cardiovascular health, increases longevity, and inhibits cancer cell proliferation.[7] Resveratrol inhibited cancer cell proliferation significantly ($p<0.01$) at 24 hours with 50, 100 and 200 µM concentrations (Figure 3A). At 48 and 72 hours, 12.5-200 µM concentrations inhibited proliferation significantly ($p<0.01$). Visibly decreased numbers of cells are evident at the 72 hour time point and an absence of viable cells is noted at 100 and 200 µM concentrations (Figure 3B). These results show that at higher concentrations and longer administration periods, resveratrol significantly inhibits DLD-1 cancer cell proliferation indicating that longer administration allows for the use of lower concentrations of resveratrol. Displayed in Table 1 are amounts of resveratrol present in food sources with high resveratrol content. It is apparent that levels utilized in this study are readily available in food sources.

DISCUSSION

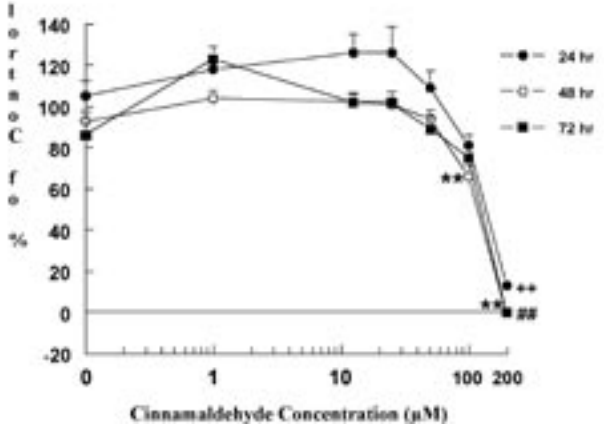
Current treatments for colon cancer are only effective in 50% of the cases.[28] In addition, these treatments (chemotherapy, surgery, and radiation) are frequently toxic to normal host cells.[2] Many plant-derived phytochemicals have been shown

to be beneficial to human health. In the present study, we sought to determine the effect of phytochemicals (common dietary agents) on cancer cell growth. Anti-proliferative effect was analyzed using a colon cancer cell line, DLD-1. The rationale of the study was that if phytochemicals exert inhibitory effects on cancer cell proliferation, then foods rich in these agents will help prevent cancer. In the current study, anti-proliferative effects on human colon cancer cells were identified for resveratrol, piperine, and cinnamaldehyde.

Amounts of resveratrol, piperine and cinnamaldehyde normally present in various foods have been reported. Detectable amounts of cinnamaldehyde in various food items have been reported from trace amounts to 31 mg/100 g body weight.[29,30] One report noted cinnamaldehyde presence in trace amounts in apple and orange juices, at 12.3 mg/100 g in an apple cinnamon cereal, and at the amount of 31.1 mg/100 g in cinnamon swirl bread.[29] Sampling of five different cereals indicated a cinnamaldehyde content of 1.8-21.9 mg/g of cereal.[29] The presence of high amounts of cinnamaldehyde in an abundance of food sources indicates that inhibition of colon cancer cell proliferation by cinnamaldehyde (Figure 1) has potential as a source of cancer prevention for human populations.

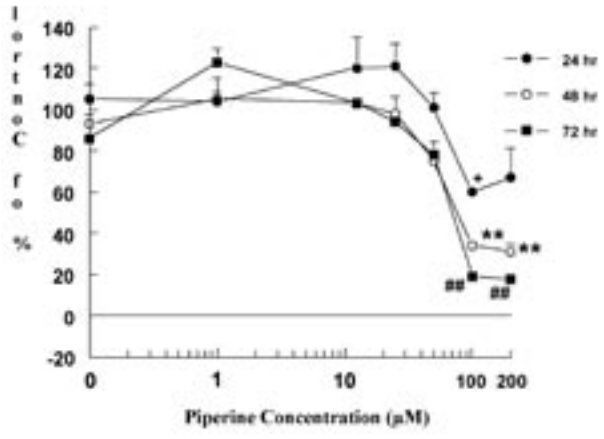
As stated earlier, major dietary sources of the polyphenol resveratrol are red wine,[7] red grapes,[7] and peanuts.[8] Amounts of resveratrol in different food items are displayed in Table 1. Examples of resveratrol content follow: in red wines, 1.92-12.9 mg/l; in red grape juice, 1.14-8.69 mg/l; in red grapes, 0.24-1.25 mg/8 oz; and in peanuts, 0.32-1.28 mg/8 oz.[31,32,33,8] As is readily apparent, variations in resveratrol concentrations are present in the literature, probably due to the types of food containing resveratrol that were assessed. The cancer preventive properties of resveratrol have been shown to be mediated by down-regulation of the cyclin D1/Cdk4 complex in the colon cancer cell line, Caco-2.[34] It has been suggested that the anti-proliferative effect of resveratrol is through an apoptosis-dependent mechanism such as has been observed in a promyelocytic leukemia cell line (HL-60)[35] and in Caco-2 cells.[34] Since Caco-2 and DLD-1 cells are both of human intestinal origin, it is interesting to speculate that the mechanism by which resveratrol inhibits DLD-1 cancer cell proliferation (Figure 3) is through a similar apoptotic mechanism as for Caco-2 cells.

Figure 1. Cinnamaldehyde elicits an anti-proliferative effect on colon cancer cells (DLD-1)



Effect of different concentrations (1, 12.5, 25, 50, 100, 200 μM) of cinnamaldehyde on the growth of DLD-1 cells at different time points (24, 48, 72 hours). All treatments were performed in quadruplicate and each experiment was performed three times (n = 3). All data were compared to the 0 μM cinnamaldehyde control and ++ = $p < 0.01$ at the 24 hour time point; ** = $p < 0.01$ at the 48 hour time point; and ## = $p < 0.01$ at the 72 hour time point.

Figure 2. Piperine elicits an anti-proliferative effect on colon cancer cells (DLD-1)

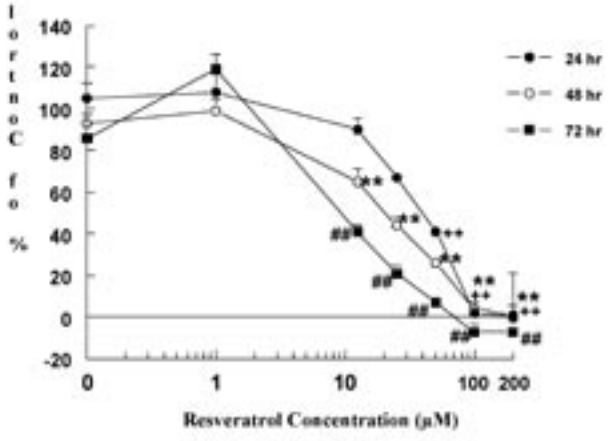


Effect of different concentrations (1, 12.5, 25, 50, 100, 200 μM) of piperine on the growth of DLD-1 cells at different time points (24, 48, 72 hours). All treatments were performed in quadruplicate and each experiment was performed three times (n = 3). All data were compared to the 0 μM piperine control and + = $p < 0.05$ at the 24 hour time point; ** = $p < 0.01$ at the 48 hour time point; and ## = $p < 0.01$ at the 72 hour time point.

Piperine has a long history of medicinal uses and has been valued as an anti-pyretic, anti-hypertensive, and anti-inflammatory agent.[12] Piperine is probably best known for its ability to increase absorbency of various drugs in human serum (e.g., increased absorbency of curcumin from the spice tumeric).[15] Piperine has been shown to be effective against sarcoma[26] and melanoma[27] *in vivo*. The *in vivo* tumor activity of piperine has been shown in mice transplanted with Sarcoma 180 tumor cells where intraperitoneal administration of piperine (50-100 mg/kg/day) inhibited solid tumor development in tumor cell-injected mice.[26] In the current study, piperine exhibited anti-proliferative activity in the DLD-1 colon cancer cell line (Figure 2).

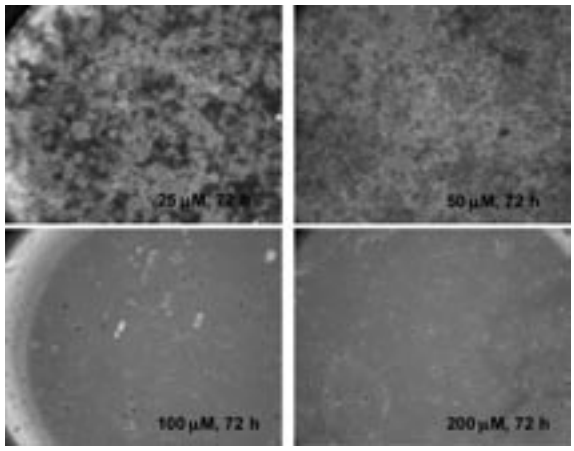
To address specificity of anti-proliferative effect of these phytoagents, more research is needed to definitively answer this question that speaks to the heart of current cancer treatments and renders effects on normal as well as cancer cells. The current paradigm for resveratrol is that the specificity of anti-proliferative effects arises from the increased susceptibility of cycling cells to its effects.[36] To directly address effects of resveratrol on non-cancer cells, review of the literature reveals that resveratrol imparts protective effects on some normal cells. For example, resveratrol protects normal cells and enhances radiation therapy effects in cancerous cells.[37] *In vivo* effects in a rat chronic colitis model indicate that resveratrol specifically attenuates colonic damage, corrects disturbances associated with colonic injury, and returns colon cells to normal (as assessed by histological and functional measures).[38] Additionally, in a primary cell culture model of Alzheimer's disease, resveratrol exhibits neuroprotective effects against amyloid-induced toxicity of hippocampal cells in a protein kinase C-dependent manner,[39] indicating that not only does resveratrol have anti-cancer effects but also cellular protective effects. Resveratrol suppresses prostate cancer progression in mice in a manner that elicits no side effects[40] which strongly suggests a specific cytotoxic action of resveratrol on cancer cells. Results of a newly published study indicate that resveratrol causes apoptosis and mitochondrial dysfunction by membrane depolarization in malignant human pancreatic cancer cells,[41] again a result that is specific. Resveratrol has protective effects against lung cancer[42] and many more anti-cancer reports are present in the literature that strongly suggest a specific cytotoxic effect on cancerous rather than non-cancerous cells.

Figure 3A. Resveratrol elicits an anti-proliferative effect on colon cancer cells (DLD-1)



Effect of different concentrations (1, 12.5, 25, 50, 100, 200 μM) of resveratrol on the growth of DLD-1 cells at different time points (24, 48, 72 hours). All treatments were performed in quadruplicate and each experiment was performed three times (n = 3). All data were compared to the 0 μM resveratrol control and ++ = $p < 0.01$ at the 24 hour time point; ** = $p < 0.01$ at the 48 hour time point; and ## = $p < 0.01$ at the 72 hour time point.

Figure 3B. Resveratrol elicits an anti-proliferative effect on colon cancer cells (DLD-1)



Crystal violet stain of live DLD-1 cells after 72 hours treatment with resveratrol (25, 50, 100, 200 μM). Lower concentrations (25 and 50 μM) of resveratrol allow for growth of some cells whereas higher concentrations (100 and 200 μM) do not. Representative wells from the 96-well microplate format were photographed.

As reviewed by Saiko and others,[36] several *in vivo* studies in animals and humans demonstrate a very low intestinal uptake of resveratrol leading to trace amounts in the bloodstream based on extensive metabolism in the gut and liver. The bulk of an intravenous dose of resveratrol is converted to sulfate conjugates within 30 minutes in humans. The undeniable *in vivo* efficacy of resveratrol, despite its low bioavailability, has led to the suggestion that its metabolites are the active agents. Oral administration of resveratrol to healthy human subjects in white wine, grape juice, and vegetable juice was the focus of a recent study.[43] Resveratrol was present in serum and urine predominantly as glucuronide and sulfate conjugates that reached peak concentrations 30 minutes after consumption. The absorption of these compounds was equivalent in aqueous and alcoholic solutions and peak plasma concentrations of individual conjugates reached 10nM-40nM. Realizing that biologic activities have been studied in much higher amounts (in the μM range), it is important to note that resveratrol binds to human serum proteins such as albumin and hemoglobin.[44] Therefore, the presence of serum in *in vivo* and cell culture studies may explain the observation that resveratrol absorption is in the nM range whereas functional effects have been reported in the μM range. Additionally, in cell culture experiments such as reported here, resveratrol effect is assessed on a single isolated cell type. It is realized that *in vivo*, a multiplicity of cells and cell types are present with resveratrol potentially binding many of the different cell types. Indeed, the complexities of resveratrol action *in vivo* remain to be elucidated.

Table 1. Resveratrol content of selected foods

Food	Serving	Resveratrol (mg)	Resveratrol (μM)
Red grapes	8 oz	0.24-1.25	4-22
Red grape juice	5 oz	0.17-1.30	5-39
Red wine	5 oz	0.03-1.07	0.9-32
White wine	5 oz	0.01-0.27	0.3-8
Peanut butter	8 oz	0.04-0.13	0.7-2.3
Peanuts (raw)	8 oz	0.01-0.26	0.2-5
Peanuts (boiled)	8 oz	0.32-1.28	6-22

The abundance of dietary compounds such as piperine, cinnamaldehyde, and resveratrol and their anti-proliferative activities specifically against cancer cells (such as DLD-1) suggest that the intake of foods with high levels of these phytochemicals may be a preventive approach to cancer treatment. Future experiments are needed that focus on mechanistic aspects of these phytochemicals and their anti-proliferative activities as well as specificity of anti-proliferative action.

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