Phytochemical Attributes Contribute to the Health-Promoting Benefits of Mangos

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Mass Spectroscopic and HPLC Characterization of Mango (*Mangifera Indica L.*) Following Enzymatic Hydrolysis

Project Summary: Mangos contain numerous compounds that have been shown to exhibit antioxidant properties. These compounds, most of which are polyphenolics, are linked to anti-cancer and anti-inflammatory activities in the body. Mangos more specifically boast a large number of high molecular weight compounds called gallotannins, composed of gallic acid units attached to glucose via a glycosidic linkage. It is unknown if these compounds are broken down into smaller molecules through the normal course of human digestion, or if food processing operations such as the addition of a gallotannin-active hydrolyases could be more effective in lowering the size of these molecules to increase the absorption and potential bioactivity. These studies will begin the process of understanding the chemical changes that occur to gallotannins derived from mangos following enzymatic hydrolysis and attempt to draw inferences relating to overall human health. Polyphenolics in mangos, cv. Ataulfo were extracted using a 1:1:1 acetone:ethanol:methanol mixture and further concentrated and clarified using a reversed phase C18 Sep-Pak cartridge. Mango extracts were treated with 20,000 U/ml and 13,000U/ml ß-glucosidase with a time course of 2, 4, 6, and 8 hours in an optimal pH 5.0 citric acid buffer, and at a constant temperature of 35°C. Changes in mango polyphenolics following enzyme hydrolysis were monitored using a Thermo Finnigan LCQ Deca XP Max MSn ion trap mass spectrometer equipped with an ESI ion source. ß-glucosidase proved to be effective in the hydrolysis of some gallotannins, but was incapable of hydrolyzing all gallotannins into free gallic acid. This was illustrated by the observance of an increase in penta, hexa, hepta-*O-* and a subsequent decrease in higher molecular weight compounds. The limitations for complete hydrolysis could be explained by the inability of ß-glucosidase to cleave the glycosidic linkage due to steric hindrance created from

having up to five gallic acid moieties attached to glucose or from the inability of the enzyme to break *m*-dipside linkages between two or more galloyl groups. Incubating mango extract with both 20,000 U/mL and 13,000 U/mL resulted in equivalent eight-fold increase in free gallic acid. Enzyme concentration was not the limiting factor in the hydrolytic reaction. Additionally, reaction time did not have a significant role in the hydrolytic rate, as the amount of free gallic remained relatively constant from 2 to 8 hours. These findings indicated that it was possible to increase low molecular weight gallotannin species following enzyme hydrolysis and will aid in future studies to understand the digestion and bioavailability mango phenolics.

Introduction

Mango (*Mangifera indica* L.) is a popular tropical fruit because of its unique taste, affordability and nutritional qualities. Mangoes are members of the family Anacardiaceae and are predominately grown in tropical and warm sub-tropical climates in places such as Asia, Africa, and the Americas. There are over one thousand different cultivars of mangos growing world-wide and mangos are considered to be one of the most widely eaten fruits. The mango is known to be an excellent source of many vitamins such as ascorbic acid, thiamine, riboflavin, and niacin, and ß-carotene. Not only are mangos rich in these nutrients, mangos are also high in non-nutrient phytochemical compounds. Recently much attention has been given to phytochemicals and the distinctive roles they play in anti-inflammatory and anti-cancer properties related to the consumption of fruits and vegetables. A need exists to chemically identify hydrolytic byproducts from mango polyphenolics in an effort to eventually relate these compounds to the absorption of mango polyphenolics following human consumption.

Mango Phytochemicals

Mangos boast a large number of high molecular weight gallotannins that range in size from 332 amu (mono-*O*-galloglucose) to over 1,852 amu (undeca-*O*-galloglucose) and possibly even larger (Figure 1). Gallotannins are composed of gallic acid units attached to glucose via a glycosidic linkage or via a *m*-dipside linkage between galloyl groups. A review of the literature shows that a compound from pomegranate (punicalagin), with a molecular weight of 1,084, was the largest biological molecule ever found to be absorbed intact using a rat model for absorption. Small intestine epithelial cells contain the enzyme β-glucosidase, with the capability of hydrolyzing gallotannins in vivo. It is unknown if gallotannins from mangos are hydrolyzed into smaller molecules through the normal course of human digestion, or if food processing unit operations such as the addition of a tannins-active hydrolyases could be more effective in lowering the size of these molecules to increase their absorption potential.

This initial study will begin the process of understanding the chemical changes that occur to gallotannins from mangos by addressing the need to characterize the end products of hydrolysis and attempt to draw inferences relating to overall human health.

Figure 1. Typical gallotannin and a hydrolytic product from tannin-active enzymes.
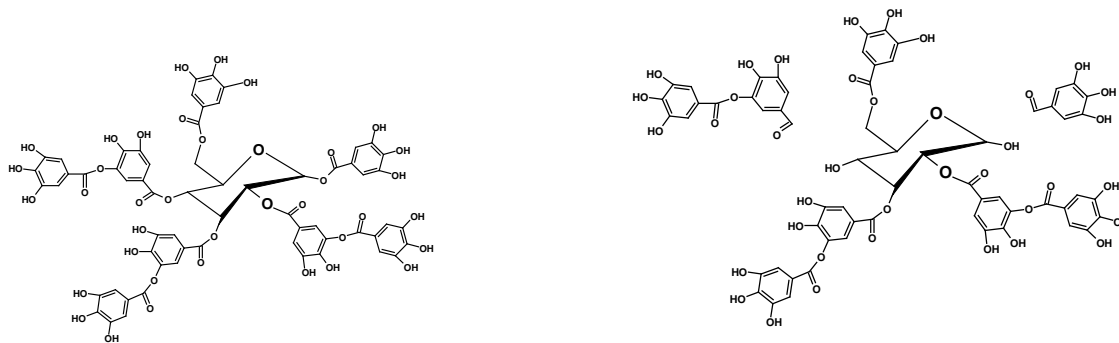
Research has been conducted to quantify and characterize compounds from kernels, peels, and leaves of the mango (Barreto and others 2008; Berardini and others 2004). Mango pulp has proven to be more difficult however, and studies have been ongoing to identify unambiguously compounds located in the pulp. Reports suggest that the phytochemical content of mango pulp consists of gallic acid, mangiferin, quercetin glycosides and many identified and uncharacterized hydrolyzable tannins (Schieber and others 2000). Other compounds in smaller concentrations include *p*-OH-benzoic acid, *m*-coumaric acid, *p*-coumaric acid, and ferulic acid (Kim and others 2007). In most mango varieties, free gallic acid, 3,4,5-trihydroxybenzoic acid, is the predominant compound present and has been shown to possess a high antioxidant capacity with numerous implications to overall human health (Shahrzad and Bitsch 1998). Gallic acid units possess three hydroxyl groups and an acid group which allow the compound to link with another gallic acid to form an ester, digallic acid (Masibo and He 2008). Gallic acid is an essential component to a group of compounds present in mango pulp called gallotannins.

Tannins

Tannins, in general, are water soluble phenolic secondary metabolites that range in size from 300 to 3000 amu (atomic mass units) and are classified into four different groups based on similar structural characteristics. Such groups include gallotannins, ellagitannins, complex tannins and condensed tannins (Mingshu and others 2006). Tannins are known for their ability to bind proteins to form either soluble or insoluble complexes (Hangerman and others 1989). Gallotannins are the predominant class of tannins identified from mango pulp and range in size from 787 – 1243 amu (Berardini and others

2004). Gallotannins consist of a sugar, primarily glucose, surrounded by several gallic acid units which can further be attached to other gallic acid units via *m*-depside bonds (Mueller-Harvey 2001). Research has suggested that the larger a polyphenolic compound is in size, the less the likelihood for intestinal absorption and subsequent bioavailability. Therefore, an important area of research is to understand the hydrolytic products of gallotannins as a means of understanding their absorption and bioactivity of the mango as a whole.

Tannin-Active Enzymes

Only by first evaluating enzyme hydrolysis techniques and understanding how these processes relate to the size and chemical composition of the resultant molecule can a later assessment of reduced molecular weight be made on potential health benefits of mangos. It is known that certain bacteria, yeasts, and molds produce enzymes that are capable of breaking down these gallotannins (Mingshu and others 2006). ß-glucosidases can also be found in the epithelial cells of the small intestine where it is thought that sugar-linked molecules are broken into smaller units that are subsequently absorbed by these cells (Nemeth and others 2003). Both ß-glucosidase and tannase have been used for hydrolysis of tannins, but further studies need to be completed in this area to determine the most effective enzyme to use, or if a combination of the two enzymes could be more effective (Kikuzaki and others 2000). It is therefore hypothesized that hydrolysis of gallotannins via the addition of ß-glucosidase will increase the concentration of free gallic acid, as well as lower molecular weight polyphenolics, and will potentially enhance the bioavailability of mango polyphenolics. Quantifying and characterizing the gallotannins and their hydrolytic by-products will aid in future research with a goal of understanding industrial food processing techniques that will aid in better or more efficient uses of nutritionally dense foods such as mango.

Materials and Procedures

Polyphenolic extraction

To extract polyphenolic compounds from mango pulp, 200 g of mango pulp was placed in a beaker with 600 mL of 1:1:1 acetone, methanol, and ethanol (v/v/v). The extract was initially filtered through cheesecloth to remove large particles and subsequently filtered through diatomaceous earth. This process was repeated twice, using the pulp remaining on the cheesecloth and extractions repeated with 400 and 200 mL, consecutively. The extraction solvents were pooled and evaporated under reduced pressure at $< 45°C$ using a rotary evaporator. Methanol was added to the extract and the extract was

centrifuged to precipitate pectin. The supernant was collected, precipitate re-extracted, and methanol was again evaporated under reduced pressure at <45°C. The mango extract was washed through a Waters Sep-Pak® Vac 35cc 10g C18 cartridge to remove sugars, residual soluble pectin, and to concentrate phenolic compounds. Bound phenolic compounds were fractioned using first 25% methanol and then 100% methanol to separate low molecular weight compounds from higher molecular weight compounds based on their affinity to C18. Each extract was brought to a total volume of 100 mL in a pH 5.0 buffer solution.

Enzyme Hydrolysis

The enzyme ß-glucosidase was purchased from MP Biomedicals and stored at -20°C. An enzyme solution was prepared immediately prior to incubation in a pH 5.0 buffer with a concentration of 13,000 U/mL and 20,000 U/ml. One unit (U) of ß-glucosidase will liberate 1.0µmol of glucose per minute at 35°C. Ataulfo mango fractions (stock, L.M.W., H.M.W) at a volume of 2.0 mL were treated with 200 µL of the prepared ß-glucosidase enzyme solution. The samples were incubated following a time course at 35°C, with samples pulled at 30 minutes, 1, 2, and 4 hrs. Following incubation, each sample was acidified to pH 3.0 to inactivate ß-glucosidase.

Chemical Analysis

Changes in mango polyphenolics following enzyme hydrolysis were monitored using a Thermo Finnigan LCQ Deca XP Max MSn ion trap mass spectrometer equipped with an ESI ion source (ThermoFisher, San Jose, CA). Separation of compounds was completed with a Dionex (Sunnydale, CA) Acclaim® 120 A, (4.6 X 250 mm; 5µM). Mobile phases used included 0.5% formic acid in water (solvent A), and 0.5% formic acid in acetonitrile (solvent B) at 0.4 mL/min. The mobile phases were run in a gradient program and began with 100% A for 5 minutes. Solvent B was changed from 0-30% in 25 minutes, to 50% in 15 minutes, to 100% in 25 minutes, back to 0% in 2 minutes. The gradient run was finished with 100% A for 8 minutes.

Results and Discussion

Several compounds were specifically identified and characterized from mango extracts by ESI (electrospray ionization) mass spectroscopy in negative ion mode. For example, free gallic acid was identified based on its molecular weight, fragmentation pattern, and spectral patterns, and comparison to an authentic standard. An ion of m/z 169 was most abundant and MS^2 showed a fragmentation pattern with a predominant ion at 125 m/z. This pattern is typical of benzoic acid derivatives (Barreto

and others 2008). Mono-galloyl glucose showed a predominate ion with m/z 331 and a loss of 162 amu in the MS^2 scan event. The loss of 162 corresponded to the loss of a glucose molecule minus a water molecule. The predominate ion in MS^2 was 169, the molecular ion for free gallic acid. Mangiferin at m/z 421 was confirmed by its MS^2 pattern at m/z 301, 331, and 403 as supported by Shieber and others (2003). For higher molecular weight compounds such as pentagalloyl glucose, a parent ion of m/z 938 was further supported by additional fragmentation to produce ions at m/z 787 that corresponded to tetragalloyl glucose. At m/z of 1242, heptagalloyl glucose created fragments at m/z 1089, 939, and 787 corresponding to fragments of hexagalloyl glucose, pentagalloyl glucose, and tetragalloyl glucose. These fragments created by inducing collision energy to the parent compound were somewhat analogous to the effects of hydrolytic enzymes (ie. ß-glucosidase), giving an indication of the strength/weakness of the chemical bonds present in gallotannins.

The enzyme ß-glucosidase proved to be effective in the hydrolysis of select gallotannins, but was not effective in complete hydrolysis of all mango gallotannins (Figure 2; Table 1). This was illustrated by the observance of an increase in moderately high molecular weight gallotannins (ie. penta, hexa, hepta-*O*-galloglucose) and a decrease in higher molecular weight compounds. This could be explained by the inability of β-glucosidase to cleave β-linkage due to steric hinderence created from having five gallic acid moieties attached to glucose. Since gallic acid can form repeating units with itself via *m*-dipside bonds (ie. digallic acid), longer chains of gallic acid may be found and resist hydrolysis with β-glucosidase. Cleavage of these longer chains from the base glucose molecule is possible, but not likely for the *m*-dipside bonds. It was hypothesized that ß-glucosidase would be effective in hydrolyzing gallic acid units attached to glucose via a glycosidic linkage, and thus an increase in gallic acid would be observed. Incubation of the polyphenolics in Auaulfo extract with 13,000 U/mL ß-glucosidase resulted in a decrease in the amount of monogalloyl glucose present in the extract by 90%, and an increase in compound gallic acid, mangiferin, and penta/heptagalloyl glucose by 88%, 40%, 50%, and 42%, respectively. The same extract was treated with 20,000 U/mL ß-glucosidase presented similar results. This study was critical in understanding the hydrolytic products from mango gallotannins in effort to product likely absorption targets in an in vivo situation.

Figure 2. Typical HPLC chromatogram of mango polyphenolics (cv. Ataulfo), <u>prior</u> to incubation with β-glucosidase.
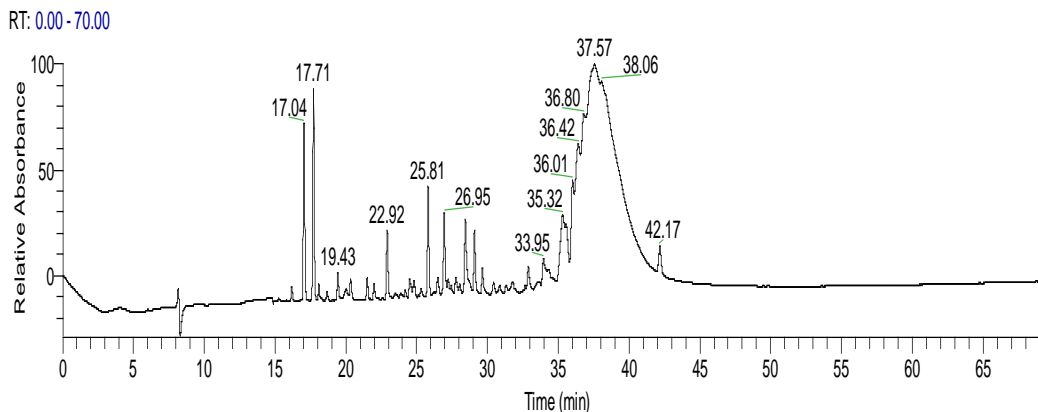

Table 1. Percent change in identified mango polyphenolic compounds after enzyme hydrolysis at two enzyme concentrations. Peaks correspond with the chromatogram in Figure 2 and 3.

Apex Time	Compound ID	% Change 13,000 U/mL	% Change 20,000 U/mL
17.71	mono-gallyol glucoside	-90	-94
19.43	gallic acid	+88	+88
28.44	mangiferin	+40	+6
32.88	penta-galloyl-glucoside	+50	+35
35.31	hepta-galloyl glucoside	+42	+32

Among the polyphenolics extracted from mango pulp, free gallic acid was present in relatively low concentrations compared to the amount of gallotannins. Mono-galloyl glucoside was considerably more abundant than free gallic acid (6-fold) and represents the lowest molecular weight compounds present in mangos. Before enzyme hydrolysis the large, resolved gallotannin "hump" was dominant and represented several gallotannins ranging from 939 to 1,852 amu. The size of these compounds is exceedingly large for intestinal absorption, and would predictably not be absorbed in their current state without significant hydrolysis during digestion. However, after hydrolysis with β-glucosidase, these compounds were significantly altered and their average size reduced to a point where many could be considered candidates for potential absorption (Figure 3; Table 2). Following the enzyme hydrolysis, mango polyphenolics were significantly altered with the higher mass compounds in the "hump" decreased to create a higher concentration of lower molecular weight polyphenolics. With the resultant

increase in both gallic acid and mono-galloyl glucose, these compounds were thought to be the primary end-products of the hydrolysis and would be presumed to be in a form that is considerably more prone to intestinal absorption. Increases were also observed for both penta-galloyl and hepta-galloyl glucosides, but points to an incomplete hydrolysis of gallotannins by the enzyme under the conditions of our evaluation. We also observed that the higher molecular weight gallotannins were able to bind to β-glucosidase and essentially reduce or eliminate its activity. Therefore, care was taken to use single strength or more dilute solutions for the enzyme incubation trials.

Figure 3. Typical HPLC chromatogram of mango pulp polyphenolics (cv. Ataulfo), <u>after</u> incubation with 13,000 U/ml β-glucosidase.
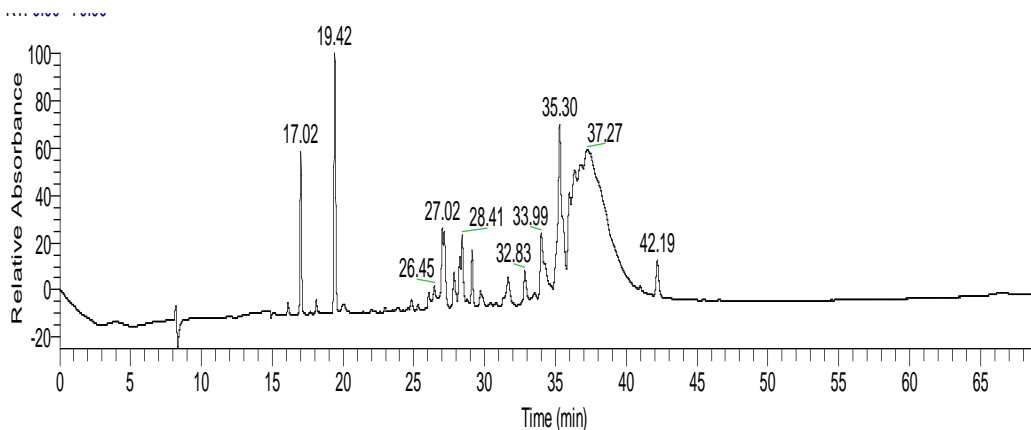

Table 2. Peaks correspond with the chromatogram in Figure 3.

Apex RT	Compound ID
17.02	mono-gallyol glucoside
19.42	gallic acid
28.41	mangiferin
32.82	penta-galloyl-glucoside
35.30	hepta-galloyl glucoside

Conclusions

The hydrolyase enzyme ß-glucosidase proved to be effective in the hydrolysis of some gallotannins, but the hydrolytic reaction was never successfully run to completion. This was illustrated by the observance of an increase in middle-weight gallotannins, (penta, hexa, hepta-gallotannins) and a decrease in high molecular weight compounds. The limitations for complete hydrolysis could be explained by the inability of ß-glucosidase to cleave the beta-linkage due to steric hindrance created

from having five gallic acid moieties attached to glucose. Incubating mango extract with both 20,000 U/mL and 13,000 U/mL resulted in an eight-fold increase in free gallic acid. Therefore, enzyme concentration did not seem to affect gallotannin hydrolysis when the difference was not greater than around 10,000 U/mL. Time did not play a significant role in the hydrolysis of gallotannins, as the amount of gallic cleaved remained relatively stable from 2 to 8 hours. These findings showed an increase in lower molecular weight species following enzyme hydrolysis and will aid in future studies to understand the digestion and bioavailability mango phenolics.

Mango Polyphenols Inhibit the Growth of Human Cancer Cells In Vitro

<u>**Anti-carcinogenic Properties of Mangos**</u>

Project Summary: Polyphenolics found in five different cultivars of mango pulp includes a wide range of galloyl glycosides of varying molecular weights, flavonol glycosides, free gallic acid, and mangiferin. Most of these compounds have shown potential to exhibit anticancer activity. The objective of these studies was to evaluate the anti-prolioferative activity of mango phenolics in several cancer cell lines. The cultivars Haden and Ataulfo were selected for additional trials based on their phenolic content and antioxidant activity. The anti-proliferative and pro-apoptotic activities of polyphenolics (mg gallic acid equivalent (GAE)/L) were tested in human Molt-4 leukemia, A-549 lung cancer, MDA-MB-231 breast cancer, LnCap prostate cancer, and SW-480 colon cancer cells. Additionally, gene expression was investigated by real-time PCR and cell cycle kinetics by flow cytometry.

Ataulfo and Haden polyphenolics inhibited the growth of human cancer cells with order of potency SW-480 = Molt-4 ≥ MDA-MB-231 > A-549 ≥ LnCap as determined by cell counting at 72 hrs. At the highest doses (5 mg GAE/L), Ataulfo inhibited the growth of colon SW-480 cancer cells by ~72% with no inhibition on the growth of non-cancerous colonic myofibroblasts CCD-18Co cells (IC_{50} was ~8 fold) as a control. Carotenoids extracted from Ataulfo also inhibited the growth of SW-480, but at relatively high concentrations. The growth inhibition exerted by Ataulfo and Haden polyphenolics in SW-480 was associated to increased gene expression of pro-apoptotic caspase 8, Bax and Bim by ~ 2, 1.3 and 1.7-fold respectively and cell cycle regulators p21 and PKMYT1 by ~ 1.3 and 1.4-fold respectively compared to the untreated cells. Furthermore, Ataulfo polyphenolics (at up to 10 mg GAE/L) induced cell cycle arrest in G2/M phase within 24 hrs and was associated with the increased PKMYT1 expression, a gene shown to negatively regulate cell cycle G2/M transition. The H_2O_2

induced production of reactive oxygen species (ROS), was also inhibited by Ataulfo and Haden polyphenolics in colon SW-480 cancer and CCD-18Co non-cancer cells; and the threshold for induction of oxidative damage was lower in SW-480 demonstrating a selective protection on CCD-18Co. Overall, mango polyphenolics may possess chemopreventive activities with great potential in treating colon cancer, since they are able to readily reach the colon and be available to the target cells.

Background and Introduction

Cancer is a leading cause of death worldwide and accounted for ~ 13% of all deaths worldwide in 2004 (*1*). Colorectal cancer is the third type of cancer leading to overall cancer mortality around the world (*2*). Even though mortality rates from colorectal cancer have declined in USA over the past two decades, is still the third most common cancer in both men and women, with an estimated 108,070 cases of colon and 40,740 cases of rectal cancer diagnosed in 2008 (*3*). Overall mortality statistics could keep decreasing by controlling most of the risk factors associated to colon cancer. Among these risk factors, diet is one of the modifiable factors that may increase the risk of colon cancer due to high consumption of red or processed meat and inadequate intake of fruits and vegetables (*3*). A diet rich in fruit and vegetables has been associated with a lower risk of cancer development (*4*) (*5*). This is supported by epidemiological studies that associate the protective effect of fruit and vegetable consumption with reduced incidence of cancer (*6*). Specifically, a protective effect of greater raw vegetable consumption has been found to be consistent for colon cancer (*7, 8*). This fact is being considered as an supplemental approach for the cancer prevention (*9*), and theraphy, enhancing the effectiveness of conventional therapies and decreasing their serious side effects (*9*). Plant polyphenolics, because of their safety and the fact that they are not perceived as "medicine," are increasingly being considered as sources of natural colon cancer chemopreventive compounds (*10*). The edible part of mango contains a mixture of phytochemicals including polyphenolics, ascorbic acid, and carotenoids that may help to protect against cancer. Polyphenolics identified in the edible part of mango have been previously characterized and include flavonoids such as quercetin and kaempferol glycosides, phenolics acids, predominately gallic acid, galloyl glycosides of varying degrees of polymerization, and mangiferin (*11, 12*) which is more closely associated to the mango peel. Low molecular weight gallotannins, hydrolytic products of gallotannins, and other polyphenolics are likely colon-available (*13-16*) and have shown to protect against the multiple stages of colon carcinogenesis in vitro (*13*). Mango could also be considered a good source of total carotenoids, mainly violaxanthin and β-carotene (*17-19*), though contents may vary with cultivar and country of origin (*20*). . However,

the colon cancer chemopreventive protection exerted by carotenoids is less relevant than that of polyphenolics, and even controversial (*21-24*).

The rational for potential health benefits of mango polyphenolics is based on a dietary increase of antioxidants that may enhance the cell defense capacity and modulate molecular pathways in target cells. As antioxidants, they protect normal cells against reactive oxygen species (ROS) and daily carcinogenic insults deriving from lifestyle (*25*). Once pre-neoplastic cells are formed, these compounds may inhibit promotion and progression stages of cancer by interfering with cell cycle regulation, regulation of signal transduction pathways,(*26*) transcription, and apoptosis (activation of pro-apoptotic genes and pro-apoptotic proteins) (*27*) (*26*). This is considered a reasonable way to prevent cancer with prolonged life expectancy , rather than treatment at the end-stage (*28*). In this study we investigated the cancer chemopreventive potential of polyphenolics extracted from different mango cultivars. Our aim was to identify the cultivar with the highest tumor growth-suppression activity and target human cancer cell lines. Some of the molecular mechanisms behind the anticarcinogenic activity are also assessed. Continued translational research will be relevant to human health with emphasis on colon cancer.

Materials and Methods

1. Plant material

Commercial varieties of mango (*Mangifera indica* L) were kindly donated by the National Mango Board, USA. The varieties Francis, Kent, Ataulfo, Tommy Atkins and Haden, were collected at a mature, firm stage. Upon arrival at Texas A&M University, fruit boxes were opened to the atmosphere and allowed to complete ripening to a light yellow and medium-soft texture by subjective evaluation whereby peels and seeds were manually removed, the pulp homogenized, and stored frozen at -20ºC until needed.

2. Extraction of polyphenolics

A representative 500 g of frozen pulp was thawed and mixed with 1.5 L of a 1:1:1 (v/v/v) ethanol/ methanol/ acetone solution and filtered with cheesecloth followed by filtration using a Whatman #1 filter paper . The solvents were completely evaporated at 40°C using a rotary evaporator (Büchi, Switzerland). The aqueous extract was mixed with methanol (1:1) and pectin was removed by centrifugation at 2000 x *g* for 10 min at 7°C. The methanol was evaporated at 40 ºC and polyphenolics concentrated on a reversed phase C18 cartridge (Waters Corporation, Milford, MA) previously conditioned with 50 mL of 100% methanol and 50 mL of nanopure water. The aqueous extract was

loaded on the C18 cartridge and washed with 50 mL of water. Polyphenolics bound to the matrix were eluted with 50 mL 100% methanol. The water from the wash was also partitioned into ethyl acetate (EtAc) in order to recover any unbound polyphenolics. The ethyl acetate phase was combined with the phenolics eluted with 100% methanol, evaporated, and re-dissolved in nanopure water to a known volume (Figure 4). The total phenolic aqueous solution was frozen at -80ºC and freeze-dried (Labconco Corporation, Kansas City, MO) at -50 ˚C and at 0.01 mBar of pressure. These extracts were then used for cell culture assays by applying different doses based on total phenolic content measured spectrophotometrically by the Folin-Ciocalteu assay (*29*) against an external standard of gallic acid (mg/L). Absorbance values were recorded on a FLUOstar Omega plate reader (BMG Labtech Inc, Durhan, NC) at 726 nm. All polyphenolic extracts were re-dissolved in culture medium and sterile filtered prior to use in cell culture experiments. Polyphenolics were also analyzed by HPLC, comparing the profile of phenolics of the freeze dried extracts and the re-dissolved in the culture medium and sterile filtered.

Figure 4: Extraction of polyphenolics
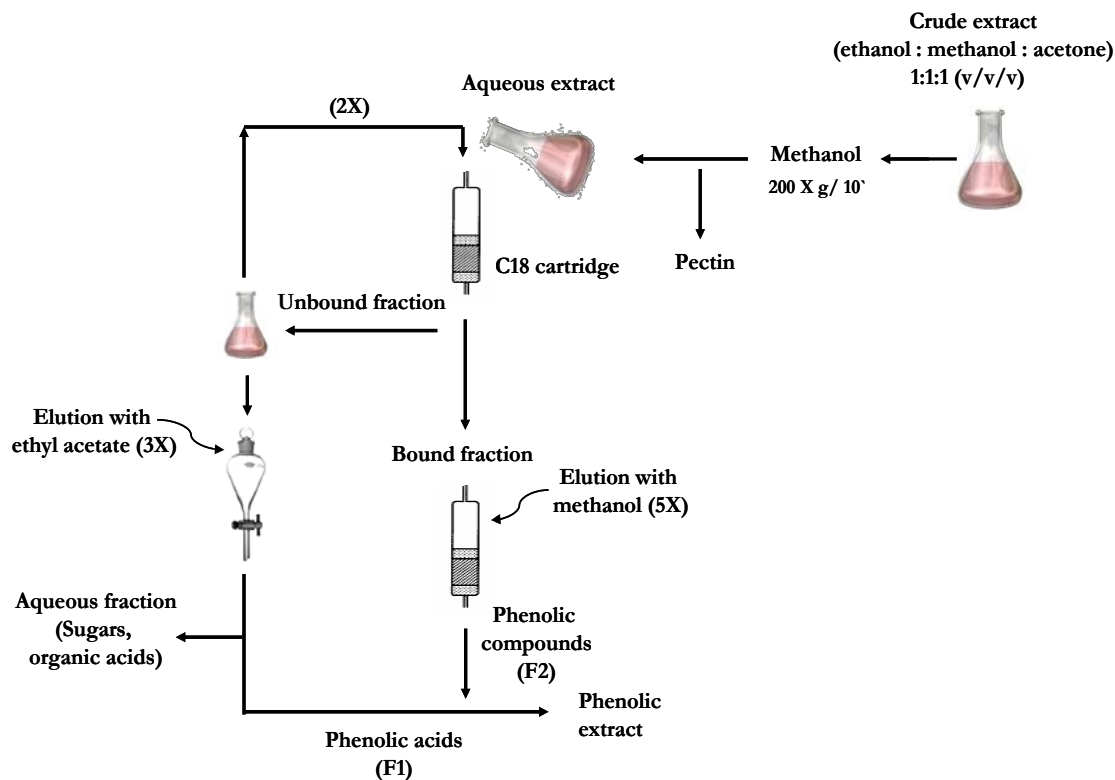
3. Extraction of carotenoids

Total carotenoids were extracted as reported by Talcott and Howard, 1999 (*30*). Briefly, 5g/25mL were extracted with acetone/ethanol (1:1). After filtering in the dark, volume was adjusted to 50 mL, and the absorbance measured using a Thermo Electron spectrophotometer (Fisher Scientific). Total carotenoids (mg/100g pulp) were calculated using the equation $(AV \times 10^6)/(A^\% \times 1000\ G)$, where A is the absorbance at 470nm, V is the total volume of extract, $A^\%$ is the extinction coefficient for a mixture of solvents arbitrarily set at 2500, and G is the sample weight in grams. For cell culture assays, carotenoids were extracted from freeze dried pulp with a solution containing methanol/hexane (1:1). The carotenoids were recovered from hexane phase, evaporated using a speed vac, re-dissolved in DMSO and sterile filter using a PVDF filter 0.22µm (Millipore, MA, USA). Total carotenoids added to the culture medium were quantified spectophotometrically (*30*) and doses were applied based on µM β-carotene equivalent.

4. Antioxidant Capacity.

Antioxidant capacity of the phenolics extracted from the different mango varieties was measured using the oxygen radical absorbance capacity (ORAC) assay as first described by Cao et al. (*31, 32*) and later modified by Ou et al. (*33*) with the use of fluorescein as fluorescent probe. Peroxyl radicals were generated by 2,2'-azobis(2-amidinopropane) dihydrochloride, and fluorescence loss was monitored on a FLUOstar Omega plate reader (BMG Labtech Inc, Durhan, NC) at 485 nm excitation and 538 nm emission. Each extract was diluted to standardize the phenolic content up to 10 mg GAE/L in pH 7.2 phosphate buffer prior to pipetting into a 96-well microplate. A 4-fold dilution factor was used in the ORAC assay that corresponded to an in-well standard concentration ranging from 6.25 to 50 µM Trolox. Results were quantified in µmol Trolox equivalents/100g pulp.

5. HPLC-DAD analysis

Polyphenolic extracts were analyzed using a Waters Alliance 2690 (Milford, MA, USA) HPLC system using a Dionex Acclaim ® 120 C18 5 µm column, 4.6 mm x 250 mm column and a 4.6 mm x 20 mm guard column. Individual phenolic compounds were separated with gradient mobile phases of acidified nanopure water: acetic acid (98:2, v/v) (phase A) and nanopure water:acetonitrile:acetic acid (68:30:2, v/v/v) (phase B) at a flow rate of 0.8 mL/min with PDA detection from 210 to 400 nm. Phenolic compounds were separated with a gradient program that ran phase A for 3 min, from 100-70% in 17 min; 70-50% in 10 min; 50-30% in 20 min; and 30-0% in 10 min, 0% in 5min, from 0-100% in 0.5 min and returning to original composition in 2 min for column equilibration. Monitoring was

performed at 280 nm (hydrolysable tannins), 250 nm (p-hydroxybenzoic acid and derivatives), 340 nm (hydroxycinnamic acids), 366 nm (mangiferin) and 370 nm (flavonols). Identification of phenolic compounds was performed by comparison of their retention times with those from standards obtained from Sigma-Aldrich (St Louis, MO) and characteristics of the UV spectra.

6. Cell lines

Cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown as recommended by the ATCC. The estrogen independent MDA-MB-231 breast cancer cells were cultured using Dulbecco's modified Eagle's medium (DMEM) high glucose, with 2 mmol/l L-glutamine, without sodium pyruvate and with phenol red. The colorectal SW-480 adenocarcinoma cells, were cultured using Leibovitz`s L-15 medium, with L-glutamine, without sodium pyruvate and phenol red. The human lung A-549 carcinoma cells were cultured in Dulbecco's modified Eagle's medium/F12 (DME/F12). The human leukemia Molt-4 cells were cultured in RPMI-1640 medium. The androgen receptor positive LnCap prostate cancer cells were cultured in RPMI-1640. All culture mediums were supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% Penicillin-Streptomycin antibiotic mix for propagation. A 2.5% charcoal stripped FBS was used in culture medium used for cell proliferation experiments. Culture mediums were supplied by Invitrogen (Gibco™, Invitrogen Corp., Grand Island, NY). Cells were maintained at 37 ºC with a humidified 5% CO2 atmosphere.

7. Generation of Reactive Oxygen Species (ROS).

The production of ROS was estimated following the method reported by (*34*) with some modifications. Briefly, human SW-480 adenocarcnoma colon cells ($1x10^5$ cells/mL) grown in 96-well plates were pre-treated with mango polyphenolics during 24h. Total polyphenolics were quantified by the Folin-Ciocalteu method (*29*), and doses were based on their phenolic content quantified as mg gallic acid equivalent (GAE)/L. Upon removal of culture medium with phenolics extracts, cells were exposed to 200µM H_2O_2 for 2h. After washing the H_2O_2, cells were stained with 10µM of 2', 7' - dichlorofluorescein diacetate (DCFH-DA) at 37 °C and incubated for 30min. The green fluorescence signal was monitored at 520 nm emission and 480 nm exitation with a FLUOstar Omega plate reader (BMG Labtech Inc, Durhan, NC). Fluorescence intensity was used as an indicator of ROS level.

8. Cell proliferation

Cell proliferation was assessed by using an electronic coulter counter (Z1™ Series, Beckman Coulter, Inc). Cells were seeded (15,000 cells/well) in a 24 well-plate and incubated for 24 h to allow cell attachment before exposure to varying concentrations of polyphenolics (mg GAE/L) or carotenoids (µM β-carotene equivalent). A pretreatment number of cells (0-time value) was established and medium was replaced containing the phenolic extracts. Phenolics extracts were re-dissolved in the culture medium and sterile-filtered before use. Each experiment was carried out in triplicate, and results were expressed as means ± SD. The difference in number of cells between the final incubation time (72h) and the 0-time represents net growth. The cell inhibition was expressed in terms of the concentration of mango phytochemicals which inhibits cell growth by 50% (IC_{50}).

9. Cell Cycle Kinetics.

Cells grown in 12-well plates (7.0×10^4) were treated with mango polyphenolics for 24 hrs. Briefly, cell pellets were obtained by tripsination and fixed with 90% ethanol at -20°C for 30 min. Then cells were re-suspended in staining solution [50 µg/mL propidium iodide, 30 units/mL RNase, 4 mmol/L sodium citrate, and Triton X-100 (pH 7.8)] and incubated at 37°C for 15 min. Sodium chloride solution was added to a final concentration of 0.15 mol/L. Stained cells were analyzed on a FACS Calibur Flow Cytometer (Becton Dickinson Immunocytometry Systems) using Cell Quest (Becton Dickinson Immun ocytometry Systems) acquisition software as previously described (35).

10. Semi-Quantitative Real-Time PCR.

Human SW-480 colon adenocarcinoma cells (5×10^5), seeded into 6-well plates, were treated with various concentrations of Ataulfo and Haden phenolics for 24h. Total RNA was extracted using RNeasy Mini kit (Qiagen), and 470ng of RNA were used to synthesize cDNA using Reverse Transcription System (Promega). Real time-PCR reactions were performed using 2µL of cDNA as previously described (35). Primers were obtained from Integrated DNA Technologies. Optimal semi-quantitative conditions were set to fall in the linear PCR product range (data not shown). As internal control, TBP (TATA-box binding protein) was amplified in each RNA sample. The sequences of the primers used were:

Fas: F: 5'- TGCCTCCTCTTGAGCAGTCA -3',

    R: 5'- TCCTGTAGAGGCTGAGGTGTCA -3'

Caspase 8: F: 5'- GGCTCCCCCTGCATCAC-3',

R: 5'- CCTGCTAGATAAGGGCATGAATCT-3',

Bax: F: 5'- CCAAGGTGCCGGAACTGA -3',

R: 5'- CCCGGAGGAAGTCCAATGT -3'

Bim: F: 5'- TGCCAGGCCTTCAACCA -3',

R: 5'- GTTCAGCCTGCCTCATGGA-3'

PKMYT1:F: 5'-CCTCTGCACTTTTAACCTTTTATCCT-3',

R: 5'- GCAGAGAAGACCATGGGAGTTC -3'

p21: F: 5'-GAG CTC TGG GTG GTC ATG GA -3'

R: 5'- ATC CTG GTG TGG GTG ACG AT -3'

TBP: F:5'-TGCACAGGAGCCAAGAGTGAA-3',

R: 5'-CACATCACAGCTCCCCACCA-3'

11. Effects of Mango polyphenolics on Transport in Caco-2 Cells

11.1 Extraction of pholyphenols

Mango frozen pulp (300 g) was thawed and mixed with 1100 mL of a 1:1:1 (v/v/v) ethanol: methanol: acetone solution, filtered with cheesecloth and with Whatman #1 filter paper . The solvents were evaporated at 40°C using a rotary evaporator. The aqueous extract was mixed with 200 mL of methanol and partionated into hexane (1:4, v/v) in order to remove carotenoids. The hexane phase was removed and the methanol phase centrifuged at 2000 x g for 10 min at 7 °C in order to precipitate pectin. The methanol was completely evaporated at 40 °C and polyphenolics were concentrated on a on a C18 Waters Sep-Pak Vac 35cc 20 cm^3 minicolumns (Waters Corporation, Milford, MA) previously conditioned with 100% methanol and nanopure water. The aqueous extract was loaded on the C18 cartridge and washed with 30 mL of water. Polyphenolics bound to the matrix were eluted with 50 mL 100% methanol (bound fraction). The water from the wash (unbound fraction) was re-eluted on the C18 cartridge one more time. The unbound fraction was partitioned twice into ethyl acetate to insure complete extraction of all polyphenolic compounds. Bound and unbound fraction were combined and evaporated under vacuum at 40 °C. Phenolics were re-dissolved in 0.1M citric acid buffer (pH 5.0) up to a known volume (50mL) (Figure 1) and storage at -20 °C until needed.

11.2. Enzymatic hydrolysis

Phenolic extract was completed with 0.1M citric acid buffer (pH 5.0) up to a known volume which corresponds to the volume in pulp (300 mL). The extracts were incubated with 0.5 mg β-glucosidase 1000 KU /mL of phenolics extract for 4 hrs at 35°C. The control was incubated without enzyme

following the same procedures describe above. In order to stop enzymatic activity, phenolic extracts were boiling for 5 min and chilled on ice immediately. The extracts were sonicated and re-eluted on a C18 Waters Sep-Pak Vac 35cc 5 cm^3 mini-columns (Waters Corporation, Milford, MA) following the same procedure described in section 11.1. The bound and unbound phases were mixed, solvents completely evaporated under vacuum at 40 ℃ and polyphenolics re-dissolved in 1 mL methanol. Extracts were evaporated in speed vac and stored at -20˚C until needed. Phenolics were re-dissolved with the same volume of HBSS buffer pH=6.0 for the transepithelial transport model assay. The same dilutions of extract were used (1,000 and 3,000 mg GAE/L) following the hydrolysis procedure.

11.3. Transepithelial Transport Model

Caco-2 colon carcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (1X) high glucose (DMEM) containing 20% fetal bovine serum (FBS), 0.4% non-essential amino acids, 100 units/mL penicillin G, 100 µg/mL streptomycin, 1.25 µg/mL amphotericin B, and 10 mM sodium pyruvate (chemicals supplied by Sigma-Aldrich Co., St. Louis, MO). Cells between passages 5-15 were seeded at a density of 100,000 cells per cm^2 onto a Costar Transwell insert, 0.4 µm pore diameter, 12 mm transparent polyester cell culture insert well plates (Transwell, Corning Costar Corp., Cambridge, MA), with 0.5 mL of DMEM 15% FBS in the apical side and 1.5 mL in the basolateral side (Figure 5). The medium was changed every 2 days. Cells were grown and differentiated to confluent monolayers for 21 days, as previously described (Hidalgo, Raub, & Borchardt, 1989). Transepithelial electrical resistance (TEER) values were monitored with an EndOhm Volt ohmmeter equipped with a STX-2 electrode (World Precision Instruments Inc., Sarasota, FL).

Figure 5. Caco-2 cell monolayer absorption system.
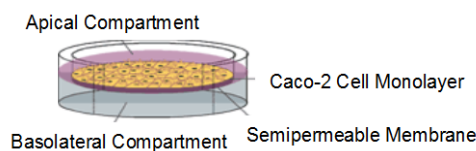
To check the cells confluence and monolayer integrity, Transepithelial electrical resistance values (TEER) was measured with an EndOhm Voltohmmeter equipped with a STX2 electrode in after 30 min of incubation with HBSS and after 2 hrs of incubation with the treatments. Additional control of

monolayer integrity was be performed using lucifer yellow, a very hydrophilic probe which can permeate the cells only trough the paracellular route. This probe (100 µg/ml) was added to the apical side, incubated for1h and the % of passage was determined by comparing with fluorescence obtained from a standard solution using excitation and emission wavelengths 485 and 530 nm, respectively. Monolayers with TEER values >450 Ω cm^2 at the beginning and >350 Ω cm^2 at the end were used for transport experiments. Prior transport experiments, cells were rinsed with Dulbecco's phosphate buffer saline (DPBS) and growth media was replaced by Hank's balanced salt solution (HBSS, Fischer Scientific, Pittsburgh, PA) containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid solution (MES, Gibco BRL Life Technology, Grand Island, NY) adjusted to pH 6.0 in the apical side, and HBSS containing *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid] buffer solution (1 M) (HEPES, Gibco BRL Life Technology, Grand Island, NY) adjusted to pH 7.4 in the basolateral side, creating a pH gradient similar to the small intestine environment. Mango polyphenolics control and enzymatic treated were diluted in HBSS adjusted to pH 6.0 and the same dilutions used (1,000 and 3,000 mg gallic acid equivalent/L) to be used in cell culture. After incubation at 37°C for 30 min of both sides of the monolayer, the HBSS adjusted to pH 6.0 from apical side of cell monolayers was replaced by 500 µL of phenolic extracts. Sample aliquots (200 µL) were taken from the basolateral compartment at 30, 60, 90, and 120 min, and immediately acidified with 5µL of 4N HCl, kept frozen (-20°C), and analyzed within one week. Samples were filtered through 0.45µm PTFE membranes (Whatman, Florham Park, NJ) and injected directly into the HPLC-ESI-MS system.

12. Statistical analysis

Quantitative data represent mean values with the respective standard deviation (SD) or standard error of the mean (SE) corresponding to 3 or more replicates. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 15.0 (SPSS Inc., Chicago, IL). Post-hoc Tukey pairwise comparisons were used ($p < 0.05$ and $p < 0.01$).

Results and Discussion

Polyphenolics. Ataulfo was found to be the highest in total phenolics (56.7 ± 0.3 mg GAE/100g), followed by Haden (19.1 ± 1.8 mg GAE/100g) (Table 3). Variations in mango polyphenolics have been associated with the level of ripening, geographic production, processing conditions (*11, 36*), seasonal changes, or microbial infections (*37*). Determination of total phenolics might be relevant

when analyzing the antioxidant and anticarcinogenic activities from fruit, since both are in part attributable to polyphenolics.

Carotenoids. Mango cultivars have been reported to be a good source of carotenoids, although the amounts may vary according to the ripening stage, cultivar origin and processing conditions (*17*) (*20*). Our screening of carotenoid confirmed such variability, with Haden and Francis containing the highest carotenoid concentration (14.2 ± 1.4 and 13.7 ± 0.9mg/100g pulp, respectively), followed by Francis, Kent, Ataulfo and Tommy Atkins (9.6 ± 0.0, 6.3 ± 0.8, and 5.3 ± 1.1 mg total carotenoid/100g pulp, respectively) (Table 3)

**Table 3. Total polyphenolics, carotenoids, and antioxidant activity (ORAC) of different mango cultivars**

Mango Cultivar	Total pholyphenols (mg GAE/kg pulp)	Antioxidant Capacity µmol TE/g	Total carotenoids (mg /100g pulp)
Ataulfo	793	8.41	6.3[c] ± 0.8
Haden	371	4.01	14.2[a] ± 1.4
Francis	604	4.58	13.7[a] ± 0.9
Kent	340	2.85	9.6[b] ± 0.0
Tommy Atkins	315	1.45	5.3[c] ± 1.1

[1]Average of three or more independent determinations ± SD.

HPLC-DAD analysis (Ataulfo and Haden)

The HPLC-DAD analysis of Ataulfo and Haden polyphenolic extracts shown the presence of phenolic acids such as gallic acid and their glycosides (gallotannins), xanthones and their glycosides such as mangiferin, hydroxycinnamic acids derivatives (which contain *p*-coumaric acid, caffeic or ferulic acids), p-OH- benzoic acid derivatives and flavonoids (Figure 6).

Figure 6: Chromatograms at 280 and 366 nm from Ataulfo (A) and Haden (B) polyphenolic extracts. Peaks 1: Galloyl glucoside, 2: Gallic acid, 3: *p*-OH-benzoic acid derivatives, 4: Hydroxycinnamic acids derivatives, and 5: mangiferin (insert).
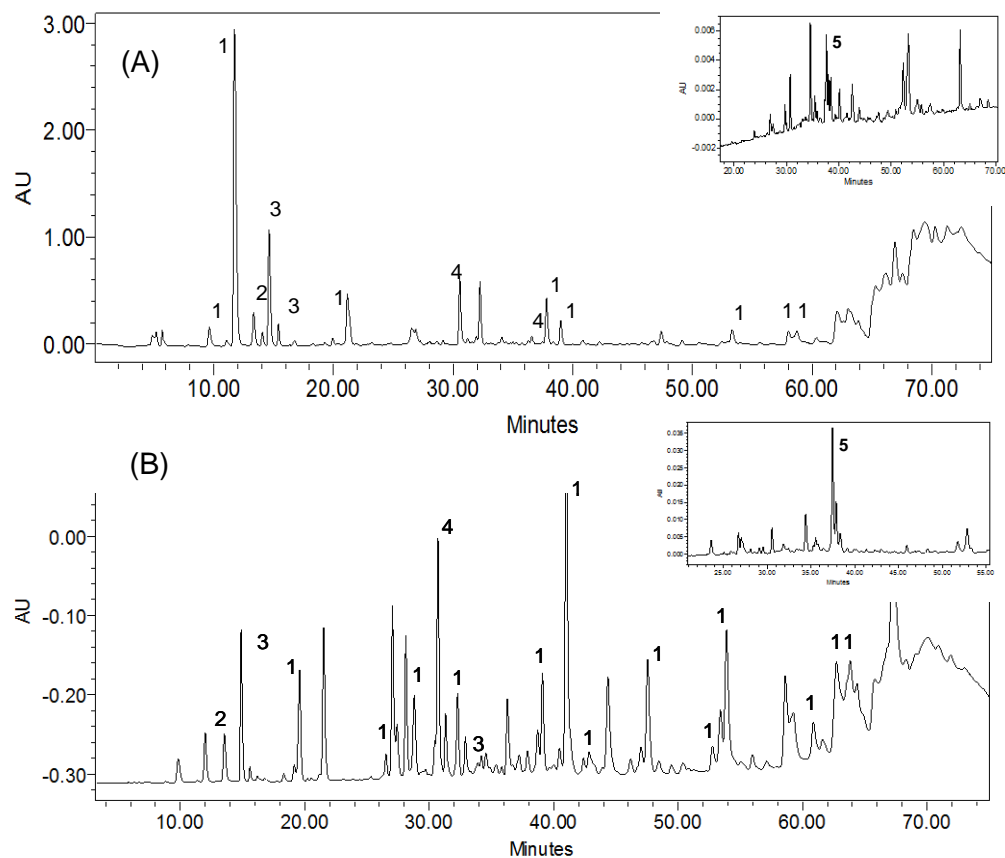

## Cancer growth-suppressive activity of Haden and Ataulfo polyphenolics

Due to their higher phenolic content and antioxidant activities, Ataulfo and Haden cultivars were selected to assess the growth inhibition activity of their polyphenolicss against the different cancer cell lines used in this study (Figure 7). Within the range of 0-42 mg GAE/L, Ataulfo polyphenolics suppressed the cancer cell growth with order of potency: SW-480 = Molt-4 = MDA-MB-231 > A-549 = LnCap. All cell lines showed a similar pattern of dose-dependent cell growth suppression when treated with Haden phenolics, although MDA-MB-231 was found to be more resistant.

Figure 7. A representative evaluation of the concentration-dependent impact of phenolic compounds extracted from the commercial varieties of mango (A) Haden and (B) Ataulfo on the net growth of Molt-4 leukemia, A-549 lung carcinoma, MDA-MB-231 estrogen independent breast cancer, SW-480 colorectal adenocarcinoma and androgen receptor positive LnCap prostate human cancer cells. Cells were incubated with various concentrations of extracts and net growth was measured at 3 days using a cell counter. Values are mean of three replicates ± SE.

A. Haden
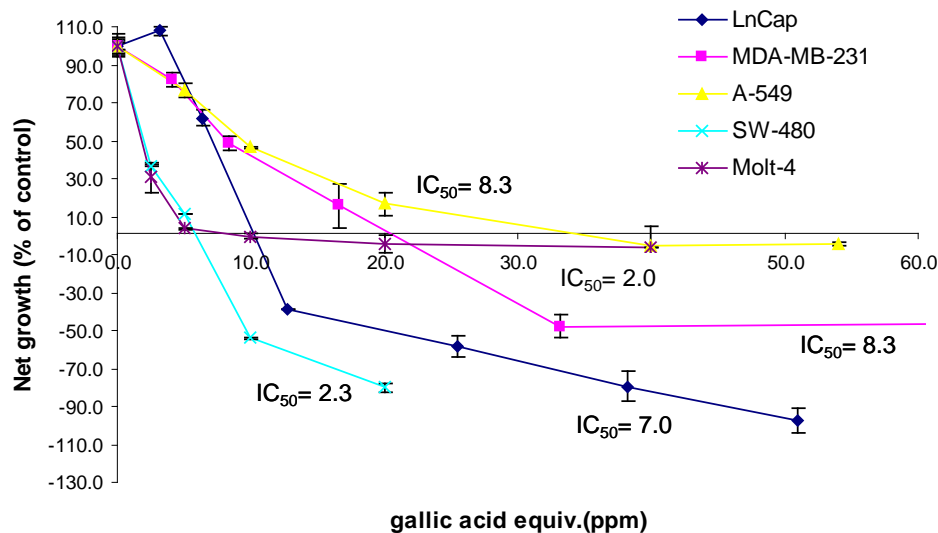
B. Ataulfo
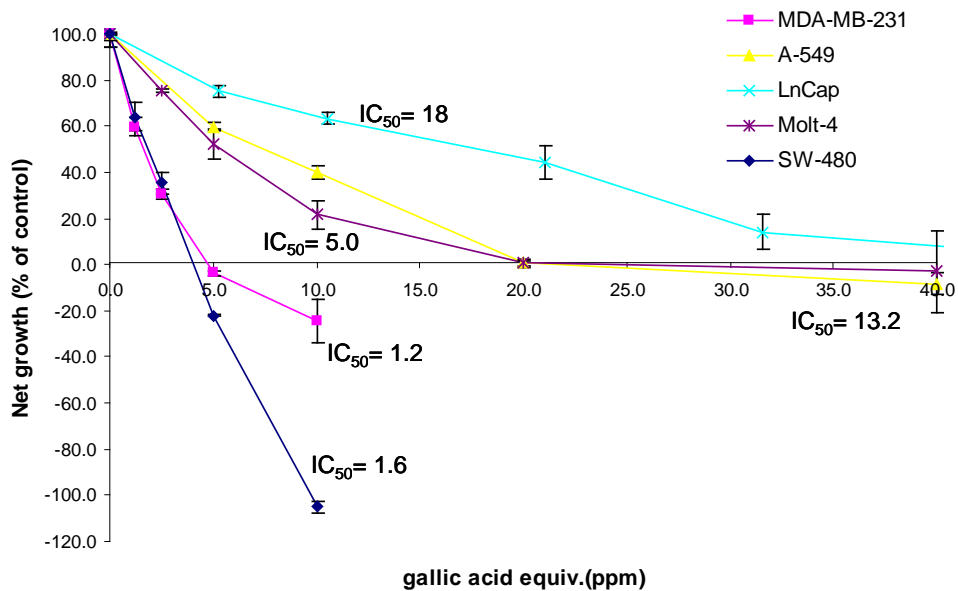
The IC_{50} values (concentration to inhibit the cancer cell growth by 50%) are summarized in Table 4, indicate that the human colorectal SW-480 carcinoma cells were inhibited by both Haden and Ataulfo polyphenolics to the same extent. Thus, lower IC_{50} values indicates that it is necessary lower concentrations of phenolic extract to have the same effect (inhibit the growth of the cancer cells by 50%).

Table 4. IC_{50} values of polyphenolics extracted from Ataulfo and Haden mango cultivars for growth suppression of different human cell lines

Mango Cultivar	IC_{50} (mg GAE/L)					
	SW-480	MDA-MB-231	LnCap	A-549	Molt-4	CCD-18Co
Ataulfo	$1.6^1 \pm 0.2^b$	$1.2^1 \pm 0.5^b$	$18.0^2 \pm 0.0^a$	$13.2^1 \pm 5.5^a$	$5.0^1 \pm 1.0^b$	$9.0^2 \pm 0.4$
Haden	$2.3^1 \pm 0.2^b$	$8.3^1 \pm 0.4^a$	$7.0^1 \pm 0^a$	$8.3^2 \pm 1.8^a$	$2.0^1 \pm 0.5^b$	ND

[1]Average of three independent determinations $\pm$ SD. [2]Average of two independent determinations. Different letters indicate statistical significance at the level $p < 0.05$. ND, no determined.

Among the polyphenolics identified in Haden pulp (*36*), the HPLC profile of extracts re-dissolved in culture medium and sterile filtered, demonstrated that the high molecular weight gallotannins (retention time over 60 min) remained in the filter (too large or complexed with proteins). Therefore, only the available low molecular weight mango polyphenolics are responsible for the in vitro cell growth-inhibition activity and other cell responses presented in this study. Interactions of individual phytochemicals such as mangiferin, quercetin 3-O-glucosides and galloyl glycosides may exert a synergistic or additive effect (*52-54*). This is relevant because mango polyphenolics are likely available in the colon (*13*). Mango gallotannins, most likely not absorbed throughout the small intestine (*16*), might reach the colon and transformed into low molecular weight galloyl glycosides derivatives, similar to those identified in our cell culture treatments. Thus, the chemopreventive protection of mango polyphenolics against colon cancer may be translated in vivo. The bioavailability of dietary polyphenolics differs significantly among low and high molecular weight compounds. Phenolic acids like caffeic acid and gallic acid are the most well absorbed polyphenolics, followed by catechins, flavanones, and quercetin glucosides. The least well-absorbed polyphenolics are large molecular weight polyphenolics such as the proanthocyanidins, the galloylated tea catechins, and the anthocyanins (*55*). These polyphenolics are more stable to gastrointestinal digestion and likely reach the colon (*13*). Later, they are extensively metabolized by the colonic microflora into a wide array of low molecular weight polyphenolic breakdown products (*56*). Results suggest that Ataulfo and Haden

cultivars may be identified as varieties with special health benefits due to their higher content of polyphenolics, antioxidant and cancer cell growth-inhibition activities.

Non-technical Summary:
After the initial chemical evaluation of mango varieties Ataulfo and Haden were selected based on their antioxidant capacity to be tested in several types of cancer cell lines. Different types of cancer may show differences in the responsiveness to the anti-cancer properties of mango compounds, therefore several types of cancer were tested. The cell growth assays indicated that the SW480 colon cancer cells were most sensitive to the treatment with both mango varieties compared to the other cancer types. Mangoes contain large and small size polyphenolics of which the larger compounds are not as likely to be absorbed, but therefore will reach the large intestines. This means that colon (cancer) cells are exposed to mango compounds through the blood as well as through the intestinal lumen.


How much Mango would we have to eat to obtain the observed effects?
The IC50, at which cell growth was reduced by 50%, is equivalent to 23 mg pulp/ mL culture medium for Ataulfo mango. If we, just as a model, assume that this concentration was needed in the colon, 23g/L intestinal volume would be needed to kill reduce 50% of colon cancer cell growth within a colon segment exposed to that 1 liter. This also assumes that none of the polyphenolics would be absorbed in the upper intestines, that the colon just contains liquid and that there is no protective mucosa covering cells. In this model, in order to obtain this concentration we would have to consume 23g of edible portion of Ataulfo mango per liter of colon volume. Given an intestinal volume of 2-3L, this would mean 46-69g of mango. From the performed experiments it cannot at all be concluded how much mango has to be consumed per day, however in cell culture the equivalent amount was incubated for 24h, where only the metabolism of the cultured cells was ongoing and nothing was excreted as it would happen in a living system*.

Cancer growth-suppressive activity of mango carotenoids

The growth-suppressive activity of carotenoids extracted from Ataulfo showed a dose-dependent response on the growth of colon SW-480 cancer cells within the range of 0 to 7.5µM β-carotene equivalent (Figure 8). Reports have shown that carotenoids, at concentrations possibly achieved in vivo (1-10 µM), do not inhibit the growth of prostate (*57*) and leukemic cancer cells (*58*). However, β-carotene has shown to inhibit the growth and induce apoptosis in caveolin-1 positive colon and prostate cancer cells (*24*). The regulation of the tumor suppressor gene caveolin-1 by β-carotene was related to the inhibition of AKT survival and activation of caspase-dependent proapoptotic pathways (*24*). However, even thought Ataulfo carotenoids were effective in inhibiting the growth of colon SW-480 cancer cells, the content of carotenoids in this cultivar is relatively low (6 mg β-carotene equivalent /100g pulp) and we needed to use significantly more plant material in order to extract enough carotenoids for the cell culture study. On the other hand, the total polyphenolics were more abundant (57 mg GAE/100g pulp), and the cell growth inhibition was achieved at lower concentrations (1.3 to 5 mg GAE/L) than carotenoids (268 – 4,026.5 mg β-carotene equivalent/L), indicating mango polyphenolics were more effective for cancer treatment and chemoprevention.

Figure 8. A representative evaluation of the concentration-dependent impact of carotenoids extracted from Ataulfo on the net growth of human SW-480 colon cancer cells. Cells were incubated with carotenoids dissolved in DMSO for 72 hrs and net growth was measured compared to the solvent treated control. Values are mean ± SE, n=3.
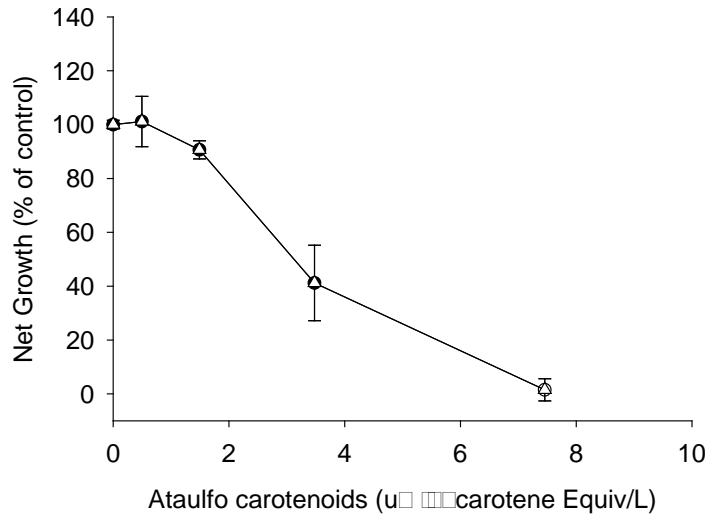
Non-technical Summary:
Mango contains carotenoids in addition to polyphenolics. In order to determine whether the contained carotenoids have anti-cancer activity, relevant to the consumption of mangoes, the carotenoid fraction was extracted from Ataulfo mangoes and colon cancer cells were incubated with different concentrations. Carotenoids were effective in inhibiting colon cancer cell growth, however the overall concentrations in mangoes is fairly low and consequently, this compound group may not be a relevant contributor to the anti-cancer effects of mangoes. The IC50, at which cell growth was reduced by 50% is equivalent to 894 mg/ml cell culture medium.

How much Mango would we have to eat to obtain the observed effects?
If we, just as a model, assume that this concentration was needed in the colon, 894g/L intestinal volume would be needed to kill reduce 50% of colon cancer cell growth within a colon segment exposed to that 1 liter. This is assuming that only carotenoids were present in mango and no other compounds. This also assumes that none of the carotenoids would be absorbed in the upper intestines, that the colon just contains liquid and that there is no protective mucosa covering cells. In this model, in order to obtain this concentration we would have to consume 894g of edible portion of Ataulfo mango per liter of colon volume to obtain the effect, which is far more than the amount needed in order to obtain the effects of polyphenolics (see above) Given an intestinal volume of 2-3L, this would mean 1788-2682g of mango. From the performed experiments it cannot at all be concluded how much mango has to be consumed per day, however in cell culture the equivalent amount was incubated for 24h, where only the metabolism of the cultured cells was ongoing and nothing was excreted as it would happen in a living system*.

Human SW-480 colon cancer growth-inhibition by polyphenolics from different mango cultivars.

Polyphenolics extracted from Ataulfo, Haden, Kent, Francis and Tommy Atkins, with descending order of potency, induced dose-dependent growth suppression in human SW-480 colon cancer cells (Figure 9). Ataulfo and Haden polyphenolics, among the five cultivars, showed the highest cell growth-inibition activity in SW-480 (lower IC_{50} values). At 1.3 and 2.5 GAE/L, Ataulfo polyphenolics suppressed the growth of SW-480 cells by 36% and 65%. The -22% net growth of SW-480 incubated with 5 mg GAE/L of Ataulfo polyphenolics indicates that the 72 hr population was lower than the 0-time population, suggesting net cell killing. Whereas, polyphenolics extracted from Haden had less substantial but concentration-dependent inhibition of SW-480 cell proliferation, with 20%, 50% and 90% of inhibition achieved at 1.3, 2.5 and 5 mg GAE/L respectively. The IC_{50} values indicate the order of potency against the growth of SW-480 cells and followed Ataulfo = Haden > Kent > Francis > Tommy Atkins (Table 5).

Figure 9. A representative evaluation of the concentration-dependent impact of phenolic compounds extracted from the commercial varieties of mango Haden, Ataulfo, Tommy Atkins, Kent and Francis on the net growth of SW-480 human colorectal adenocarcinoma cells. Cells were incubated with various concentrations of extracts and net growth was measured at 3 days using a cell counter. Values are mean of three replicates ± SE.
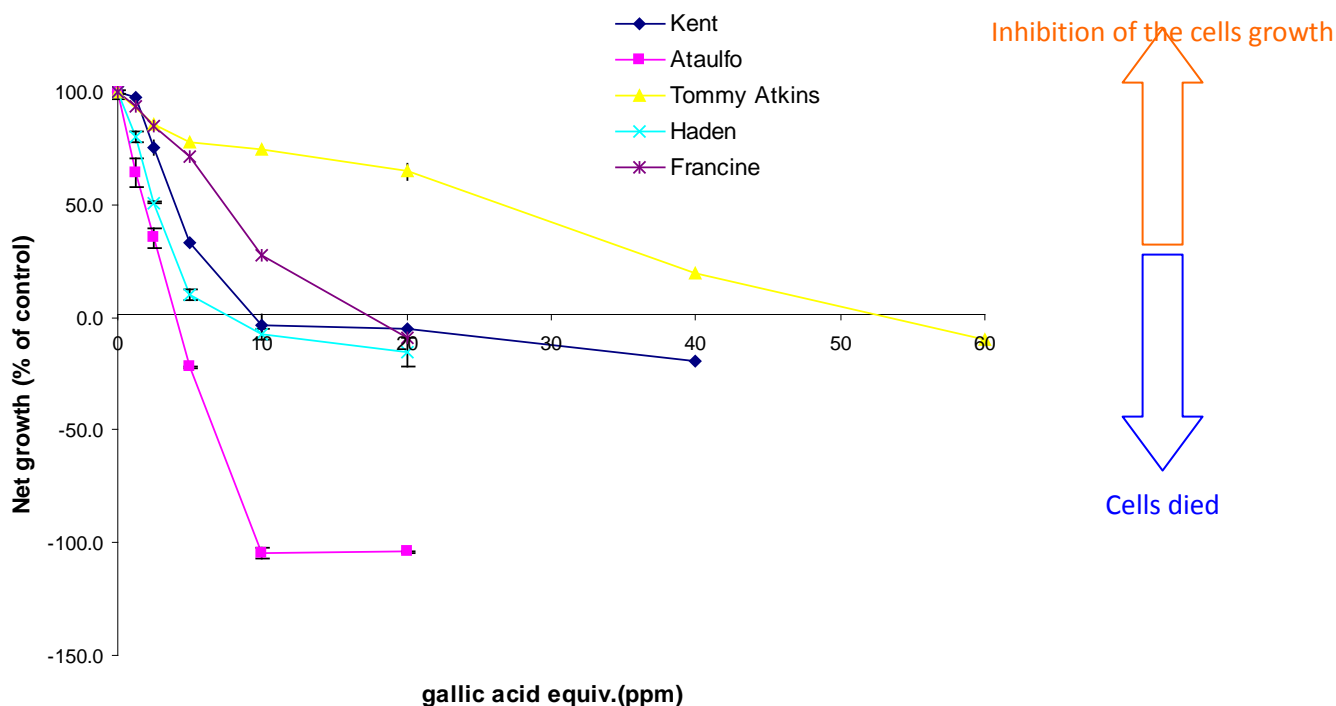
Table 5. IC_{50} values of polyphenolics extracted from mango cultivars for growth suppression of human SW-480 colon cancer cells

Mango Cultivar	IC_{50} (mg GAE/L)
Ataulfo	$1.6^{1} \pm 0.2^{a}$
Haden	$2.3^{1} \pm 0.2^{a}$
Kent	$5.0^{2} \pm 1.4^{a,b}$
Francis	$8.2^{1} \pm 2.6^{b}$
Tommy Atkins	$27.3^{2} \pm 0.4^{c}$

[1]Average of three independent determinations $\pm$ SD. [2]Average of two independent determinations. Different letters indicate statistical significance at the level $p < 0.05$.

Related studies reported galloylated polyphenolics as the most effective in inhibiting proliferation of colon cancer cells (*59*). However the IC_{50} values reported for SW-480 (from 134-190 μM) (*60*), when compared to the IC_{50} value of Ataulfo polyphenolics, lead us to identify the mixture of polyphenolics found in Ataulfo as colon cancer growth-suppressing natural agents with high potency. Taken together, results from the cell proliferation study, quantification and characterization of polyphenolics, and their antioxidant activities, Ataulfo and Haden cultivars may be identified as varieties with potential for colon cancer treatment and prevention.

Non-technical Summary:
After selecting SW-480 colon cancer cells based on their high sensitivity to mango compounds, several mango cultivars were tested within this cell line and it was demonstrated that the Tommy Atkins variety is more than 10-fold less effective than Ataulfo. Effects from Kent and Francis were less than Ataulfo and Haden but more than Tommy (see table above).

Cell growth-suppressive activity of Ataulfo polyphenolics against non-cancer colonic myofibroblasts CCD-18Co cells

The non-cancer colonic myofibroblasts CCD-18Co cells were more resistant to the polyphenolic-mediated growth suppression than SW-480 colon cancer cells. At the highest dose (5 mg GAE/L), in which Ataulfo polyphenolics suppressed the growth of SW-480 by ~ 79 %, the growth of CCD-18Co was not inhibited. Likewise, the IC_{50} value for CCD-18Co was 9.0 ± 0.4 mg GAE/L, therefore Ataulfo polyphenolics exert a relatively lower toxicity on non-cancer colon cells since the concentration

needed to inhibit the growth by 50% is 5.6 fold of that needed to inhibit the growth of SW-480 at the same extent (Figure 10).

Figure 10. Representative curves showing the concentration-dependent growth suppression of Ataulfo polyphenolics on the human SW-480 colon cancer cells and colonic myofibroblasts CCD-18Co cells. Cells were incubated with extracts and net growth was measured at 3 days. Values are mean ± SE, n=3. Asterisk indicates a significant difference compared to untreated control (*) $p \leq 0.01$.
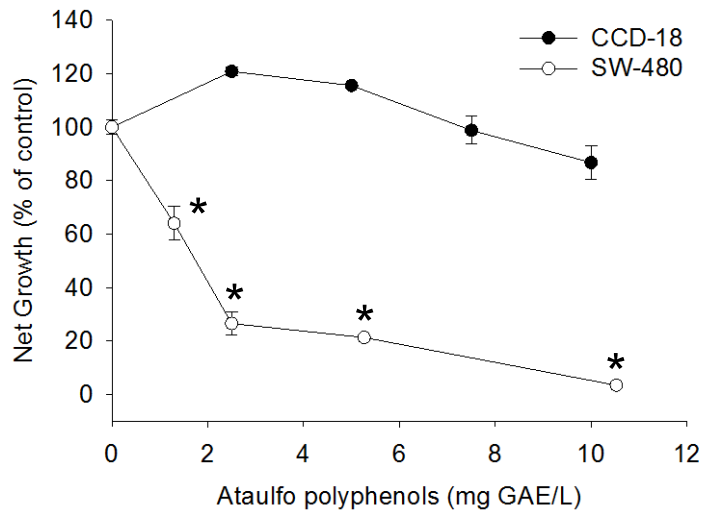
These results suggest that polyphenolics present in mango with bioactive properties were mainly galloyl glycosides, whereas flavonol-O-glycosides (*36*) were also shown to have potential as chemopreventive and chemotherapeutic natural compounds against colon cancer since they preferentially target the SW-480 cancer cells with low toxicity to normal cells. Previous studies have reported the colon cancer growth-inhibitory and cytotoxicity effects of plant extracts against colon cancer cells (*10, 61-63*). However, these studies failed to assess the toxicity of the active polyphenolics on non-cancerous cells. These results are relevant because the effective dosage levels are close to the reported range of high molecular weight polyphenolics that remains in the gastrointestinal tract and passes through to the colon (*14*). Therefore the potential preventive effects in colorectal cancer could be translated in vivo.

Non-technical Summary:
When we compared the effects of Ataulfo in non-cancer colon cells, we found that the polyphenolic mango compounds did not negatively affect the growth of normal cells, only of cancer cells. This means that the consumption of Mango is not expected to have a negative effect on normal colon cells.

Transcriptional regulation in human SW-480 colon cancer cells by polyphenolic from Ataulfo and Haden cultivars. Haden and Ataulfo polyphenolics regulated transcription of cell cycle control and pro-apoptotic genes at dose-range 0-10 mg GAE/L (Figure 11).

Results indicate that both the extrinsic death receptor and the intrinsic mitochondria proapoptotic pathways are being targeted at the transcription levels by Ataulfo and Haden polyphenolics. The Fas-receptor activation happens at post-translational level, leading to activation of caspase-8, an initiator of the caspase cascade resulting in resulting in proteolysis of cellular proteins and death by apoptosis or programmed cell death (*45*). On the other hand, natural plant extracts and phytochemicals appear to target the intrinsic mitochondrial pathway (*30*). This pathway may be activated by several stimuli, including stress by ROS and cytotoxic compounds. Ataulfo polyphenolics up-regulated the transcription of the mitochondria related proapoptotic genes Bax and Bim, whereas Haden polyphenolics seem not to be effective in targeting Bim gene expression. When the mithocondrial pathway is activated, the proapoptotic Bax form channels and contributes to regulate preexisting channels that permeabilize the outer mitochondrial membrane, which results in the release of proteins from the inter-membrane space. These proteins, once released into the cytosol, induce or promote apoptosis (*46*). Bim on the other hand, has been related to death stimuli activation and acts as a 'death ligand' which can neutralize certain members of the pro-survival Bcl-2 sub-family of proteins (*47*).

Regarding cell cycle control genes, results show that Ataulfo polyphenolics at low doses (2.5 mg GAE/L) repressed the expression of p21(Cip1/WAF1) (p21) and this effect was reversed at higher doses; the same trend was found in cells treated with Haden polyphenolics. The p21 gene encodes a potent cyclin-dependent kinase inhibitor, and functions as a regulator of cell cycle progression at G1 phase. Finally, both Ataulfo and Haden polyphenolics up-regulated the protein kinase-membrane associated tyrosine/threonine 1 (PKMYT1) gene expression. The protein encoded by this gene is a member of the serine/threonine protein kinase family. This kinase preferentially phosphorylates and inactivates cell division cycle 2 protein (CDC2), and thus negatively regulates cell cycle G2/M transition (*48*).

Figure 11. mRNA expression of SW-480 colon cancer cells treated with (A) Ataulfo and (B) Haden polyphenolics during 24 hours and analyzed by real time PCR as ratio to TATA-binding protein (TBP) mRNA. Values are means ± standard errors (n=3). Different letters indicate significance at p < 0.05.
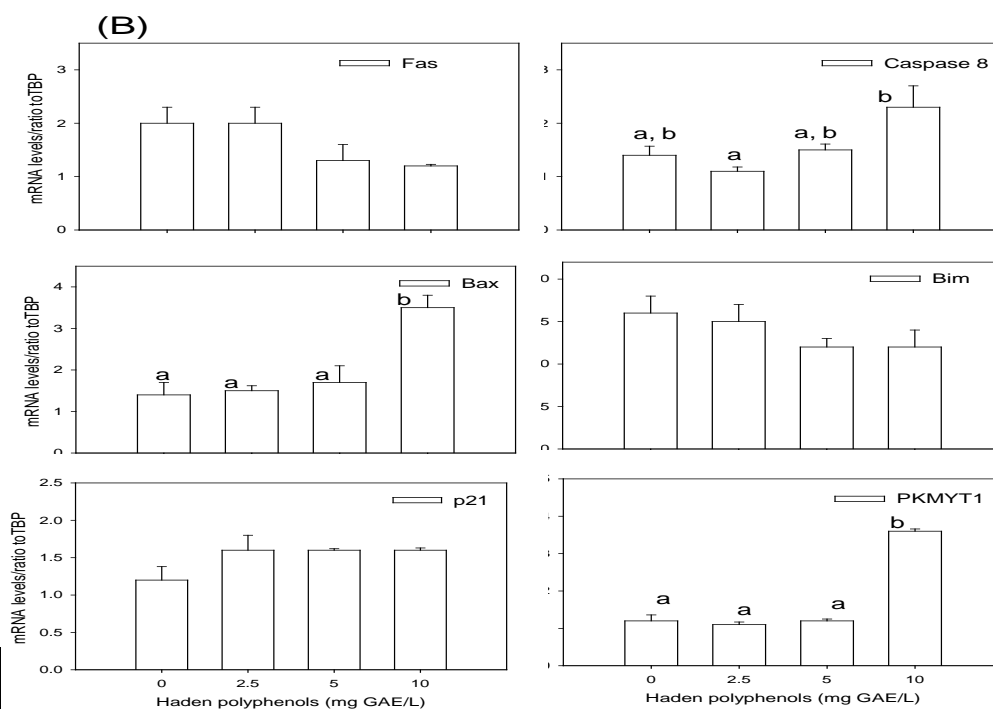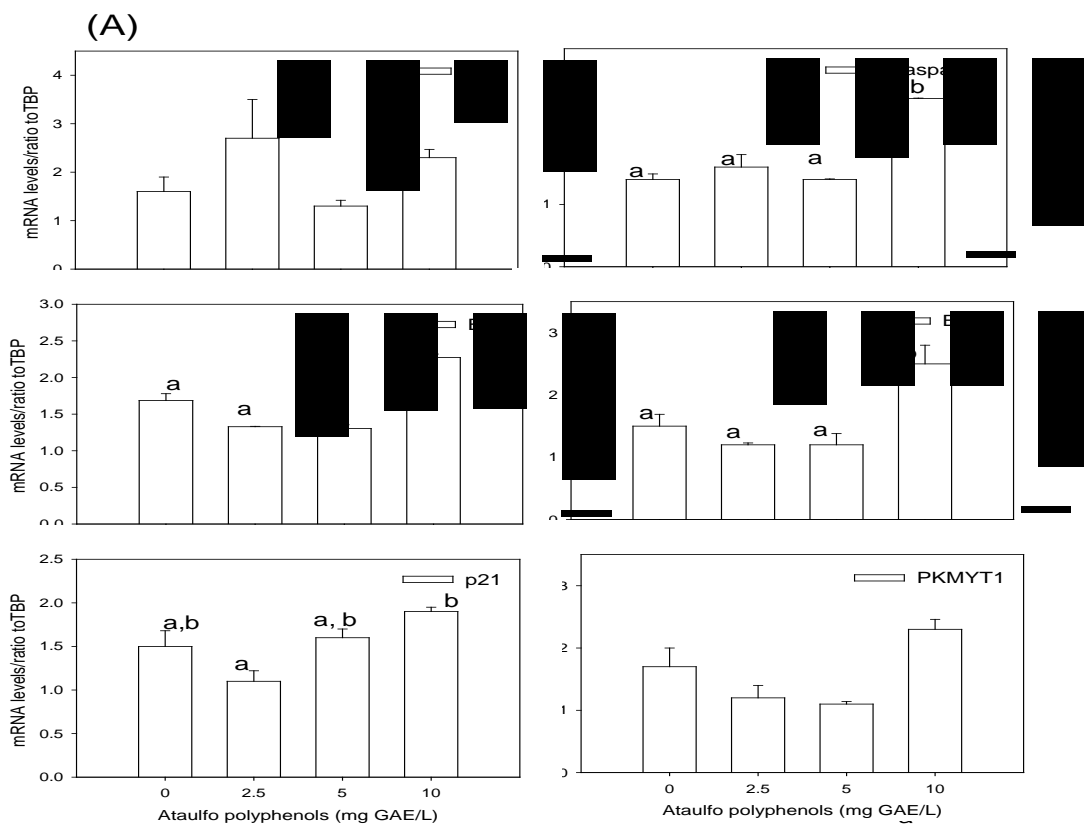
Cell Cycle Regulation

The cell cycle, or cell-division cycle, is the series of events that take place in a cell leading to its division and duplication (replication). The Cell Cycle includes five stages: G1, S, G2, (interphase), M (mitosis), C (cytokinesis). However, the cell cycle can be divided in two brief periods: interphase, during which the cell grows, accumulating nutrients needed for mitosis and duplicating its DNA, and mitosis, (M) phase, during which the cell splits itself into two distinct cells, often called "daughter cells". There are three stages of interphase, each phase ends when a cellular checkpoint checks the accuracy of the stage's completion before proceeding to the next. The stages of interphase are:

- G_1 (Gap 1), in which the cell grows and functions normally. The first phase within interphase, from the end of the previous M phase until the beginning of DNA synthesis is called G_1 (G indicating *gap*). During this time, a lot of protein synthesis occurs and the cell grows (to about double its original size) - more organelles are produced and increasing the volume of the cytoplasm. If the cell is not to divide again, it will remain in this phase.
- **Synthesis** (S), in which the cell duplicates its DNA (via semi-conservative replication).
- G_2 (Gap 2), in which the cell resumes its growth in preparation for mitosis.

M phase is itself composed of two tightly coupled processes: mitosis, in which the cell's chromosomes are divided between the two daughter cells, and cytokinesis, in which the cell's cytoplasm divides forming distinct cells. Additionally cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called G_0 phase, which is a stage separate from interphase and an extended G_1 phase. Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called G_0 phase. A tumor formation can happen due to a disregulation of the cell cycle components. In a tumor, the proportion of cells that are in active cell division (versus quiescent cells in G0 phase) is much higher than that in normal tissue.

Figure 12 shows the percentage of cells in each cell cycle phase. The percentage of cells treated with Ataulfo polyphenolics in G0/G1 phase is lower than control (no treatment) acts, indicating that Ataulfo polyphenolics suppressed the number of cells in G0/G1 phase. Besides, considering we have a increasing in the number of cells in G2/M phase after treatment with Ataulfo extracts, polyphenolics from this cultivar also induced G2/M phase arrest, though the G2/M phase arrest effect was reversed at the highest dose (15 mg GAE/L). Since it occurs, polyphenolics from Ataulfo are preventing the progression of the cell cycle and consequently, the tumor formation. On the other hand, Haden polyphenolics did not regulate cell cycle on G0/G1 but the effect on arresting cell cycle on G2/M phase was achieved at higher doses (10-15 mg GAE/L).

Figure 12. Flow cytometry analysis of SW-480 human colorectal adenocarcinoma cells. Cells were treated with 0 (control), 5, 10 and 15 mg gallic acid equivalent/L of Ataulfo (B) and Haden (A) extracts for 24h. Different letters indicate significance at P < 0.05.
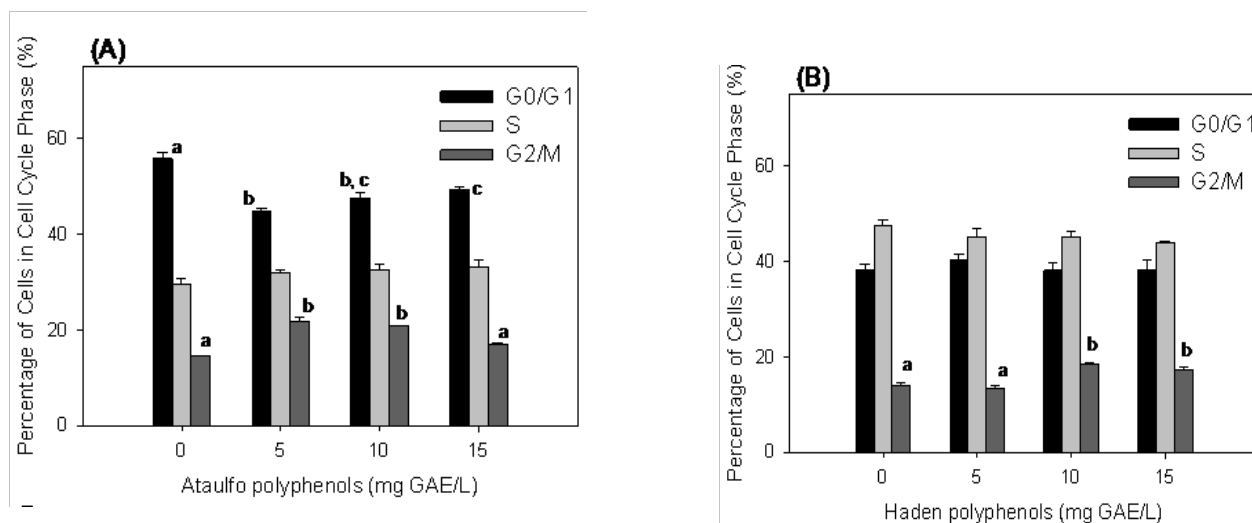
Non-technical Summary:
We found that the genes affected by mango polyphenolics are involved in the regulation of cell division and growth and that mango compounds induce a so-called suicide death of colon cancer cells. Which means that the compounds trigger mechanisms which cause the cancer cells to self-destruct, which is a common mechanisms for anticancer effects of natural compounds.

HPLC-DAD analysis of polyphenolics extracted from Ataulfo for Caco-2 Cell Monolayers

After hydrolysis with β-glucosidase the phenolics profile changed significantly, as was observed in earlier studies to confirm gallotannin effects following enzyme hydrolysis. The total phenolic content had a reduction of 24%, which corresponds to reduction in galloyl glycosides, as confirmed by HPLC (retention times longer than 60 min). These high molecular weight compounds (hexa galloyl glycosides or higher) could be bound with the enzyme making a complex. Indeed, a precipitated was formed and retained during elution of phenolics on C18 cartridge. The hydrolysis increase the concentration of phenolic acids, such as gallic acid (1327.3% or 13.3 fold) and xanthones, such as mangiferin (40.4% or 1.4 fold). Besides, others phenolic acids were liberated from their glycosides, such as caffeic, p-coumaric, vannilic, p-OH-benzoic and ferulic acids (Figure 13).

Figure 13. Chromatograms at 280 nm (A), 250nm (B) and 320nm (C) of Ataulfo extract without treatement (c) and hydrolyzed with beta-glucosidase (e).
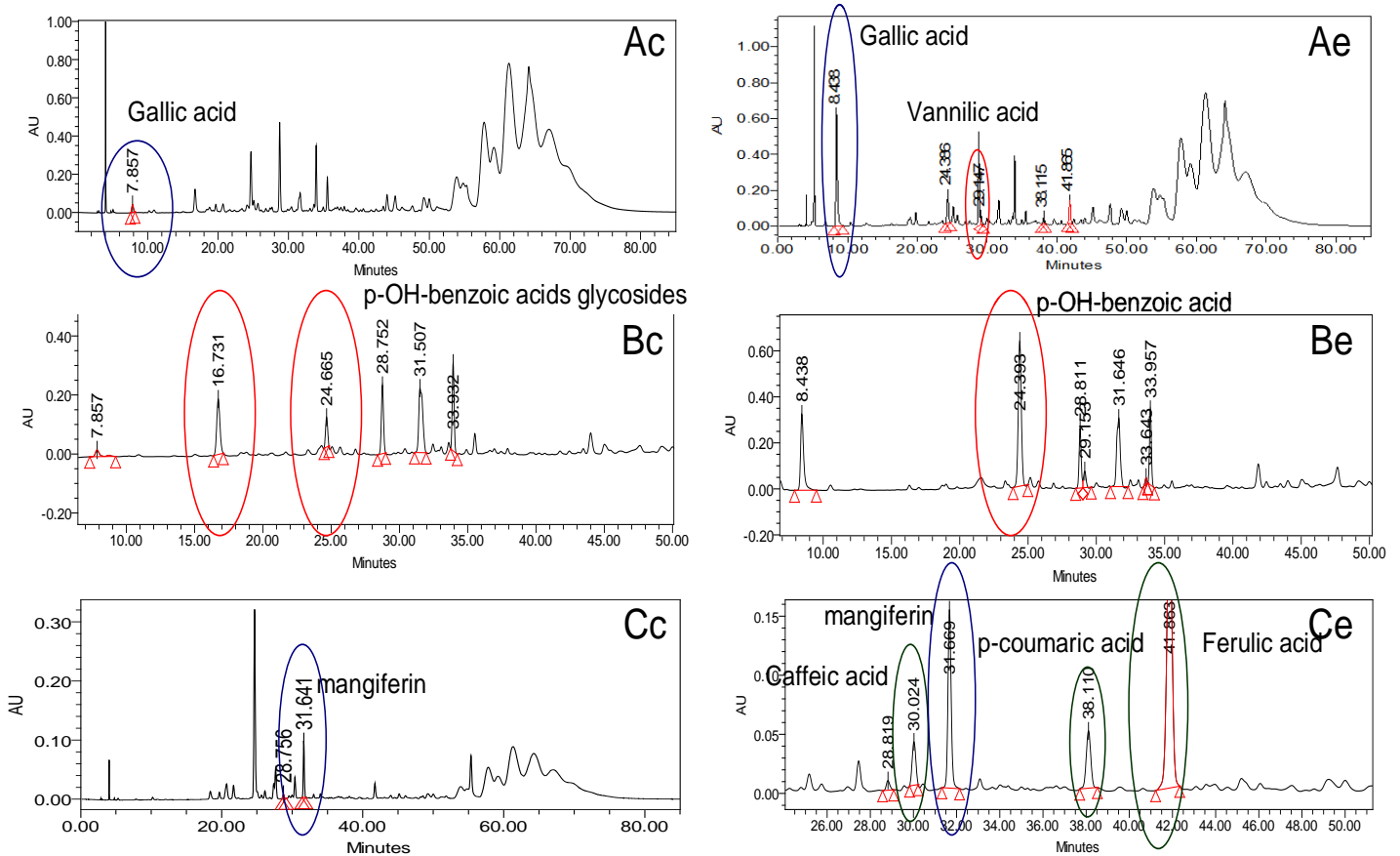
Phenolic compounds absorption by caco-2 cells monolayer

For both ataulfo extracts (control and enzyme treatment), high molecular weight compounds such as gallotannins, xanthone glycosides and flavonoid glycosides were not found in the basolateral side, which suggests these compounds were not absorbed intact by caco-2 cells. Also, mangiferin in its intact form was not found in the basolateral side after 2 hrs of incubation with both extracts. On the

order hand, all phenolic acids in their aglycone forms were absorbed. Caffeic acid absorption was detected by HPLC at 320 nm, even in low concentration (Figure 14).
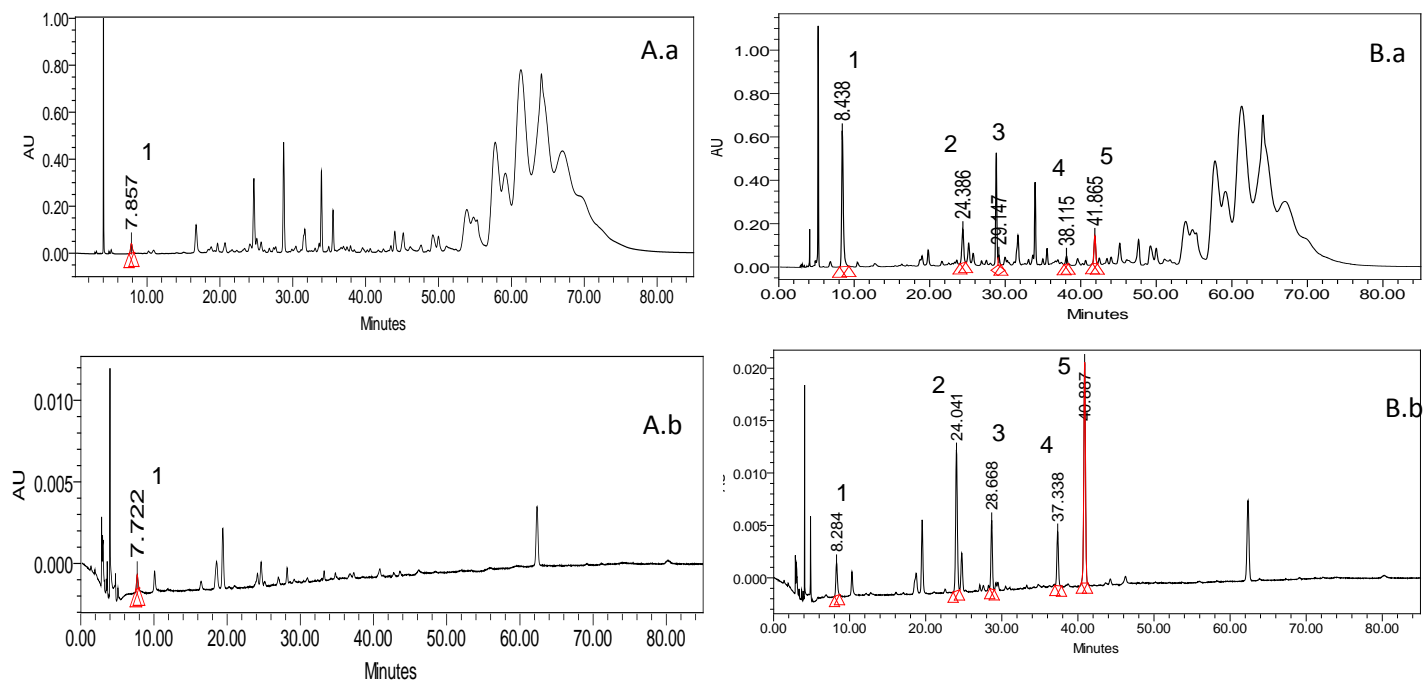
Figure 14. Chromatograms at 280 nm of Ataulfo phenolic extract without treatment (A) and hydrolyzed (B) dissolved in HBSS pH 6.0 (apical side) (a) or compounds analyzed in the basolateral side after 120 min of absorption (b) . (1) gallic acid; (2) p-hydroxybenzoic derivatives; (3) vanilic acid; (4) p-coumaric acid and (5) ferulic acid.

Conclusions

Among the five mango cultivars studied, Ataulfo and Haden were identified for their enhanced health benefits due to their antioxidant and anticancer activities. Polyphenolics identified in the edible part of these mango cultivars comprise a range of low molecular weight galloyl glycosides, flavanol glycosides, mangiferin, and high molecular weight gallotannins. The cancer growth-suppressive activity exerted by mango phytochemicals is attributed to the polyphenolics fraction, since the carotenoids fraction exerted a dose-response inhibitory effect at relatively high doses, not feasible to be achieved after mango consumption. The bioactive low molecular weight galloyl glycosides found in Ataulfo and Haden at concentrations available in physiological conditions preferentially inhibited the growth of the human SW-480 colon cancer cells over the other cancer cell lines studied and the non-cancer CCD-18Co colon cells. Moreover, at the same doses (5 mg GAE/L), Ataulfo inhibited the growth of SW-480 colon cancer cells by ~79%, without affecting the growth of the colonic myofibroblasts CCD-18Co cells.

The SW-480 gene trancription regulation induced by Ataulfo and Haden polyphenolics suggests induction of apoptosis through intrinsic and extrinsic mechanisms. The extrinsic pro-apoptotic pathway might be mediated by caspase 8, whereas the intrinsic pro-apoptotic pathway by Bax and Bim. Likewise cell cycle arrest in G2/M was related to upregulation of PKMYT1 gene expression, which has been reported to negatively regulate cell cycle G2/M transition. Finally, Ataulfo and Haden polyphenolics may exert protection during the initiation and promotion stages of carcinogenesis. At initiation, these compounds protected the non-cancer CCD-18Co colon cells by lowering the ROS generation that may cause DNA damage and mutation. During the promotion stage, they decreased the ROS signals needed to drive proliferation of tumor cells. When applied at higher concentrations that may resemble paharmacological doses, these polyphenolics might exert the ROS production on colon cancer cells over an established threshold, thus contributing to kill cancer cells selectively. These results have important clinical implications because polyphenolics found in mango are very likely to be available in the colon and metabolized by the gut microflora, thus releasing the active compounds in the target tissue. Overall, polyphenolics from mango have anti-cancer properties in several cell lines, with an overall efficacy comparable to pomegranate dietary hydrolysable tannins, with anticancer effects attributed to their ellagic acid units, which within a concentration of 1-30 μM GAE have also shown to induce to induce cell cycle arrest and apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway, with no effect on normal colon cells.

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