

Anticarcinogenic Effects of Polyphenolics from Mango (*Mangifera indica*) Varieties

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Many polyphenolics contained in mango have shown anticancer activity. The objective of this study was to compare the anticancer properties of polyphenolic extracts from several mango varieties (Francis, Kent, Ataulfo, Tommy Atkins, and Haden) in cancer cell lines, including Molt-4 leukemia, A-549 lung, MDA-MB-231 breast, LnCap prostate, and SW-480 colon cancer cells and the noncancer colon cell line CCD-18Co. Cell lines were incubated with Ataulfo and Haden extracts, selected on the basis of their superior antioxidant capacity compared to the other varieties, where SW-480 and MOLT-4 were statistically equally most sensitive to both cultivars followed by MDA-MB-231, A-549, and LnCap in order of decreasing efficacy as determined by cell counting. The efficacy of extracts from all mango varieties in the inhibition of cell growth was tested in SW-480 colon carcinoma cells, where Ataulfo and Haden demonstrated superior efficacy, followed by Kent, Francis, and Tommy Atkins. At 5 mg of GAE/L, Ataulfo inhibited the growth of colon SW-480 cancer cells by ~72% while the growth of noncancer colonic myofibroblast CCD-18Co cells was not inhibited. The growth inhibition exerted by Ataulfo and Haden polyphenolics in SW-480 was associated with an increased mRNA expression of pro-apoptotic biomarkers and cell cycle regulators, cell cycle arrest, and a decrease in the generation of reactive oxygen species. Overall, polyphenolics from several mango varieties exerted anticancer effects, where compounds from Haden and Ataulfo mango varieties possessed superior chemopreventive activity.

KEYWORDS: Mango varieties; *Mangifera indica*; cancer prevention; polyphenolics

INTRODUCTION

Consumer interest in mango fruits (*Mangifera indica*) and derived products has been increasing in recent years on the basis of olfactory properties and also a potential in the prevention of chronic diseases (1). Moreover, mango fruits have found their way into the unofficial classification as a so-called superfruit. Cancer is one of the leading chronic diseases and causes of death worldwide. Major types of cancer include lung, colorectal, breast, and prostate cancer. Colon cancer is the third most common cancer in the USA for both men and women, with an estimated 108 070 cases of colon and 40 740 cases of rectal cancer diagnosed in 2008. Overall mortality associated with colon cancer might be decreased by controlling risk factors. Among these risk factors, diet is one of the factors which may be modified through an increased intake of fruits and vegetables (2).

Plant polyphenolics are increasingly being considered as sources of natural colon cancer chemopreventive compounds on the basis of safety and efficacy assessments. The edible portion of mango contains polyphenolics and carotenoids that may protect against cancer. Polyphenolics identified in the edible part

of mango have been previously characterized and include flavonoids such as quercetin and kaempferol glycosides, phenolic acids, predominately gallic acid, galloyl glycosides, in part polymerized within a wide range of degree, and mangiferin (3). The absorption of gallotannins seems to be limited due to their high molecular weight, and they are likely to be present in the large intestine, where they may be protective in colon carcinogenesis.

Few studies have shown the anticancer activity of mango extracts, and they suggest cell line specificity; e.g., mango extracts were shown to protect against prostate cancer in in vitro and in vivo models (4) and to inhibit the cell cycle in the G(0)/G(1) phase of HL-60 cells (5). In contrast, mango extracts were not effective against the breast cancer MCF-7 cell line (6). In addition, some of the phytochemicals found in mango, i.e. the triterpenes lupeol and mangiferin, have been shown to exhibit anticancer activity. Mangiferin exerted a chemopreventive and chemotherapeutic action against lung carcinogenesis induced by benzo(a)pyrene in Swiss albino mice (7). The chemopreventive action of mangiferin may be related to the induction of permeability in mitochondria (8). The overall anticancer potential of mango polyphenolics may at least in part be based on antioxidant properties that may enhance cell defense capacity and modulate molecular pathways in target cells. These compounds may inhibit

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promotion and progression stages of cancer by interfering with cell cycle regulation, signal transduction pathways, transcription, and activating apoptosis (activation of pro-apoptotic genes and pro-apoptotic proteins) in neoplastic cells. The availability of mango polyphenolics may vary according to specific compounds: i.e., the most well absorbed are gallic acid, followed by catechins and quercetin glucosides (9). The least well absorbed might be the gallotannins, which in comparison to ellagitannins are not absorbed as such but are metabolized by the intestinal flora (10). Therefore, limited bioavailability of gallotannins might not represent a limitation in the exertion of possible health benefits, since large molecular compounds may have potential anticancer effects in the large intestine.

The objective of this study was to compare the cancer chemopreventive potential of polyphenolics extracted from different mango cultivars in several types of cancer, while potential molecular mechanisms involved in anticancer activities are also assessed for the most efficacious mango varieties.

MATERIALS AND METHODS

Plant Material. Commercial varieties of mango (*Mangifera indica* L.) were kindly donated by member growers of the National Mango Board. The varieties Francis (Haiti), Kent (Mexico), Ataulfo (Mexico), Tommy Atkins (Mexico), and Haden (Mexico) were obtained at a mature, green stage. Fruit were allowed to ripen at room temperature (23 °C) until subjectively determined to be a full ripe stage, as determined by a soft texture and aroma development. Fruit were manually peeled to remove skin and seeds; homogenized pulp was obtained from three or more fruits per variety, prior to storage at -80 °C until needed.

Extraction of Polyphenolics. Fruit pulp was thawed and homogenized in a ratio of 500 g of pulp to 1.5 L of a solvent mixture (ethanol/methanol/acetone, 1/1/1), filtered through cheesecloth followed by Whatman #1 filter paper. Only the weight of the thawed pulp was recorded. The solvents were removed under reduced pressure at 40 °C, and the aqueous residue was centrifuged at 2000g to remove insoluble precipitates. Polyphenolics were partitioned in a 20 cm³ Waters C18 cartridge (Waters Corporation, Milford, MA) previously conditioned with 50 mL of 100% methanol and 50 mL of nanopure water and eluted with 50 mL 100% methanol containing 0.01% HCl. Compounds not adsorbed to the cartridge were partitioned into ethyl acetate using a separatory funnel. The ethyl acetate phase was combined with the methanol eluate from the C18 cartridges, and the solvents were removed under reduced pressure (Figure 1). The residual water from the extracts was removed by lyophilization at -50 °C at 0.01 mBar of pressure (Labconco Corp., Kansas City, MO). The dried extracts were reconstituted in cell culture media and used for cell culture assays by applying different concentrations based on total phenolic content measured spectrophotometrically by the Folin-Ciocalteu assay against an external standard of gallic acid (11).

Antioxidant Capacity. The antioxidant capacity of the phenolics extracted from the different mango varieties was measured using the oxygen radical absorbance capacity (ORAC) assay as described by Ou et al. (12) with the use of fluorescein as the 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., Durham, NC) at 485 nm excitation and 520 nm emission. Each mango polyphenolic extract was diluted to up to 10 mg of gallic acid equivalents (GAE)/L in pH 7.0 phosphate buffer prior to pipetting into a 96-well microplate against a standard curve from 3.1 to 50 μM Trolox. Results were quantified in μmol of Trolox equivalents/100 g of pulp.

HPLC-DAD and HPLC-ESI/MSⁿ Analysis. Individual polyphenolics were characterized using a Waters Alliance 2690 (Milford, MA) HPLC system using a 4.6 × 250 mm Dionex Acclaim 120 C₁₈ column (5 μm) and a 4.6 mm × 20 mm guard column. A gradient elution program ran phase A (water and acetic acid; 98/2) and phase B (water, acetonitrile, and acetic acid; 68/30/2) at a flow rate of 0.8 mL/min with detection from 210 to 400 nm. The gradient initially ran 100% phase A for 3 min and changed from 100 to 70% in 17 min, 70 to 50% in 10 min, 50 to 30% in 20 min, 30 to 0% in 10 min, and then isocratic at 0% phase A for 5 min

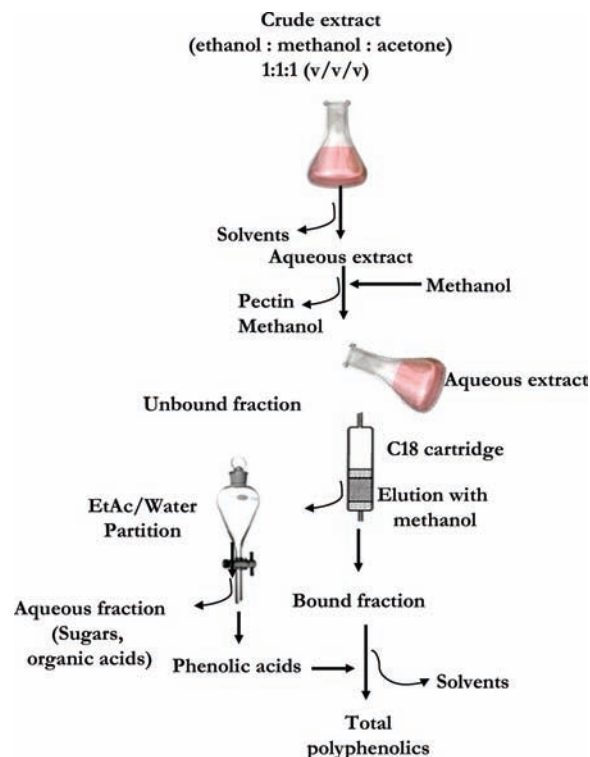


Figure 1. Overview of extraction method of total polyphenolics from mango.

prior to column re-equilibration. Identifications of polyphenolics were made by comparison of their retention times and UV spectra with those of authentic standards (Sigma-Aldrich, St. Louis, MO). Additional identification by mass spectrometric analyses was performed on a Thermo Finnigan LCQ Deca XP Max MSn ion trap mass spectrometer equipped with an ESI ion source (Thermo Fisher, San Jose, CA). Ionization was conducted in the negative ion mode under the following conditions: sheath gas (N₂), 40 units/min; auxiliary gas (N₂), 5 units/min; spray voltage, 5 kV; capillary temperature, 350 °C; tube lens offset, 10 V.

Cell Culture. Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured as recommended by the ATCC. Estrogen-independent MDA-MB-231 breast cancer cells were cultured using Dulbecco's modified Eagle's medium (DMEM) high glucose, with 2 mmol/L of L-glutamine, without sodium pyruvate and with phenol red. The colorectal SW-480 adenocarcinoma cells were cultured using Liebovitz's L-15 medium, with L-glutamine and 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 and the androgen receptor positive LnCap prostate cancer cells were cultured in RPMI-1640 medium. All culture media were supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotic mix. A 2.5% charcoal stripped FBS was used in culture media used for cell proliferation experiments. Culture mediums were supplied by Invitrogen (Gibco, Invitrogen Corp., Grand Island, NY). Cells were maintained at 37 °C with a humidified 5% CO₂ atmosphere. Cells were seeded for cell proliferation assay in 24-well plates (15 000 cells/well), for the ROS study in 96-well plates (10 000 cells/well), for the cell cycle kinetics in 12-well plates (70 000 cells/well), and for gene expression studies in 6-well plates (500 000 cells/well). Cells were grown for 24 h to allow cell attachment before exposure to varying concentrations of mango polyphenolics (mg of GAE/L) dissolved in culture media and sterile-filtered before use.

Generation of Reactive Oxygen Species (ROS). The production of ROS was analyzed as previously performed with some modifications (13). Briefly, cells were pretreated with mango polyphenolics over 24 h (from 1.5 to 10 mg of GAE/L) followed by 200 μM H₂O₂ for 2 h. After the residual H₂O₂ was washed, cells were exposed to 10 μM of 2',7'-dichlorofluorescein diacetate (H₂DCF-DA) at 37 °C and incubated for 30 min. The fluorescence signal was monitored at 520 nm emission and 480 nm excitation with

a FLUOstar Omega plate reader (BMG Labtech Inc., Durham, NC). Fluorescence intensity was used as an indicator of the generation of ROS.

Cell Proliferation. The cell proliferation assay was used because it is one of the most accurate and straightforward methods of quantifying viable cells and, therefore, the anticancer activity of mango polyphenolics that might result from a combination of growth suppression and apoptosis induction. Cell proliferation was assessed by using an electronic counter (Z2 Series, Beckman Coulter, Inc., Fullerton, CA), where the difference in cell number between final incubation time (72 h) and incubation start (0 h) represents net growth. The cell growth inhibition expressed as IC₅₀ is the concentration of total polyphenolics expressed in mg of GAE/L or μ M GAE/L which inhibited cell growth by 50%. Since we quantified total phenolics against a standard curve of gallic acid, the formula weight of gallic acid was used to convert mg of GAE/L to μ M GAE/L.

Cell Cycle Kinetics. Cells were treated with mango polyphenolics (5 and 10 mg of GAE/L) for 24 h and harvested for flow cytometer analysis. Briefly, cell pellets were obtained by trypsination and centrifugation and fixed with 90% ethanol at -20°C for 4 h. Cells were resuspended in staining solution (50 μ g/mL of propidium iodide, 30 units/mL of RNase, 40 mmol/L of sodium citrate, and Triton X-100 (pH 7.8)) and incubated at 37°C for 15 min. Stained cells were analyzed on a FACS Calibur Flow Cytometer (Becton Dickinson Immunocytometry Systems) using Cell Quest (Becton Dickinson Immunocytometry Systems) acquisition software.

Quantitative RT-PCR. Human SW-480 colon adenocarcinoma cells were pretreated with Ataulfo and Haden polyphenolics (2.5–10 mg of GAE/L) for 24 h. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA), and 470 ng of RNA was used to synthesize cDNA using a Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY). Real-time PCR reactions were performed using 2 μ L of cDNA and SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). Three independent cell culture replicates were analyzed for gene expression ($n=3$).

Optimal semiquantitative conditions were set to fall in the linear PCR product range (data not shown), and TBP (TATA-box binding protein) was used as internal control. The sequences of the primers (Integrated DNA Technologies, Coralville, IA) used were

Fas	F	5'-TGCCTCCTCTTGAGCAGTCA-3'
Fas	R	5'-TCCTGTAGAGGCTGAGGTGTC-3'
Caspase 8	F	5'-GGCTCCCCCTGCATCAC-3'
Caspase 8	R	5'-CCTGTAGATAAGGGCATGAATCT-3'
Bax	F	5'-CCAAGGTGCCGGAAGTGA-3'
Bax	R	5'-CCCGGAGGAAGTCCAATGT-3'
Bim	F	5'-TGCCAGGCCCTCAACCA-3'
Bim	R	5'-GTTTCAGCCTGCCTCATGGA-3'
PKMYT1	F	5'-CCTCTGCACTTTTAACTTTATCCT-3'
PKMYT1	R	5'-GCAGAGAAGACCATGGGAGTTC-3'
p21	F	5'-GAGCTCTGGGTGGTCATGGA-3'
p21	R	5'-ATCCTGGTGTGGGTGACGAT-3'
TBP	F	5'-TGCACAGGAGCCAAGAGTGA-3'
TBP	R	5'-CACATCACAGCTCCCAACCA-3'

Statistical Analysis. Quantitative data represent mean values with the respective standard deviation (SD) or standard error of the mean (SE) corresponding to three or more replicates. For chemical analysis, standard deviation values indicated variability of the assay performed out of a composite sample (number of fruits ≥ 3). In the cell culture assays, the SD or SE indicates the variability of the cell response when exposed to different concentrations of mango polyphenolics. Data were analyzed by one-way analysis of variance (ANOVA) using posthoc multiple comparisons Tukey's-b ($p < 0.05$ and $p < 0.01$) and Pearson correlations using SPSS version 12.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Total Polyphenolics and Antioxidant Activity of Mango Cultivars. Yields of polyphenolics extracted from different mango cultivars varied, with Ataulfo presenting the highest yield. The yields (mg of freeze-dried extract/g of mango pulp) are as follows:

Table 1. Total Polyphenolics and Antioxidant Activity (ORAC) of Different Mango Cultivars

mango cultivar	total polyphenolics ^a (mg of GAE/100 g of pulp)	antioxidant activity ^a (μ mol of TE/100 g of pulp)
Ataulfo	56.7 \pm 0.3a	326.6 \pm 4.8a
Haden	19.1 \pm 1.8b	225.8 \pm 0.6b
Francis	17.8 \pm 0.3b,c	219.0 \pm 1.0b
Kent	16.4 \pm 0.9c	150.0 \pm 0.9c
Tommy Atkins	15.3 \pm 1.6c	156.6 \pm 1.1c

^a Average of three or more independent determinations \pm SD. Different letters indicate significance at the $p < 0.05$ level.

11.2 \pm 0.2, 7.0 \pm 0.2, 6.7 \pm 0.4, 6.0 \pm 0.3, and 3.7 \pm 0.1 mg/g for Ataulfo, Francis, Kent, Tommy, and Haden, respectively. The total polyphenolic content of extracts prepared from mango pulps assessed by the Folin–Ciocalteu assay (Table 1) demonstrated that Ataulfo extracts also contain the highest amount of total phenolics normalized to gallic acid equivalents (GAE) (56.7 \pm 0.3 mg of GAE/100 g). Previous studies showed total phenolic content in mango pulp may range from 48.4 to 209 mg of GAE/100 g (14) and may be influenced by ripening, cultivar, environmental, and handling factors (15).

Antioxidant Capacity (AOX). As natural antioxidant compounds, mango polyphenolics may interrupt radical chain reactions by scavenging reactive oxygen species and free radicals. Our results show that Ataulfo followed by Haden extracts were the highest in ORAC values (326.6 \pm 4.8 and 225.8 \pm 0.6 μ mol of Trolox equivalents/100 g of mango pulp, respectively) (Table 1). The AOX of polyphenolics extracted from different mango cultivars correlated with their total phenolic content ($r = 0.91$). In this study, polyphenolic isolates did not contain ascorbic acid, soluble proteins, and reducing sugars. This might explain why a previous study reported higher AOX values for mango pulp extracts (326–1002 μ mol of TE/100 g with a slightly higher correlation between ORAC and total phenolic content ($r = 0.98$) (16).

HPLC-DAD and HPLC-ESI-MSⁿ Analysis of Ataulfo and Haden Polyphenolic Extracts. HPLC analysis of Ataulfo and Haden polyphenolic extracts confirmed the presence of free gallic acid and mangiferin (Figure 2). Both compounds were found in all cultivars, with the exception of Francis, which did not contain mangiferin. Mangiferin peaks are shown in insets A₁ and B₁ of Figure 2, with elution time at approximately 40 min and detection at 366 nm. Previous studies also confirmed our findings (15). Moreover, hydrolyzable tannins composed of gallic acid units attached to glucose via a glycosidic linkage (3) were structurally characterized by HPLC-ESI-MSⁿ analysis (Table 2). Galloyl glucosides identified by MS in Haden presented chemical structure ranging from 1 (mono-*O*-galloylglucoside) to 9 (nona-*O*-galloylglucoside) units; whereas the characterization of Ataulfo polyphenolics revealed the presence of higher molecular weights of gallotannins (molecular weights ranged from 5 (penta-*O*-galloylglucose) to 11 (undeca-*O*-galloylglucose). This pattern corresponds to compounds eluted after 60 min in HPLC spectra, with an elution pattern from lower to higher degrees of galloylation. Similarly, hydrolyzable tannins with 4–7 galloyl groups were previously identified in mango pulp from the cultivar Tommy Atkins (17), though the abundance of gallotannins of different degree of polymerization in mango pulp was not reported.

Cell-Growth-Suppressive Activity of Mango Polyphenolic Extracts. On the basis of their superior antioxidant capacity, Ataulfo and Haden were selected to assess the growth inhibition activity of their polyphenolics in different cancer cell lines (Figure 3). All

cell lines showed cell growth suppression in a dose-dependent manner when treated with Ataulfo and Haden extracts. The concentrations that inhibit growth by 50% (IC_{50} values, **Table 3**) show that Ataulfo polyphenolics suppressed the cancer cell growth in the following order of efficacy: SW-480 = Molt-4 =

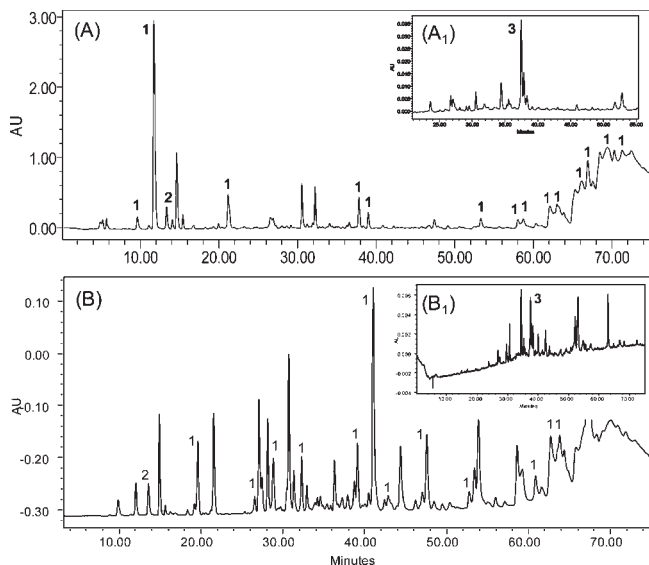


Figure 2. Representative chromatograms at 280 nm of Ataulfo (A) and Haden polyphenolics (B). Representative chromatograms at 366 nm of Ataulfo (A₁) and Haden (B₁): peak 1, galloyl glycosides; peak 2, gallic acid; peak 3, mangiferin.

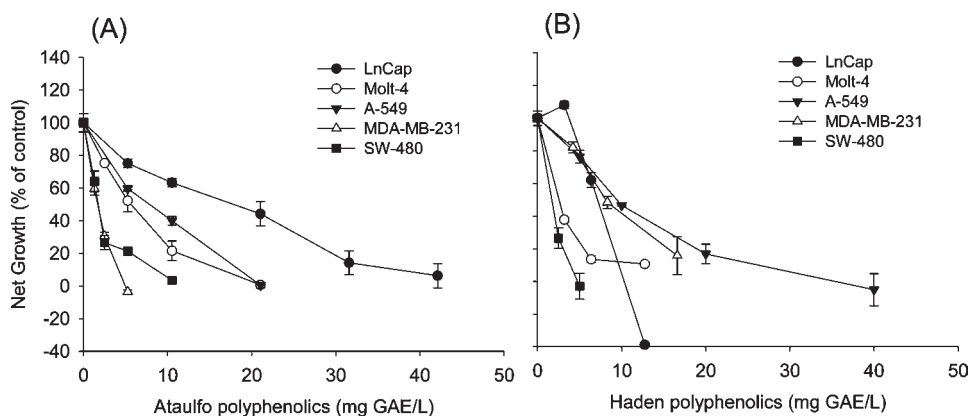


Figure 3. Cell proliferation of prostate LnCap, lung Molt-4, leukemia A-549, breast MDA-MB-231, and colon SW-480 cancer cells treated with Ataulfo (A) and Haden (B) polyphenolics. Cells were treated with different concentrations of extracts, and cell growth was assessed after 72 h of incubation. Values are means \pm SD, $n = 3$.

Table 2. Polyphenolics Profile from Mango Cultivars Ataulfo, Haden, Kent, Francis, and Tommy Atkins Determined by HPLC-ESI-MS^a

class	polyphenolics	Ataulfo	Haden	Kent	Francis	Tommy Atkins
phenolic acid	gallic acid	d	d	d	d	d
xanthone	mangiferin	d	d	d	nd	d
hydrolyzable tannins	mono- <i>O</i> -galloylglucose	d	d	d	d	d
	di- <i>O</i> -galloylglucose	nd	d	nd	nd	nd
	tetra- <i>O</i> -galloylglucose	nd	d	d	d	nd
	penta- <i>O</i> -galloylglucose	d	d	d	d	d
	hexa- <i>O</i> -galloylglucose	d	d	d	d	d
	hepta- <i>O</i> -galloylglucose	d	d	d	d	d
	octa- <i>O</i> -galloylglucose	d	d	d	d	d
	nona- <i>O</i> -galloylglucose	d	d	nd	d	d
	deca- <i>O</i> -galloylglucose	d	nd	nd	nd	nd
undeca- <i>O</i> -galloylglucose	d	nd	nd	nd	nd	

^a Legend: d, determined to be present; nd, not determined or not detected.

MDA-MB-231 > A-549 = Ln Cap within a range of 0–42 mg of GAE/L. Likewise, IC_{50} values (**Table 3**) indicate that human colorectal SW-480 carcinoma cells were inhibited by both Haden and Ataulfo polyphenolics to a similar extent. Overall, mango polyphenolics have a low bioavailability, in part due to the presence of large molecular weights of present polyphenolics (18). Thus, the chemopreventive protection of mango polyphenolics, specifically those with greater molecular weight, may be relevant to in vivo situations relevant to the prevention of colon cancer. These results suggest that Ataulfo and Haden cultivars had antiproliferative effects in all tested types of cancer cell lines, where the SW-480 colon cancer cell line seemed to be most susceptible to the treatment.

On the basis of their increased sensitivity to mango polyphenolics and the likely presence of mango polyphenolics in the colon, SW-480 cells were selected for the following analyses. Polyphenolics extracted from Ataulfo, Haden, Kent, Francis, and Tommy Atkins, in descending order of efficacy, induced a dose-dependent growth suppression in human SW-480 colon cancer cells (**Figure 4**). Among the five cultivars, Ataulfo and Haden polyphenolics showed the highest cell growth inhibition in SW-480. At 1.3 and 2.5 mg of GAE/L, Ataulfo polyphenolics suppressed the growth of SW-480 cells by 36% and 65% at 72 h of incubation, respectively. On incubation with 5 mg of GAE/L of Ataulfo polyphenolics, net cell growth was decreased by 22%, indicating that the number of cells at 72 h was lower than at the baseline (0 h), suggesting net cell killing (**Figure 4**). Polyphenolics from Haden reduced cell growth in a concentration-dependent manner, with 20%, 50%, and 90% of inhibition achieved at 1.3, 2.5, and 5 mg of GAE/L, respectively. The order of efficacy for the

Table 3. IC₅₀ Values of Polyphenolics Extracted from Ataulfo and Haden Mango Cultivars for Growth Suppression of Different Human Cell Lines^a

mango cultivar	IC ₅₀ (mg of GAE/L)					
	SW-480	MDA-MB-231	LnCap	A-549	Molt-4	CCD-18Co
Ataulfo	1.6 ^b ± 0.2b	1.2 ^b ± 0.5b	18.0 ± 0.0a	13.2 ± 5.5a	5.0 ^b ± 1.0b	>10 ^c
Haden	2.3 ^b ± 0.2b	8.3 ^b ± 0.4a	7.0 ± 0a	8.3 ± 1.8a	2.0 ^b ± 0.5b	ND

^a Different lower-case Roman letters indicate statistical significance at the level $p < 0.05$. ND = not determined. ^b Average of three independent determinations ± SD. ^c Average of two independent determinations.

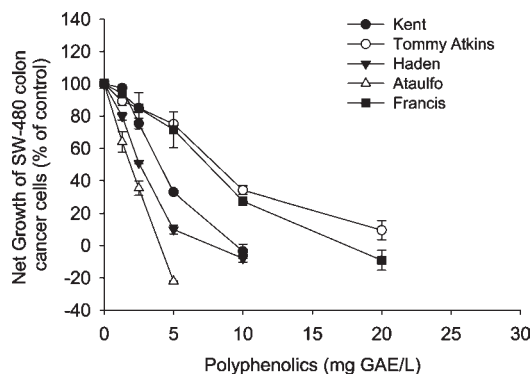


Figure 4. Representative evaluation of the concentration-dependent impact of polyphenolics extracted from Kent, Tommy Atkins, Haden, Ataulfo, and Francis mango cultivars on colon SW-480 cancer cells. Cells were incubated with extracts, and net growth was measured after 72 h of incubation. Values are mean ± SD, $n = 3$.

tested mango extracts was: Ataulfo = Haden ≥ Kent ≥ Francis > Tommy Atkins on the basis of their IC₅₀ values (Table 4).

A previous study reported the low cytotoxic efficacy of mango aqueous extracts in MCF-7 breast cancer cells in comparison to other plant-based foods consumed in Mexico (6). Polyphenolics in that study were extracted with water and did not contain water-insoluble compounds such as higher molecular weight gallotannins found in our study, as demonstrated by Percival et al. (5). As a comparison to another tannin-containing fruit, pomegranate juice contains ellagitannins, which are bioactive and have been shown to inhibit prostate cancer in vitro and in vivo (19). This effect was demonstrated in prostate cancer cells and in human prostate cancer xenografts and mediated, at least in part, through their Nuclear Factor Kappa-B inhibitory effects. Similar studies with hazel polyphenolic extracts (20) have shown that fractions with the highest percentage of galloylation were the most effective in inhibiting human HT-29 colon cancer cell proliferation, arresting the cell cycle in S phase, and inducing apoptosis. Apparently, the degree of galloylation may play a role in the cytotoxic effects in colon cancer cells (20), which may indicate that some of the anticancer effects may be influenced by the high degree of galloylation of mango polyphenolics.

In summary, SW-480 colon cancer cells were affected most by the treatment with the selected mango polyphenolics, and extracts from Ataulfo and Haden were assessed as being the most efficacious in the induction of cell death in comparison to other mango varieties.

Growth-Suppressive Activity of Ataulfo Polyphenolic Extract in Cancer Compared to Normal Cells. The growth inhibition effects of mango polyphenolics were investigated in noncancerous colonic myofibroblasts CCD-18Co cells compared to SW-480 cells. At 5 mg of GAE/L, Ataulfo polyphenolics suppressed the growth of SW-480 cells by ~79%, while the growth of CCD-18Co cells was not inhibited at the same concentration. Even at the highest concentration of Ataulfo polyphenolics (10 mg of GAE/L), the growth inhibition for CCD-18Co cells was only

Table 4. IC₅₀ Values of Polyphenolics Extracted from Mango Cultivars for Growth Suppression of Human SW-480 Colon Cancer Cells^a

mango cultivar	IC ₅₀ (mg GAE/L)	IC ₅₀ (μM GAE/L)
Ataulfo	1.6 ± 0.2a	10 ± 0a
Haden	2.3 ± 0.2a	13.52 ± 1.2a
Kent	5.0 ± 1.4a,b	29.4 ± 8.2a,b
Francis	8.2 ± 2.6b	48.2 ± 15b
Tommy Atkins	27.3 ± 0.4c	160 ± 2.35c

^a Different lower-case Roman letters indicate statistical significance at the level $p < 0.05$. ^b Average of three independent determinations ± SD. ^c Average of two independent determinations.

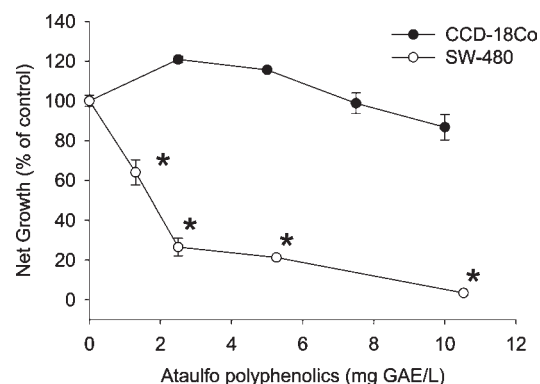


Figure 5. Concentration-dependent growth suppression of Ataulfo polyphenolics on the human SW-480 colon cancer cells and colonic myofibroblast CCD-18Co cells. Cells were incubated with extracts, and the net growth was measured after 48 h. Values are mean ± SD, $n = 3$. Asterisks indicate a significant difference compared to untreated control, $p \leq 0.01$.

13 ± 6% (Figure 5). Previous studies have reported that the growth-inhibitory effects of plant extracts were greater in colon cancer compared to nontumorigenic colon cells. In general, bioactive natural compounds which are growth suppressive in cancer cells but do not affect normal cells may have a potential in chemoprevention. These data indicate that polyphenolic mango extracts may have a potential in chemoprevention.

Gene Expression Regulation. The expression of genes involved in the regulation of apoptosis, referred to as “programmed cell death”, was investigated. Two pathways involved in the activation of caspases have been studied (21), one of them is the extrinsic death receptor pathway or Fas-mediated apoptosis induced by ligation of the Fas receptors. The Fas-receptor activation leads to activation of caspase-8, an initiator of the caspase cascade, resulting in proteolysis of cellular proteins and death by apoptosis (21). The other is the intrinsic or mitochondrial pathway mainly regulated by the Bcl-2 family members, which include pro-apoptotic and pro-survival proteins. Some pro-apoptotic proteins, such as Bax, form channels and contribute to regulate preexisting channels that permeabilize the outer mitochondrial membrane, which releases caspase-activating proteins into the cytosol, whereas others, such as Bim, neutralize certain pro-survival Bcl-2 proteins (22).

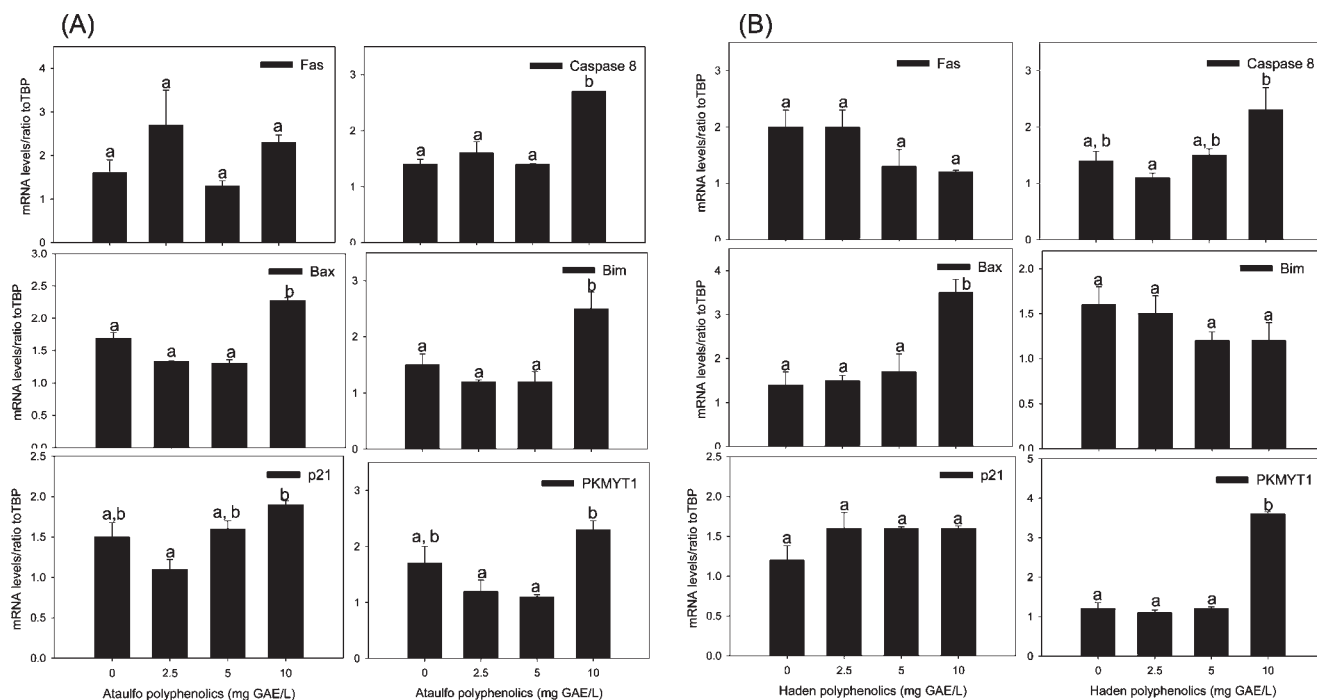


Figure 6. mRNA expression of SW-480 colon cancer cells treated with Ataulfo (A) and Haden (B) polyphenolics after 24 h and analyzed by real-time PCR as a ratio to TATA-binding protein (TBP) mRNA. Values are means \pm SE ($n = 3$). Different letters indicate significance at $p < 0.05$.

The effects of Haden and Ataulfo polyphenolics on the expression of genes involved in apoptosis were investigated within the concentration range 0–10 mg of GAE/L in SW-480 cells after 24 h of incubation. The gene expression response was achieved at higher doses of polyphenolics due to the shorter incubation time compared to doses required for cell growth inhibition in 72 h incubation.

Results indicate that both the extrinsic death receptor and the intrinsic mitochondria proapoptotic pathways were targeted at the transcriptional level by Ataulfo and Haden polyphenolics after 24 h of treatment (Figure 6). Caspase 8 gene expression was upregulated by Ataulfo at the highest dose (10 mg of GAE/L) by 2-fold, suggesting the contribution of the extrinsic pathway to the induction of apoptosis. However, the expression of Fas was not regulated within the time frame and concentration range used and/or might be being targeted at the post-translational level.

Ataulfo polyphenolics, when used at the highest concentrations, upregulated the transcription of mitochondria-related proapoptotic Bax and Bim by 1.3- and 1.7-fold, respectively, whereas Haden polyphenolics only increased Bax mRNA (2.6-fold), without any effect on Bim gene expression.

Natural plant extracts and phytochemicals appear to target the intrinsic mitochondrial pathway (23), and a cross-talk between the intrinsic and extrinsic pathways occurs. This is the first study reporting gene expression regulation of mango polyphenolics on colon SW-480 cancer cells. However, the main polyphenolics identified in mango, i.e. gallotannins, have been shown to regulate the pro-apoptotic and cell cycle related proteins on colon cancer cells (24).

In this study, the effects of mango polyphenolics on the expression of genes encoding the cell cycle regulator protein p21(Cip1/WAF1) (p21), which regulates the cell cycle in the G_0/G_1 phase, and the protein kinase membrane associated tyrosine/threonine 1 (PKMYT1), involved in the regulation of the G_2/M phase, were analyzed. Results showed that Ataulfo and Haden polyphenolics nonsignificantly increased the expression of

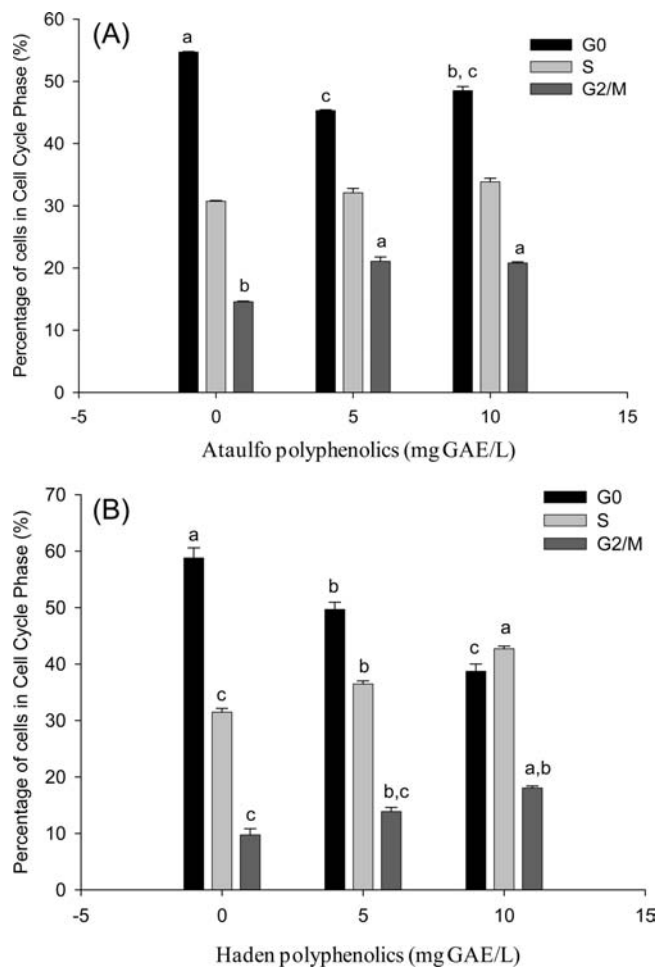


Figure 7. Cell cycle analysis of SW-480 colon cancer cells treated with Ataulfo and Haden polyphenolics for 24 h. Values are means \pm SE ($n = 3$); different letters indicate significance at $p < 0.05$.

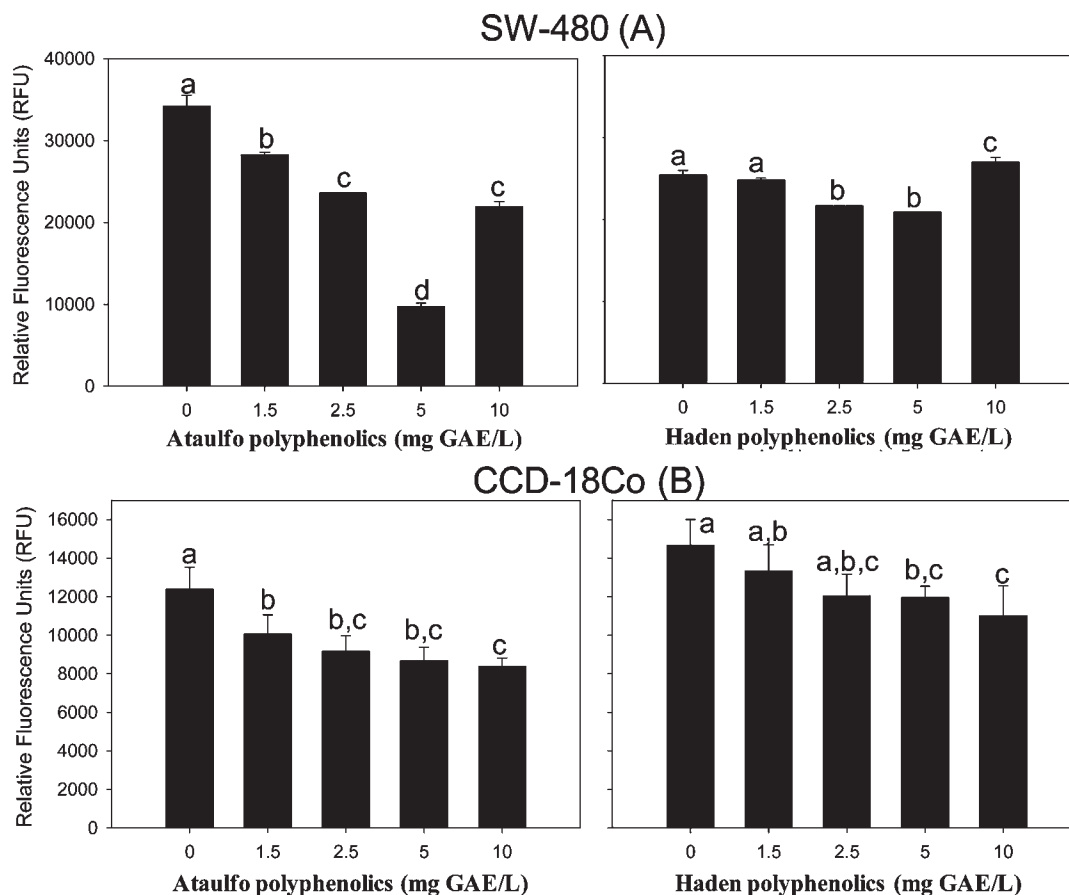


Figure 8. Generation of reactive oxygen species (ROS) after H_2O_2 -induced oxidative damage in SW-480 colon cancer cells (A) and noncancer colonic myofibroblasts CCD-18Co cells (B). Cells were pretreated with Ataulfo and Haden polyphenolics for 24 h before induction of ROS. Values are means \pm SD ($n = 6$); different letters indicate significance at $p < 0.05$.

p21 mRNA (Figure 6). Both Ataulfo and Haden polyphenolics upregulated PKMYT1 gene expression by 1.4- and 3-fold, respectively. The protein encoded by the PKMYT1 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 in G₂/M transition (25).

Individual phytochemicals found in mango have been reported to modulate genes involved in cell cycle regulation: i.e., penta-*O*-galloyl- β -D-glucose induced S- and G₁-cell cycle arrests in prostate cancer cells (26). As observed with many polyphenolics, the influence on gene expression by mango polyphenolics in SW-480 colon cancer cells indicates that growth inhibition is at least in part mediated by the regulation of pro-apoptotic genes and cell cycle control genes.

Cell Cycle Regulation. Ataulfo and Haden polyphenolic extracts induced G₂/M cycle arrest (Figure 7). Haden polyphenolics at 10 mg of GAE/L were more effective than Ataulfo in increasing the number of cells in the G₂/M phase by 1.9-fold. These results were accompanied by upregulation of PKMYT1 gene expression, a suppressor of G₂/M transition. Haden polyphenolics also induced cell cycle arrest in the S phase in a dose-dependent manner, whereas Ataulfo polyphenolics did not show significant effect at this phase. Previous studies demonstrated that gallic acid was able to interfere with the cell cycle in Caco2 colon cancer cells with an increased percentage of cells in the S and G₂/M phases (27). Similarly, grape polyphenolics with a high percentage of galloylation showed to be effective in arresting the cell cycle in the G₂/M phase and inducing apoptosis in HT29 human colon cancer cells (28). Overall, the cell cycle modulating properties of

gallic acid, galloylated derivatives and other polyphenolics in mango, which have previously been demonstrated, are likely to have contributed to the anticancer properties in SW-480 cancer cells.

Protective Effects against Reactive Oxygen Species (ROS). Oxidative damage by ROS seems to be a crucial event in the initiation of cancer; therefore, the potential of mango polyphenolics in protecting colon cells against reactive oxygen species (ROS) was investigated. Pretreatment of SW-480 cells with 1.5, 2.5, and 5 mg of GAE/L Ataulfo polyphenolics for 24 h significantly reduced the 200 μ M H_2O_2 induced ROS levels by ~17%, 31%, and 72%, respectively. Haden polyphenolics, when used at the same concentrations, were less effective in protecting against H_2O_2 -induced oxidative damage, with ~2.5%, 15%, and 18% reduction of ROS production, respectively, in comparison to untreated control. However, the dose-dependent protection of SW-480 against ROS production was reversed at the highest dose (10 mg GAE/L) by both Ataulfo and Haden polyphenolics (Figure 8A). Previous findings suggest that cancer cells use ROS signals to drive proliferation and other events required for tumor progression (29). Therefore, the ROS generation inhibition may contribute to slowing down the tumor progression. The observed increase of ROS in cancer cells caused by mango polyphenolics at higher concentrations may have been caused by an increased basal oxidative stress which may have driven them beyond a tumor-sustaining threshold, making them more sensitive to oxidative stress that finally ends in cell death (29).

In contrast, when the noncancer colonic myofibroblast CCD-18Co cells were pretreated with Ataulfo and Haden polyphenolics at the same concentration range (1.5–10 mg of GAE/L), a

protection against ROS production was achieved in a dose-dependent manner, including the highest concentration of 10 mg/L. Within 1.5–10 mg of GAE/L, Ataulfo and Haden reduced the ROS generation by 18–32% and 6–25%, respectively (Figure 8B). These results demonstrate that at concentrations which induce oxidative damage in cancer cells (10 mg of GAE/L), mango polyphenolics still protect the noncancer cells against ROS. Overall, Ataulfo polyphenolics were more potent compared to Haden in the protection of noncancer colon CCD-18Co cells against ROS. Several studies have previously demonstrated how natural compounds may target the production of ROS in their cytotoxic effects in cancer cells but not normal cells (30).

In conclusion, among five studied mango cultivars, Ataulfo and Haden were identified as the most efficacious in their anticancer effects. Polyphenolics identified in the edible part of these mango cultivars comprise a range of low-molecular-weight galloyl glycosides, flavonol glycosides, mangiferin, and higher molecular weight gallotannins. Ataulfo and Haden polyphenolics inhibited the growth of human SW-480 colon cancer cells, which were more sensitive to the treatment than the other cancer cell lines studied and noncancer CCD-18Co colon cells. SW-480 gene regulation influenced by Ataulfo and Haden polyphenolics suggested the induction of apoptosis through intrinsic and extrinsic mechanisms in addition to cell cycle arrest. Likewise, cell cycle arrest in the G₂/M phase was related to the upregulation of PKMYT1 gene expression. Ataulfo and Haden polyphenolics exerted protection of noncancer CCD-18Co colon cells by lowering the ROS generation in a dose-dependent manner. On the basis of their anticancer effects and protective effects in normal cells, mango polyphenolic extracts may have a high potential as chemopreventive agents.

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LITERATURE CITED

- Masibo, M.; He, Q. Major mango polyphenols and their potential significance to human health. *Compr. Rev. Food Sci. Food Saf.* **2008**, *7* (4), 309–319.
- American Cancer Society Cancer Facts & Figures, 2008; <http://www.cancer.org/downloads/STT/2008CAFFfinalsecured.pdf> (03/13/09).
- Barreto, J. C.; Trevisan, M. T.; Hull, W. E.; Erben, G.; de Brito, E. S.; Pfundstein, B.; Wurtele, G.; Spiegelhalter, B.; Owen, R. W. Characterization and quantitation of polyphenolic compounds in bark, kernel, leaves, and peel of mango (*Mangifera indica* L.). *J. Agric. Food Chem.* **2008**, *56* (14), 5599–5610.
- Prasad, S.; Kalra, N.; Shukla, Y. Induction of apoptosis by lutein and mango extract in mouse prostate and LNCaP cells. *Nutr. Cancer* **2008**, *60* (1), 120–130.
- Percival, S. S.; Talcott, S. T.; Chin, S. T.; Mallak, A. C.; Lounds-Singleton, A.; Pettit-Moore, J. Neoplastic transformation of BALB/3T3 cells and cell cycle of HL-60 cells are inhibited by mango (*Mangifera indica* L.) juice and mango juice extracts. *J. Nutr.* **2006**, *136* (5), 1300–1304.
- Garcia-Solis, P.; Yahia, E. M.; Morales-Tlalpan, V.; Diaz-Munoz, M. Screening of antiproliferative effect of aqueous extracts of plant foods consumed in Mexico on the breast cancer cell line MCF-7. *Int. J. Food Sci. Nutr.* **2009**, 1–15.
- Rajendran, P.; Ekambaram, G.; Sakthisekaran, D. Effect of mangiferin on benzo(a)pyrene induced lung carcinogenesis in experimental Swiss albino mice. *Nat. Prod. Res.* **2008**, *22* (8), 672–680.
- Pardo-Andreu, G. L.; Dorta, D. J.; Delgado, R.; Cavalheiro, R. A.; Santos, A. C.; Vercesi, A. E.; Curti, C. Vimang (*Mangifera indica* L. extract) induces permeability transition in isolated mitochondria, closely reproducing the effect of mangiferin, Vimang's main component. *Chem. Biol. Interact.* **2006**, *159* (2), 141–148.
- Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81* (1 Suppl), 230S–242S.
- Espin, J. C.; Gonzalez-Barrio, R.; Cerda, B.; Lopez-Bote, C.; Rey, A. I.; Tomas-Barberan, F. A. Iberian pig as a model to clarify obscure points in the bioavailability and metabolism of ellagitannins in humans. *J. Agric. Food Chem.* **2007**, *55* (25), 10476–10485.
- Swain, T.; Hillis, W. The phenolic constituents of *Prinus domestica*. I. The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* **1959**, *10*, 63–68.
- Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001**, *49* (10), 4619–4626.
- Meng, Q.; Velalar, C. N.; Ruan, R. Effects of epigallocatechin-3-gallate on mitochondrial integrity and antioxidative enzyme activity in the aging process of human fibroblast. *Free Radical Biol. Med.* **2008**, *44* (6), 1032–1041.
- Rocha Ribeiro, S. M.; Queiroz, J. H.; Lopes Ribeiro de Queiroz, M. E.; Campos, F. M.; Pinheiro Sant'ana, H. M. Antioxidant in mango (*Mangifera indica* L.) pulp. *Plant Foods Hum. Nutr.* **2007**, *62* (1), 13–17.
- Ribeiro, S. M. R.; Barbosa, L. C. A.; Queiroz, J. H.; Knodler, M.; Schieber, A. Phenolic compounds and antioxidant capacity of Brazilian mango (*Mangifera indica* L.) varieties. *Food Chem.* **2008**, *110* (3), 620–626.
- Kim, Y.; Brecht, J. K.; Talcott, S. T. Antioxidant phytochemical and fruit quality changes in mango (*Mangifera indica* L.) following hot water immersion and controlled atmosphere storage. *Food Chem.* **2007**, *105* (4), 1327–1334.
- Berardini, N.; Carle, R.; Schieber, A. Characterization of gallotannins and benzophenone derivatives from mango (*Mangifera indica* L. cv. 'Tommy Atkins') peels, pulp and kernels by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2004**, *18* (19), 2208–2216.
- Coates, E. M.; Popa, G.; Gill, C. I.; McCann, M. J.; McDougall, G. J.; Stewart, D.; Rowland, I. Colon-available raspberry polyphenols exhibit anti-cancer effects on in vitro models of colon cancer. *J. Carcinog.* **2007**, *6*, 4.
- Heber, D. Multitargeted therapy of cancer by ellagitannins. *Cancer Lett.* **2008**, *269* (2), 262–268.
- Lizarraga, D.; Tourino, S.; Reyes-Zurita, F. J.; de Kok, T. M.; van Delft, J. H.; Maas, L. M.; Briede, J. J.; Centelles, J. J.; Torres, J. L.; Cascante, M. Witch hazel (*Hamamelis virginiana*) fractions and the importance of gallate moieties—electron transfer capacities in their antitumoral properties. *J. Agric. Food Chem.* **2008**, *56* (24), 11675–11682.
- Vermeulen, K.; Van Bockstaele, D. R.; Berneman, Z. N. Apoptosis: mechanisms and relevance in cancer. *Ann. Hematol.* **2005**, *84* (10), 627–639.
- O'Connor, L.; Strasser, A.; O'Reilly, L. A.; Hausmann, G.; Adams, J. M.; Cory, S.; Huang, D. C. Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J.* **1998**, *17* (2), 384–395.
- Miura, T.; Chiba, M.; Kasai, K.; Nozaka, H.; Nakamura, T.; Shoji, T.; Kanda, T.; Ohtake, Y.; Sato, T. Apple procyanidins induce tumor-cell apoptosis through mitochondrial pathway activation of Caspase-3. *Carcinogenesis* **2008**, *29* (3), 585–593.
- Al-Ayyoubi, S.; Gali-Muhtasib, H. Differential apoptosis by gallotannin in human colon cancer cells with distinct p53 status. *Mol. Carcinog.* **2007**, *46* (3), 176–186.
- Liu, F.; Rothblum-Oviatt, C.; Ryan, C. E.; Piwnicka-Worms, H. Overproduction of human Myt1 kinase induces a G2 cell cycle delay by interfering with the intracellular trafficking of Cdc2-cyclin B1 complexes. *Mol. Cell. Biol.* **1999**, *19* (7), 5113–5123.
- Hu, H.; Zhang, J.; Lee, H. J.; Kim, S. H.; Lu, J. Penta-O-galloyl-beta-D-glucose induces S- and G(1)-cell cycle arrests in prostate cancer cells targeting DNA replication and cyclin D1. *Carcinogenesis* **2009**, *30* (5), 818–823.
- Salucci, M.; Stivala, L. A.; Maiani, G.; Bugianesi, R.; Vannini, V. Flavonoids uptake and their effect on cell cycle of human colon

- adenocarcinoma cells (Caco2). *Br. J. Cancer* **2002**, *86* (10), 1645–1651.
- (28) Lizarraga, D.; Lozano, C.; Briede, J. J.; van Delft, J. H.; Tourino, S.; Centelles, J. J.; Torres, J. L.; Cascante, M. The importance of polymerization and galloylation for the antiproliferative properties of procyanidin-rich natural extracts. *FEBS J.* **2007**, *274* (18), 4802–4811.
- (29) Schumacker, P. T. Reactive oxygen species in cancer cells: live by the sword, die by the sword. *Cancer Cell* **2006**, *10* (3), 175–176.
- (30) Wu, X. J.; Hua, X. Targeting R. O. S. selective killing of cancer cells by a cruciferous vegetable derived pro-oxidant compound. *Cancer Biol. Ther.* **2007**, *6* (5), 646–647.

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