

MICHELE CORRÊA BERTOLDI

ANTIOXIDANT CAPACITY, ANTICANCER EFFECTS AND ABSORPTION
OF MANGO (*Mangifera Indica* L.) POLYPHENOLS *IN VITRO*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 9 de dezembro de 2009.

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To my son Vitor, for his unconditional love.
To my husband Hernani, for his love, care and friendship.
To my family, for their constant support.

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BIOGRAPHICAL SKETCH

MICHELE CORRÊA BERTOLDI was born in September 12th, 1980, in Juiz de Fora, Minas Gerais, Brazil. She earned the degree of Food Engineering through Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil, in 2004. At the same year, she was admitted by the Graduate Program in Food Science and Technology at the Department of Food Technology of the Universidade Federal de Viçosa and received a fellowship of National Counsel of Technological and Scientific Development (CNPq). She completed her Master degree in Food Science and Technology with a minor in Chemistry, Physics, Physical chemistry and Biochemistry of Food, in 2006. Following that, she was admitted by the same Graduate Program at the Department of Food Technology of the Universidade Federal de Viçosa, and her studies were funded by the Foundation Coordination for the Improvement of Higher Education Personnel (CAPES). She received a fellowship of this Governmental Foundation to conduct part of her Doctoral studies at the Centeq Research Parkway and at the Department of Food Science and Nutrition of Texas A&M University, College Station, USA (2008-2009). In 2009, she pursued the degree of Doctor of Science.

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RESUMO

BERTOLDI, Michele Corrêa, D.Sc., Universidade Federal de Viçosa, dezembro, 2009.
Capacidade antioxidante, efeitos anticarcinogênicos e absorção de polifenóis de manga (*Mangifera indica* L.) *in vitro*. Orientador: Paulo Cesar Stringheta. Co-orientadores: Susanne U. Mertens-Talcott e Tânia Toledo de Oliveira.

Polifenóis presentes em polpa de manga, incluindo galotaninos, glicosídeos de flavonóides, ácido gálico, derivados da benzofenona, e mangiferina, têm demonstrado propriedades anticarcinogênicas. A etapa de deglicosilação por β -glicosidases tem se mostrado necessária ao metabolismo e à absorção de polifenóis derivados da dieta pelo organismo humano, o que poderia influenciar suas propriedades anticarcinogênicas. O objetivo deste estudo foi elucidar os efeitos anticarcinogênicos de polifenóis extraídos da polpa de manga de diferentes variedades (Francis, Kent, Ataulfo, Tommy Atkins e Haden) em diferentes tipos de câncer. Os efeitos antiproliferativos de polifenóis de manga foram estudados utilizando modelos *in vitro* de cultura de células cancerosas, incluindo as linhagens celulares de câncer humano Molt-4 (leucemia), A-549 (câncer de pulmão), MDA-MB-231 (câncer de mama), LnCap (câncer de próstata), SW-480 (câncer de cólon) e células de cólon não cancerosas CCD-18Co. Os mecanismos moleculares envolvidos nas propriedades anticarcinogênicas de polifenóis de manga foram investigados. O efeito do tratamento com polifenóis na expressão gênica, na regulação do ciclo celular e na produção de espécies reativas de oxigênio em células cancerosas de cólon humano SW-480 foi investigado por RT-PCR, citometria de fluxo e quantificação da intensidade de fluorescência, respectivamente. Além disso, o efeito da hidrólise de polifenóis de manga pela enzima β -glicosidase na atividade antioxidante, na supressão do crescimento tumoral e na absorção intestinal *in vitro* através da monocamada de células de adenocarcinoma de cólon humano Caco-2 foi avaliado. Ademais, o efeito antiproliferativo de frações fenólicas enriquecidas com polifenóis de baixo e elevado peso molecular em células cancerosas de cólon (SW-480) e mama (MDA-MB-231) foi estudado. Células cancerosas foram tratadas com extratos fenólicos das variedades Ataulfo e Haden, as quais foram selecionadas em razão da maior capacidade antioxidante quando comparada a outras variedades. Polifenóis de Ataulfo e Haden inibiram o crescimento de todas as linhagens celulares. SW-480 (câncer de cólon), MOLT-4 (leucemia) e MDA-MB-231 (câncer de mama) apresentaram

igualmente maior sensibilidade ao tratamento com polifenóis de Ataulfo, enquanto SW-480 e MOLT-4 mostraram-se mais sensíveis ao tratamento com polifenóis de Haden, segundo resultados obtidos por contagem de células. O efeito antiproliferativo dos extratos fenólicos de todas as variedades de manga foi avaliado em células cancerosas de colón humano (SW-480). As variedades Ataulfo e Haden demonstraram maior efeito supressor, seguidas de Kent, Francis e Tommy Atkins. Quando células cancerosas de colón SW-480 foram tratadas com 5 mg GAE/L de polifenóis de Ataulfo, o crescimento celular foi inibido em ~79%, enquanto a proliferação de miofibroblastos não cancerosos CCD-18Co não foi inibida. A supressão do crescimento celular pelo tratamento com polifenóis de Ataulfo e Haden em células de câncer de colón SW-480 foi associada com o aumento na expressão gênica de biomarcadores de apoptose (caspase 8, Bax e Bim) e reguladores do ciclo celular (PKMYT1), atraso do ciclo celular e alteração na produção de espécies reativas de oxigênio. Os extratos fenólicos da polpa de manga continham ácido gálico, mangiferina, derivados de ácidos fenólicos e galotaninos, os quais foram caracterizados por análises em HPLC-DAD e HPLC-ESI-MSⁿ antes e após a hidrólise enzimática (0.17 mg β-glicosidase 1000 KU/g polpa de manga / 4 h / 35°C). Ácidos fenólicos incluindo ácido gálico, caféico, ferúlico, *p*-coumárico e *p*-hidroxibenzóico consistiram os principais compostos derivados da hidrólise enzimática. Monocamadas de células Caco-2 foram incubadas por 2h no compartimento apical com extratos controle e hidrolisado. Quando incubadas com o extrato hidrolisado, ácido gálico, caféico, ferúlico, *p*-coumárico, vanílico e *p*-hidroxibenzóico foram detectados no compartimento basolateral, enquanto apenas ácido gálico foi detectado quando as células foram tratadas com o extrato controle. Polifenóis de elevado peso molecular, incluindo mangiferina e galotaninos, não foram transportados. Polifenóis de polpa (controle) de todas as variedades inibiram a proliferação de células humanas de câncer de colón HT-29 (0-27 µg de ácido gálico equiv./mL) e de mama MDA-MB-231 (0-24 µg GAE/mL) em até 99.8 e 89.9 %, respectivamente. Apesar da hidrólise enzimática ter aumentado a absorção de polifenóis de manga, não houve aumento significativo na atividade antioxidante, no conteúdo fenólico e na supressão do crescimento de células cancerosas de colón e mama. Ademais, ambas as frações fenólicas enriquecidas com polifenóis de baixo (138-194 Da) e elevado (422; 788-1852 Da) peso molecular inibiram o crescimento de células cancerosas de colón e mama na mesma extensão (0-

20 µg GAE/mL), o que poderia indicar que a atividade anticarcinogênica de polifenóis de manga seria independente da hidrólise enzimática. Estes resultados corroboram resultados obtidos em estudos *in vivo*, que sugerem que a grande parte dos polifenóis não seriam absorvidos em sua forma intacta através do intestino delgado, mas poderiam ser hidrolisados por enzimas intestinais em ácidos aromáticos de baixo peso molecular, os quais seriam posteriormente absorvidos; ou ainda, quando não absorvidos, poderiam alcançar o intestino grosso, modulando a microflora intestinal e, desta forma, contribuiriam para reduzir o risco de câncer de cólon. Desta forma, polifenóis de polpa de manga de diferentes variedades exibiram efeitos anticarcinogênicos em modelos de cultura de células, os quais poderiam não ser necessariamente dependentes da hidrólise enzimática pela β -glicosidase.

ABSTRACT

BERTOLDI, Michele Corrêa, D.Sc., Universidade Federal de Viçosa, December, 2009.
Antioxidant capacity, anticancer effects and absorption of mango (*Mangifera indica* L.) polyphenols *in vitro*. Adviser: Paulo Cesar Stringheta. Co-advisers: Susanne U. Mertens-Talcott and Tânia Toledo de Oliveira.

Polyphenols found in mango pulp, including gallotannins, flavonol glycosides, gallic acid, benzophenone derivatives and mangiferin have shown anticancer activity. Biological activities of polyphenols have been related to their bioavailability. Deglycosylation by β -glucosidases is a critical step in the metabolism and absorption of dietary polyphenols in humans, which might influence their anticancer properties. The objective of this study was to elucidate the anti-cancer effects of mango polyphenols of several varieties (Francis, Kent, Ataulfo, Tommy Atkins and Haden) in different types of cancer. The antiproliferative effects of mango polyphenols were studied *in vitro* using different cancer cell lines including Molt-4 leukemia, A-549 lung, MDA-MB-231 breast, LnCap prostate, SW-480 colon cancer cells and the non-cancer colon cell line CCD-18Co. Molecular mechanisms involved on the anti-cancer activities of mango polyphenols were assessed. The effect of mango polyphenols on gene expression, cell cycle regulation and reactive oxygen species production on colon cancer cells SW-480 were investigated by RT-PCR, flow cytometry and fluorescence intensity measurement, respectively. The effect of the hydrolysis of mango polyphenols with β -glucosidase on their antioxidant activity, cancer cell-growth suppression activity and *in vitro* intestinal absorption through human colon adenocarcinoma Caco-2 cell monolayers was evaluated. In addition, the antiproliferative effect of high and low molecular weight polyphenols rich-fractions on colon (SW-480) and breast (MDA-MB-231) cancer cells was studied. Cell lines were incubated with Ataulfo and Haden phenolic extracts, which were selected based on their superior antioxidant capacity compared to the other varieties. Ataulfo and Haden polyphenols inhibited the growth of all human cancer cell lines. SW-480 (colon cancer), MOLT-4 (leukemia) and MDA-MB-231 (breast-cancer) were statistically equally most sensitive to Ataulfo, whereas SW-480 and MOLT-4 were the most sensitive cell lines to Haden, as determined by cell counting. The efficacy of phenolic extracts from all mango varieties in inhibiting cell growth was tested on SW-480 colon carcinoma cells. Ataulfo and Haden demonstrated superior efficacy, followed

by Kent, Francis and Tommy Atkins. At 5 mg GAE/L, Ataulfo inhibited the growth of colon SW-480 cancer cells by ~79% while the growth of non-cancer colonic myofibroblasts CCD-18Co cells was not inhibited. The growth inhibition exerted by Ataulfo and Haden polyphenols on SW-480 cells was associated with an increased mRNA expression of pro-apoptotic biomarkers (caspase 8, Bax and Bim) and cell cycle regulators (PKMYT1), cell cycle arrest and an alteration in the generation of reactive oxygen species. Phenolic extracts from mango pulp contained gallic acid, mangiferin, phenolic acid derivatives and gallotannins, which were characterized by HPLC-DAD and HPLC-ESI-MSⁿ analysis before and after enzymatic hydrolysis (0.17 mg β -glucosidase 1000 KU/g mango pulp/ 4 h / 35°C). Phenolic acids including gallic, caffeic, ferulic, *p*-coumaric and *p*-hydroxybenzoic acids consisted the main compounds derived from enzymatic hydrolysis. Caco-2 cell monolayers were incubated for 2h on the apical side with hydrolyzed and non-hydrolyzed mango extracts. Gallic, caffeic, ferulic, *p*-coumaric, vanillic and *p*-hydroxybenzoic acids were detected on the basolateral side for hydrolyzed extract but only gallic acid was detected for the non-hydrolyzed extract. High molecular weight polyphenols, mangiferin and gallotannins, were not transported. Mango pulp polyphenols (control) from all varieties inhibited the proliferation of HT-29 colon (0-27 μ g of gallic acid equiv/mL) and MDA-MB-231 breast (0-24 μ g GAE/ mL) human cancer cells by up to 99.8 and 89.9 %, respectively. Despite enhanced absorption facilitated by enzymatic hydrolysis, a significant increase in antioxidant activity, phenolic content and antiproliferative effects on breast and colon cancer cells was not observed. Additionally, both high (422; 788-1852 Da) and low (138-194 Da) molecular weight polyphenols rich-fractions equally inhibited cell proliferation of colon and breast cancer cells at the same extent (0-20 μ g of gallic acid equiv/mL), which may indicate that the anti-cancer efficacy of mango polyphenolics is not dependent on enzymatic hydrolysis. These results corroborate previous findings from *in vivo* studies, which suggest that the most of mango polyphenols are not absorbed intact through the small intestine, but may be hydrolyzed by intestinal enzymes into low molecular weight aromatic acids, which would be later absorbed; or when polyphenols are not absorbed, they likely reach the large intestine, modulating the gut microflora, and thus they contribute to reduce the risk of colon carcinogenesis.

Overall, polyphenols from several mango varieties exerted anti-cancer effects, and these effects may not require enzymatic hydrolysis by β -glucosidase.

INTRODUCTION

Carcinogenesis

Cancer is one of the leading chronic diseases and causes of death worldwide (1). In 2008, 12 million of new cases of cancer and around 7 million of deaths were estimated, with the highest incidence for lung, breast and colon cancer (1). Colon cancer is the third most common cancer in USA for both men and women, with 108,070 estimated cases of colon and 40,740 cases of rectal cancer diagnosed in 2008 (2). According to INCA (National Institute of Cancer) (3), the occurrence of 489.270 new cases of cancer is expected to 2010-2011 in Brazil, with the highest incidence for skin (114,000 cases), prostate (52,000), breast (18,000) and colon (28,000).

Neoplasia consists in an abnormal, uncontrolled and exaggerated proliferation of cells as a result of damage in mechanisms of cell cycle regulation or alteration in genes that regulate the growth and differentiation of cells, which may be benign or malignant (4-5). Cancer is a term used for diseases in which abnormal cells divide without control and are able to spread through the blood and lymph systems and invade others tissues in the body. The process is initially characterized in cell mutation (initiation) as a result of a change in the genetic material of the cell primes. This alteration may occur spontaneously or by an agent that causes cancer (carcinogen). Development of cancer includes invasion, which refers to the direct migration and penetration by cancer cells into neighboring tissues, and metastasis, which is the ability of cancer cells to penetrate into lymphatic and blood vessels, circulate through the bloodstream, and then invade normal tissues elsewhere in the body. Cancerous cells present uncontrolled growth, evasion of apoptosis, self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, limitless replicative potential, sustained angiogenesis, invasion of adjacent tissues, and sometimes metastasis, process by which cells spread to other locations in the body via lymph or blood. These malignant properties are hallmarks of cancer and differentiate cancer cells from benign tumors, which are self-limited, and do not invade or metastasize (6-10).

The inadequate functioning of genes which regulate the proliferation of transformed cells is a major factor in neoplasia. Cancer formation is a multi-step

process that may involve the sequential activation of oncogenes as well as the inactivation of tumor suppressor genes often in the same clone of cells. These genetic changes cause phenotypic alterations in tumor cells that allow them to continue to survive and expand. Both tumor suppressor genes and oncogenes are responsible for proliferation control or differentiation after mutation, which may result in an overexpression of proteins and tumor formation (11). In normal cells, oncogenes often are underexpressed or inactivated (12). Cancer cells are generated, when DNA is damaged, and cells do not undergo apoptosis but instead oncogenes are activated to prevent cell death, resulting in continued and disorderly proliferation (11). Tumor suppressor genes (anti-oncogenes) normally function to limit cell proliferation. Their loss of function facilitates cancer development, usually in combination with other genetic changes (13). Tumor suppressor genes, or more precisely, the proteins they encode, either have a dampening or repressive effect on the regulation of the cell cycle or promote apoptosis, and sometimes both. The products of oncogenes and tumor suppressor genes have therefore become an important target for new anti-cancer drugs. Thus, knowledge regarding the cancer molecular biology, following the identification and functional characterization of many oncogenes and tumor suppressor genes are critical for therapeutic approach in targeting and eliminating cancer cells (11, 14).

Cell culture models have been extensively used as *in vitro* trials to evaluate the biological activities of several drugs and natural compounds, including polyphenols (15-19). Because the great availability of different types of human cancer cell lines, including colon, prostate, breast, and so on (20), the use of cell culture models permits evaluation of the potential anticancer effects with higher specificity, reduced cost, and in a shorter period of time.

Polyphenols in cancer prevention

Clinical and epidemiological studies have been related the fruit and vegetable consumption to the reduced risks of several chronic diseases, including cancer. Therefore, the risk of mortality associated with cancer might be reduced by diet modification (21-23). The health benefits ascribed to fruit and vegetables are mainly related to their bioactive compounds, which includes vitamins C and E, tocopherol,

beta-carotene, and phenolic compounds, which comprise the major antioxidant compounds derived from diet (24).

Phenolic compounds include a complex mixture of secondary plant metabolites, with high diversity in chemical structure and reactivity. Chemically, they are composed by aromatic rings with one or more hydroxyl groups, including their functional derivatives (25). Currently, more than 8000 polyphenols have been identified (17), including phenolic acids, flavonoids, lignans, stilbenes, coumarins and tannins, and their consumption has been estimated in 1 g/day (26).

Polyphenols are associated not only with color and sensory properties, but also are increasingly being considered as natural cancer chemopreventive compounds based on safety and efficacy assessments (27-29). These compounds may prevent cancer by interfering in enzymatic expression (30), improving intestinal health (31), reducing oxidative stress (32-33), interfering in synthesis and repair of DNA (34), as well as inducing apoptosis (35).

Although many studies have been shown *in vitro* and *in vivo* cancer-inhibitory activities from individual compounds, the combination of a variety of phytochemicals in fruits and vegetables may be increased in cancer prevention due to synergistic effects (16, 35-36). Therefore, studies with complex mixtures of these compounds might represent the health-benefits derived from food consumption.

Bioactive effects of mango phytochemicals

Mangoes (*Mangifera indica* L.) are among the most important tropical fruits marked in the world, with a global production exceeding 33 million tons in 2007. Moreover, mangoes consist one of the most consumed fruits in Brazil due to sensorial characteristics and nutritional value, ranking the seventh position worldwide in terms of production in 2007 (37).

Consumer interest in mango fruits (*Mangifera indica*) and derived products has been increasing in recent years based on fruit olfactory properties, attractive color, nutritional content, and health-promoting phytochemicals, which confer them great potential in preventing chronic diseases (38). The consumption of mangoes in Brazil is characteristic of high-volume producer countries, and has been estimated to be 2.8

kg/person/year (39). Furthermore, there has been increasing interest in understanding the mango phytochemicals biological properties due to their health-promoting characteristics such as antioxidant, antitumoral, anti-inflammatory, and immunomodulatory activities (40-45). Recently, mango fruit has been listed as a nutrient-rich fruit into the unofficial classification and so-called superfruit due to its considerable content of carotenoids, specially β -carotene, vitamin C and polyphenols, including gallic acid, gallotannins, quercetin and kaempferol glycosides as well as xanthone-C-glucosides (46-48). Gallotannins (Figure 1) represent the major high molecular weight polyphenols found in mango pulp, with molecular weights ranging from 332 (mono-*O*-galloyl-glucose) to over 1852 Da (undeca-*O*-galloyl-glucose) (47).

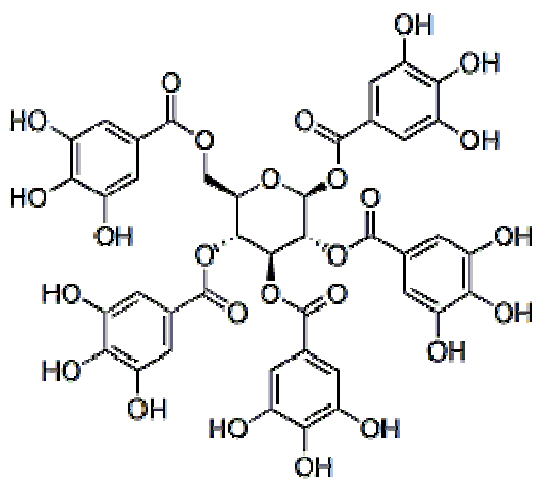


Figure 1. Chemical structure of 1,2,3,4,6-penta-*O*-galloyl- β -D-glucose (PGG), the precursor of gallotannins.

In traditional medicine, the use of mango extracts as herbal drugs is widespread. *Vimang*, a mango stem bark extract, have been used at least 10 years in Cuban medicine with effectiveness against several diseases, like cancer (45). Moreover, studies *in vitro* and *in vivo* performed with individual polyphenols found in mangoes have been shown antitumor activities, such as induction of apoptosis, inhibition of tumor growth and angiogenesis (49-54). Mangiferin has shown radioprotective (55-56), antioxidant, antiviral, anti-inflammatory (57) and anticarcinogenic effects (58). This xanthone

demonstrated chemopreventive action against lung carcinogenesis induced by benzo(a)pyrene in Swiss albino mice (59), which might be related to induction of mitochondria permeability (60). Lupeol and related terpenoids exerted varied cytotoxicity against several cancer cell types, including B16 2F2 mouse melanoma, leukemia HL60, U937, K562 melanoma G36, human lung carcinoma (A-549) and human colon adenocarcinoma (DLD-1) (61). Gallic acid showed to inhibit human cancer cell lines including esophageal, gastric, breast, cervix and colon cancer cells HT-29, Colo201 and colon 26 (mouse colon cancer). In esophageal cancer cells, apoptosis-related molecular mechanism included up-regulation of the pro-apoptotic protein Bax, PARP cleavage induction and caspase-cascade activity, down-regulation of antiapoptotic proteins Bcl-2 and Xiap and the survival Akt/mTOR pathway (62). The effect of gallic acid and its alkyl esters is known to induce cell death or cell cycle arrest in a variety of cancer cells, including colon cancer (63). This phenolic acid (100µM) significantly increased the number of cells in G2/M phase, with a consequent reduction of cells in G1 and S phases on human colon adenocarcinoma cells (Caco2) (64). Additionally, several studies have been shown the anticancer-related activities of gallotannins. 1,2,3,4,6-penta-O-galloyl-beta-d-glucose (PGG), which is the precursor of gallotannins, showed to induce cell cycle arrest, cell proliferation and apoptosis in a cell type-dependent manner (51-53), and to suppress tumor growth *in vivo* via angiogenesis inhibition and stimulation of apoptosis (65).

Recently, studies performed with mango pulp extracts have been demonstrated their chemopreventive potential (66-70), although conclusion regarding the biological activities of the mango phenolic fraction is still not so clear (67-68), since other bioactive compounds like L-ascorbic acid and carotenoids have been conjunctly evaluated (66-68). Studies performed with mango extracts have suggested cell line specificity; e.g. mango extracts have been shown to protect against prostate cancer in *in vitro* and *in vivo* models (69) and to inhibit the cell cycle in the G(0)/G(1) phase of HL-60 cells (66), although their lack of effectiveness against MCF-7 breast cancer cells has also been reported (67). Moreover, anticancer effects of mango phytochemicals may vary according to the variety as a result of changes in mango composition. It was demonstrated the superior phenolic content and antioxidant activity *in vitro* of mango pulp Ubá as compared to the varieties Palmer, Haden and Tommy Atkins (71).

The overall anti-cancer potential of mango polyphenols might be in part associated to their antioxidant properties (72), which may contribute to enhance cell defense capacity and modulate molecular pathways in target cells. These compounds might also inhibit promotion and progression stages of cancer by interfering in cell cycle regulation, signal transduction pathways, transcription and activating apoptosis (activation of pro-apoptotic genes and pro-apoptotic proteins) in neoplastic cells (73).

Oxidative stress and cancer

Free radicals are highly reactive chemical species which contains one or more unpaired electrons. These species comprise reactive oxygen species (ROS) and reactive nitrogen species (RNS), which may attack molecules *in vivo* including proteins, lipids and DNA (74). Superoxide, hydroxyl radical, hydrogen peroxide, hypochlorite and singlet oxygen includes the most important reactive oxygen species (75), whereas reactive nitrogen species constitute the molecules derived from nitric oxide (76).

Reactive oxygen species are produced as natural byproducts of oxygen reduction during the normal metabolism and play an important role in cell signaling (77-78). However, during times of environmental oxidative stress, ROS may cumulated and result in significant damage to cell structures (DNA, RNA and proteins), which is caused by an imbalance between the production of ROS and a biological system's ability to readily detoxify the reactive intermediates or repair the resulting damage. ROS can potentially cause DNA damage and promote tumor progression. Thus, oxidative stress has been considered a hallmark of many tumors (79). In addition to causing genomic instability, ROS are know to increase tumorigenesis by activating signaling pathways that regulate cellular proliferation, angiogenesis, and metastasis (78, 80-81). Low or transient levels of ROS can activate cellular proliferation or survival signaling pathways, while high levels of ROS can initiate damage or cell death (78).

Polyphenols are known to present anticancer activity due to their ability to modulate expression/activity of antioxidative and phase II drug-metabolizing enzymes as well as scavenging free radicals (82-84). In cancer cells, these bioactive compounds may induce cell cycle arrest and apoptosis by causing generation of ROS to trigger

signal transduction (72). Interestingly, the induction in ROS generation by some dietary anticancer agents is tumor cell specific, which may not occur in normal cells (85-87).

Bioavailability of polyphenols

Polyphenols are extensively metabolized either in tissues, once they are absorbed by small intestine, or by the colonic microflora, that transform the non-absorbed fraction or the fraction re-excreted in the bile (Figure 2). Once absorbed, polyphenols are conjugated to form *O*-glucuronides, sulphate esters and *O*-methyl ether in the small intestine and later in the liver, and no free aglycones are found in the plasma. Conjugation (methylation, sulfation and glucuronidation) reduces polyphenols potential toxicity, enhances their hidrophilicity and facilitates their biliary and urinary elimination. Trough enterophatic recirculation, conjugated compounds are excreted by the liver as components of bile into the intestine, while deconjugated compounds are transformed by microbial enzymes before being absorbed (31, 88-90).

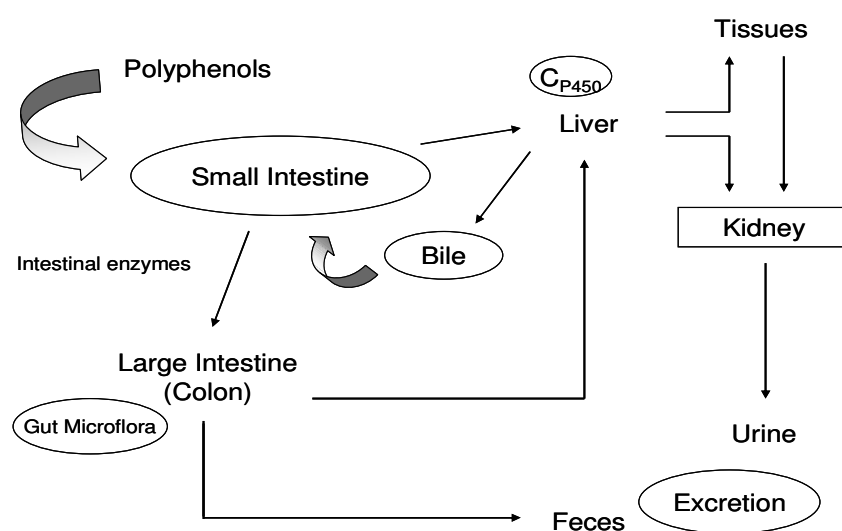


Figure 2. Routes of absorption and metabolism of dietary polyphenols in human body.

Most of polyphenols are found in food in the form of esters, glycosides, or polymers that cannot directly be absorbed in their intact form through small intestine.

Thus, exogenous (luminal) deglycosylation by several β -glucosidases in small intestine, or coordination between epithelial transporters and intracellular β -glucosidases facilitate the absorption of some phenolic compounds such as flavonoid glycosides (90-91). Because only aglycones and some glucosides can be absorbed in the small intestine, most of dietary polyphenols need to be firstly transformed in the colon by intestinal microflora before their metabolites may be absorbed (31, 88). Thus, gut bacteria can hydrolyze glycosides, glucuronides, sulfates, amides, esters and lactones. They also are responsible for reduction, decarboxilation, ring-cleavage, demethylation, and dehydroxylation reactions. However, when the microflora is involved, the efficiency of absorption is often reduced because the flora also degrades the aglycones into simple aromatic acids. In addition, polyphenols, which present antimicrobial activities, can interact specifically in a structure-dependent way with certain types of microorganisms in the gut, also modulating the microbial population of the gastrointestinal tract. This has effects on gastrointestinal health as well as in polyphenolic metabolism (92-93). Therefore, the biotransformation by gut bacteria may result in metabolites with higher or lower biological activity than the parent compounds (94-95).

Structural parameters of polyphenols including molecular weight, glycosylation and esterification have a major impact on intestinal absorption (96-97). According to the Lipinski's Rule of 5 (98), compounds with five or more hydrogen bond donors (OH and NH groups), ten or more hydrogen bond acceptors (N and O), molecular weight higher than 500, and Log P greater than 5 are usually poorly absorbed following oral administration. In general, low molecular weight phenolics such as caffeic acid are directly absorbed through the gut barrier by passive diffusion, whereas high molecular weight polyphenols such as flavonoids and proanthocyanidins are typically transformed in the colon by intestinal microflora into a wide array of bioactive low molecular weight metabolites, which are later absorbed through the colonic barrier (90, 99). Thus, the enzyme and microbial-mediated biotransformation and active efflux are factors that also limit the availability of polyphenols. Because of their unfavorable structure as substrates for the cytochrome P450s, most polyphenolics are not subject to phase I metabolism and, therefore, these compounds can directly undergo methylation, glucuronidation and sulfation steps during phase II metabolism (90).

In addition to molecular weight, glycosylation has a considerable impact in the intestinal absorption of dietary polyphenols, because it influences chemical, physical and biological properties of polyphenols and it directly affects its polarity and partition coefficient (26). The aglycones are able to be absorbed by the small intestine. Thus, a commonly accepted concept regarding polyphenols intestinal absorption is that glycosylated polyphenols need to be converted to the aglycone by glycosidases in the food or gastrointestinal mucosa, or from the colon microflora in order to be absorbed by passive diffusion (100). Esterification has also an impact in polyphenols absorption. Esterified polyphenols are, in general, lower absorbed than their non-esterified forms. Galloylated catechins, for example, were better recovered in human urine after black tea consumption than non-galloylated catechins, demonstrating their low availability (101).

Therefore, as a result of structural impediment and molecular size, high molecular weight compounds like tannins and flavonoids are not absorbed through intestinal gut barrier, but hydrolyzed by gut microflora (26, 31). It has been confirmed by several *in vitro* and *in vivo* studies. As for the smaller oligomers, it was reported that radiolabeled monomers, dimers, and trimers can be transported across *in vitro* cell layer of Caco-2 cells, whereas the larger oligomers adhere to the cell surface (102-103). Deprez *et al.* (2001) (103) demonstrated that proanthocyanidins dimers, trimers and polymers were hardly absorbed through the human colon carcinoma (Caco-2) monolayer at higher transepithelial electric resistance (TEER values= 509 $\Omega\cdot\text{cm}^2$), and absorption of proanthocyanidins polymers with an average polymerization degree of 6 (molecular weight 1,740) was about 10-fold lower. According Holt *et al.* (2002) (104), dimeric procyanidins were detected in human plasma as early as 30 min after the consumption of a cocoa, whereas no evidence of absorption of oligomeric procyanidins was found. Similarly, Prasain *et al.* (2009) (105) showed that monomeric catechins and their methylated metabolites, as well as proanthocyanidins up to trimers were detected in blood samples after oral consumption of grape seed proanthocyanidin-rich extracts. Although the absorption of procyanidin dimers has been demonstrated using animal models (106), Appeldoorn *et al.* (2009) (107) showed that these compounds are also metabolized by human microbiota. Interestingly, even though punicalagin was detected in plasma of rats after oral ingestion and it is known to be the highest molecular weight compound (1084 Da) ever absorbed in animals so far (108), studies showed that this

water soluble ellagitannin found in pomegranates was hydrolyzed into ellagic acid *in vitro* across the mitochondrial membrane (109), and converted *in vivo* by gut microflora to 3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one (urolithin A, UA) derivatives (18).

Caco-2 cell monolayers are among the most functional *in vitro* models in the field of drug absorption and permeability, which has been used to predict the *in vivo* intestinal absorption and transport of various polyphenols including phenolic acids (110), flavonoids (111), and procyanidins (103). Several studies have been shown that β -glucosidase activity is related to the intestinal absorption of glycosides *in vivo* and in caco-2 cell monolayers (112-117). The β -glucosidases (β -glucan glucohydrolase; EC 3.2.1.21) are a widespread group of enzymes that hydrolyze a broad variety of glycosides including aryl- and alkyl- β -D-glycosides, where β -D-galactoside and β -D-glucoside substrates can be hydrolyzed with comparable efficiencies (118). In mammals, several β -glucosidases have been characterized, including the lysosomal β -glucosidase (also called acid β -glucosidase or glucocerebrosidase), lactase phlorizin hydrolase (LPH) and cytosolic (or broad-specificity) β -glucosidase (118). The human cytosolic β -glucosidase (hCBG) constitutes a group of nine enzymes with similarities in the amino-acid and structural features related to their substrate specificities. They are found in the cytosol of liver, spleen, kidney, small intestine, and lymphocytes of mammals and can catalyze the hydrolysis of O-linked β -glycosidic bonds at the non-reducing end of carbohydrates with retention of anomeric configuration (118).

Gallotannins represent the major compounds found in mango pulp, with molecular weight ranging from 332 (mono-*O*-galloyl-glucose) to up to over 1852 Da (undeca-*O*-galloyl-glucose) or large (47). Considering that punicalagin (1084 Da) was reported to be the highest molecular weight compound absorbed intact through the gut barrier after oral ingestion (108), it is assumed that higher molecular weight polyphenols would not be absorbed intact through human gut. However, limited bioavailability of these compounds might not limit their biological properties, since the highest local concentration of these compounds is found in the gut lumen, where polyphenols may be protective against colon carcinogenesis (26, 31, 103). Therefore, it is relevant to measure the biological activity of polyphenols on cultured cells or isolated tissues in their form present in food, reflecting the potential health benefits associated with the consumption of the entire fruit. At the same time, it is also important to identify

their metabolites and test their respective biological activities, since the hydrolysis of glycosides and further gut bacterial transformation of aglycones may lead to the production of more or less biologically active compounds, which may protect against carcinogenesis (100, 119).

Apoptosis

Apoptosis or Programmed cell death is a physiological process that leads to cellular self-destruction, which is an essential process in development, maintenance of homeostasis and host defense in multicellular organisms. Apoptosis involves typical morphological characteristics including plasma membrane blebbing, cell shrinkage, nuclear chromatin condensation and fragmentation. In contrast to necrosis, apoptosis is a tightly regulated process, which requires the activation of the intracellular machinery by energy requiring (120). Dysregulation of this process contribute to various diseases, including cancer (121-122).

A family of cystein-dependent aspartate-directed proteases, called caspases, plays an essential role in apoptosis. Caspases are synthesized in normal cells as inactive proenzymes (123), and can be activated by apoptosis signaling, which includes autoproteolytic cleavage or cleavage of other caspases at specific residues of aspartic acid (124). During apoptosis, initiator caspases function as upstream signal transducers and proteolytically activate downstream caspases ('effector' caspases). The initiator caspases-2, -8, -9 and -10 are involved in interactions with adaptor proteins, whereas effector caspases-3, -6 and -7 act on a variety of substrates resulting in proteolysis of cellular proteins, leading to the apoptotic features (internucleosomal DNA fragmentation, and culminating in cell death by apoptosis. Two pathways involved in the activation of caspases have been extensively studied: the extrinsic death receptor pathway, and the intrinsic or mitochondrial pathway (125-129).

Extrinsic death receptor pathway

Extrinsic death receptor pathway is characterized by external apoptosis signalling, which is induced by several plasma membrane receptors belong to the

tumour necrosis factor (TNF)-receptor superfamily. This family includes Fas (Apo-1 or CD95), TNF-receptor-1 (TNF-R1), death receptor-3, TNF-related apoptosis inducing ligand receptor-1, TRAIL-R2 and DR6.

The death receptor Fas leads to receptor trimerisation and recruitment of specific adaptor proteins to form an apoptotic signaling complex (*130-131*). The apoptosis signaling mediated by Fas involves the ligation of a death ligand (Fas ligant) to the transmembrane death receptor (Fas), which contains a death domain (DD) in cytoplasm region that interacts with the adaptor protein (FADD), forming a death-receptor-induced signalling complex (DISC). FADD, which contains a death effector domain (DED), recruits the pro-caspase 8 into the disc, which will be proteolytically activated into caspase-8. Thus, the effector caspase (Caspase-8) is an initiator of the caspase cascade, because activate downstream effector caspases, including the apoptosis executioners pro-caspases -3 and -7 into caspases -3 and -7, which cleavage proteins and activate others caspases, resulting in cell death by apoptosis (*129*). Caspases -8 and -3 can establish a link between the death receptor and the mitochondrial pathways by cleavage and activation of Bid, which translocates from the cytosol to mitochondria and leads to the release of cytochrome-c and apoptosis induction (*132*). The overview of Fas-mediated extrinsic death receptor pathway is presented in Figure 3.

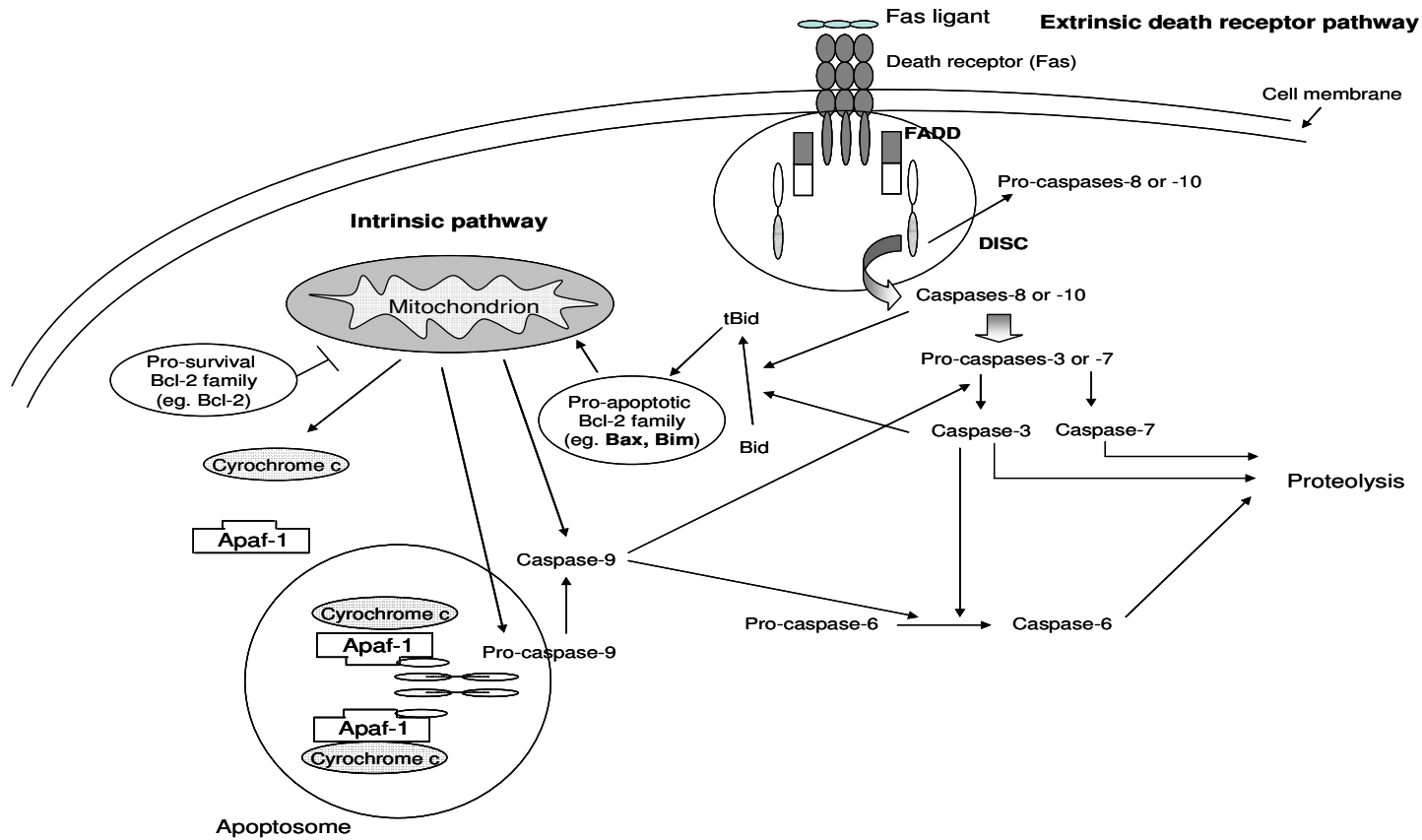


Figure 3. Pathways involved in apoptosis signaling: Fas-mediated extrinsic death receptor pathway and mitochondrial pathway; (—|) inhibition; (—>) activation.

Mitochondrial pathway

The second pathway involves the participation of mitochondria in apoptosis, which release caspase-activating proteins into the cytosol (Figure 3). The release of cytochrome c from the mitochondria results in the activation of the apoptotic protease activating factor-1 (Apaf-1). In the presence of cytochrome c and ATP, Apaf-1 bind and activate procaspase-9, thus forming the mitochondrial DISC, also designated as 'apoptosome' (133). Following activation, the apoptosome-associated caspase-9 will in turn activate downstream caspases like caspase- 3, -6 and -7 (132).

Intrinsic or mitochondria pathway is mainly regulated by proteins members of the B cell lymphoma (Bcl-2) family, which plays multiple roles in carcinogenesis process. These proteins normally reside in the intermembrane space of mitochondria and, in response to a variety of apoptotic stimuli, can be released to the cytosol. It has been suggested that changes in the concentration or function of Bcl-2 protein family may lead to the cytochrome c release by modulating the permeabilization of the inner and/or outer mitochondria membranes (134-135).

Bcl-2 family includes pro-apoptotic proteins, which facilitate this physiological process of cell death, and pro-survival proteins, that inhibit apoptosis (136). The homology between members of the Bcl-2 family is greatest within four small segments, designated Bcl-2 homology (BH) regions, some of which contribute to the interactions between Bcl-2 family members. Even though conclusion regarding whether the ability to associate with other family members is central to apoptosis regulation, previous studies suggested that the ability of Bcl-2 in inhibiting cell death requires binding to a pro-apoptotic family member. Bcl-2 family can be divided into three groups according to BH domains, mitochondrial anchorage (MA) and pro or anti-apoptotic action of their proteins. They consist in anti-apoptotic (Bcl-2 and Bcl-x_L) members, which contain BH1, BH2, BH3 and BH4 domains; pro-apoptotic members with multidomains including BH1, BH2 and BH3 (Bax and Bak), and cytosolic BH3-only pro-apoptotic members (Bid, Bad and Bim) (137-138).

Bax is known to induce apoptosis by interacting with pro-apoptotic members, (Degli Esposti and Dive, 2003; Putcha *et al.*, 2002). This protein forms channels and contributes to regulate preexisting channels that permeabilize the outer mitochondrial

membrane, thus releasing caspase-activating proteins into the cytosol (139-140). Likewise, Bim acts as sensor for cytoskeleton integrity and induce apoptosis by neutralizing certain pro-survival Bcl-2 sub-family of proteins (141). Recently, it was demonstrated the direct interaction between Bim and Bax domains, which suggest a possible Bax activation by Bim (142).

Cell cycle regulation

Cell cycle consists in the series of events which occur in a cell that lead to its division and replication. In eukaryotes, cell cycle is divided in interphase, process of high expression of cell growth proteins which are required to mitosis; and mitosis, the process of nuclear division. Mitosis includes four stages: prophase, metaphase, anaphase and telophase, whereas interphase includes G1, S and G2 phases. DNA Replication occurs in S phase, which is preceded by a gap called G1, a phase characterized by cell growth and high biosynthetic activity of enzymes that are required in S phase. Likewise, RNA transcription and protein synthesis rates are high in G2 phase, during which the cell prepares for mitosis (143-144). Cells in G1 can, before commitment to DNA replication, enter a resting state called G0. The major part of the non-growing, non-proliferating cells in the human body is found in G0 phase. Although the duration of cell cycle in tumor cells is equal to or longer than that of normal cell cycle, the proportion of cells that are in active cell division (versus quiescent cells in G₀ phase) in tumors is much higher than that in normal tissue. Thus there is a net increase in cell number, whereas the number of cells that die by apoptosis or senescence may remain the same (145).

Cell cycle, a highly conserved and regulated process, is essential to cell survival because includes the repair of genetic damage as well as the prevention of uncontrolled cell division. The mutation in some genes is the main cause of cell cycle dysregulation, which may lead to an uncontrolled cellular proliferation, tumor formation and carcinogenesis development (143, 146).

The transition from one cell cycle phase to another is regulated by cell cycle genes and their protein products, which include cyclins, cyclin dependent kinases (Cdks), Cdk inhibitors (CKI) and extracellular factors (growth factors) (147). The

regulation mechanisms involve controlled expression and destruction of cyclins, activation and inhibitory phosphorylation and dephosphorylation of the cyclin-dependent kinases (Cdks), cyclins, and other proteins; the binding of a number of Cdk inhibitory proteins; and the expression and destruction of inhibitory proteins associated with Cdks, or Cdk/cyclin complexes (*143, 146, 148-150*).

Cyclins are targets for extracellular signaling and frequently are dysregulated during oncogenesis. These proteins are produced or degraded as needed during interphase in order to drive the cell through the different stages of cell cycle. They control the progression of cell cycle by activating cyclin-dependent kinase (Cdk) enzymes. Cyclins consist in cell cycle regulators which are activated by Cdks at specific points: at the G1 phase (Cyclin D), at G1/S transition (Cyclin E), at S phase (Cyclin A), at G2/M phase transition (Cyclin A) and in mitosis (Cyclin B) (*145, 151*).

Likewise, cyclin-dependent kinases are cell cycle regulatory proteins from a serine/threonine protein kinases family. Some of these proteins are known to induce downstream processes by phosphorylating specific proteins upon becoming active by forming clyclin complexes with other proteins, or by phosphorylation during different cell cycle phases (*148*). In humans, Cdk4, Cdk6, Cdk2 are know to regulate G1 phase, Cdk2 is active at S phase, while Cdk1 controls mitosis (*152*).

Phosphorylation plays a central role in regulating the formation, activation and inactivation of CDK/cyclin complexes. Protein kinases phosphorylate proteins involved in mRNA production, such as cyclin-dependent Kinases (CDKs), which become active upon binding to a cyclin (*153*). In mammals, cell division control protein 2 homolog (p34, cdc2, cyclin B or Cdk1) is a cyclin-dependend kinase from the serine/threonine protein kinase family, which is encoded by the CDC2 gene (*154*). This protein is a catalytic subunit of the highly conserved protein kinase complex known as maturation promoting factor (MPF), composed of cyclin B and Cdk1 (cdc2). MPF controls the transition from G2/M phase to mitosis by phosphorylating multiple proteins needed during mitosis. Thus, these phosphorylated proteins are responsible for specific events during cycle division such as microtubule formation and chromatin remodeling (*155-156*). When complexed to cyclins, Cdc2 can be phosphorilated and inactivated by the membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase (PKMYT1), an enzyme of the serine/threonine protein kinase family which is encoded

by the PKMYT1 gene in humans (157). Therefore, this enzyme plays an important role in carcinogenesis, because negatively regulates cell cycle G2/M transition by cdc-2 inactivation (157), which may leads to G2/M cell cycle arrest and cell growth suppression.

The activity of cyclin-dependent kinase (Cdk) can be counteracted by cell cycle inhibitory proteins, known as cyclin-dependent kinase inhibitors (CKI), which are produced by genes called tumor suppressor genes. CKIs can halt cell cycle and, consequently, the tumor progression, by binding Cdk alone or Cdk/cyclin complexes. CKIs are distributed in two major families: the INK4a/ARF (Inhibitor of Kinase 4/Alternative Reading Frame) family, composed of inhibitors (p15, p16, p18 and p19) of single Cdk enzyme before cyclin binding (158); and the cip/kip family, composed of CDKs (p21, p27 and p57) that inactivate CDK-cyclin complexes (159). In addition, they inhibit the CDK/cyclin complexes at G1 phase, and to a lesser extent, Cdk1/cyclin B complexes (160).

The cyclin-dependent kinase inhibitor 1A (Cip1/WAF1 or p21), a protein encoded by CDKN1A gene, mainly regulate cell cycle at G1 phase by inhibiting the activity of Cyclin/Cdk2 complexes (161). The expression of p21 is mainly under transcriptional control of the p53 tumor suppressor gene, although may be activated by a response to a variety of stress stimuli (162). When overexpressed, p21 showed to induce cell cycle arrest at G1 (161), G2 (163) and S (164) phases of cancer cells, which contributed to cell growth suppression (165-167). In addition to cell cycle arrest, p21 expression can mediate cellular senescence (G0 phase) by interacting with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor, thus blocking DNA synthesis during cell cycle progression at S phase (168). Additionally, this protein was reported to induce apoptosis because p21 can be specifically cleaved by caspase-3, which thus leads to a dramatic activation of CDK2 and caspase cascade activation (166). On the other hand, when p21 is repressed, different results can appear depending on the context. Studies have shown that p21 repression contributed or not to cancer progression by apoptosis inhibition. However, the mechanism involved is still not completely understood (165).

Anticarcinogenic effects of polyphenols in normal as compared to cancer cells

Tumor cells present several aspects which make them different from normal cells. Cancerous cells do not depend on growth factors like normal cells do, because they are able to produce their own growth factors to stimulate cell proliferation. Normal cells need to keep in contact with the extracellular medium to grow, whereas cancer cells are not dependent on anchorage. Moreover, in cell culture, normal cells grow in a monolayer and may be inhibited by contact with other cells, whereas cancerous cells grow to form several monolayers. Tumoral cells present less adherence than normal cells. Additionally, normal cells interrupt cell proliferation as soon as they reach certain density, whereas tumoral cells keep growing. Normal cells remain in the area where they belong and do not spread to other parts of the body. Cancer cells, otherwise, may spread through the body (metastasize), by direct invasion and destruction of the organ of origin, or spread through the lymphatic system or bloodstream to distant organs such as the bone, lung, and liver (10).

Several in vitro studies have been demonstrating the chemopreventive effects of polyphenols in cancer cells but not in normal cells (169-173). Previous studies have reported that growth-inhibitory effects of polyphenols were greater in colon cancer as compared to non-tumorigenic colon cells (169-170). The isogenic human colon cancer cell lines HCT-116(p53 +/+) (IC₅₀ value \square 45 μ g/mL) and HCT-116 (p53 -/-) (IC₅₀ value \square 30 μ g/mL) showed to be more sensitive to the treatment with gallotannins than normal human intestinal epithelial cells FHs 74Int (IC₅₀ value > 60 μ g/mL) (173).

Overall, cancer cells have been demonstrated to be more sensitive to polyphenol-induced apoptosis than normal cells, which often undergo apoptosis only with higher polyphenol-concentrations. Although the mechanism for this phenomenon are still not well elucidated, possible differences between normal and cancer cells may explain in part the higher sensibility of cancer cells. These include membrane fluidity, expression and activity of non-mutated proteins, oncogenes, as well as intracellular signal transduction (174-177).

Objectives

The overall objective of this study was to elucidate the anti-cancer effects of mango polyphenols of several varieties in different types of cancer.

Based on previous published data which state that individual polyphenols found in mango pulp present anticancer properties including antiproliferative effects, induction of apoptosis, regulation of cell cycle and inhibition of angiogenesis, we hypothesize that mango phenolic extracts present chemopreventive properties, and might represent the health benefits derived from the consumption of the entire fruit instead of isolated compounds. The antiproliferative effects *in vitro* of polyphenols from different mango varieties (Francis, Kent, Ataulfo, Tommy Atkins and Haden) were compared on different cancer cell lines (Molt-4 leukemia, A-549 lung, MDA-MB-231 breast, LnCap prostate, SW-480 colon cancer cells and the non-cancer cell line CCD-18Co). Molecular mechanisms involved on the anti-cancer activities of mango polyphenols on colon cancer cells were assessed by studying the effect of mango polyphenols on gene expression, cell cycle regulation and reactive oxygen species production.

Based on previous publications indicating the increased bioavailability of polyphenolics after metabolism by β -glucosidase, we hypothesize that the enzymatic hydrolysis of mango polyphenols could enhance their absorption in a selected cell culture model. This hypothesis was tested using Caco-2 human coloncarcinoma cell monolayers *in vitro* model. In order to elucidate the *in vivo* intestinal absorption, mango polyphenols were prior hydrolyzed using the enzyme β -glucosidase, commonly found in gut microflora. The effect of enzymatic hydrolysis on antioxidant activity, phenolic content and cell-growth suppressive activity of mango polyphenols was evaluated on MDA-MB-231 breast and HT-29 colon human cancer cells. The antiproliferative activity of high molecular weight polyphenols-rich fraction, characterized by mangiferin (422.35 Da) and gallotannins (788-1851 Da), and low molecular weight polyphenols-rich fraction, comprising mainly phenolic acids (138-194 Da), was studied using cell culture models.

Results from this study provide valuable insight into the anti-cancer effects of mango polyphenols and the mechanisms underlying those effects. Furthermore, information regarding the antioxidant and cancer-inhibitory activities of mango

polyphenols contributes to the ongoing evaluation of health benefits derived from fruit consumption.

Overall, the performed studies elucidate the preventive effects of polyphenols extracted from mango on carcinogenesis process.

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ANTICARCINOGENIC EFFECTS OF POLYPHENOLS FROM DIFFERENT MANGO (*MANGIFERA INDICA* L.) VARIETIES

ABSTRACT

Polyphenols found in different cultivars of mango pulp, including gallotannins, flavonol glycosides, gallic acid, benzophenone derivatives and mangiferin have shown anticancer activity. The objective of this study was to compare the anti-cancer properties of phenolic extracts from several mango varieties (Francis, Kent, Ataulfo, Tommy Atkins and Haden). The antiproliferative effects of mango polyphenols were studied *in vitro* using different cancer cell lines including Molt-4 leukemia, A-549 lung, MDA-MB-231 breast, LnCap prostate, SW-480 colon cancer cells and the non-cancer colonic myofibroblasts cell line CCD-18Co. Additionally, gene expression was investigated by RT-PCR and cell cycle kinetics was studied by flow cytometry. Cell lines were incubated with Ataulfo and Haden phenolic extracts, which were selected based on their superior antioxidant capacity compared to the other varieties. Ataulfo and Haden polyphenols inhibited the growth of all human cancer cell lines. SW-480, MOLT-4 and MDA-MB-231 were statistically equally most sensitive to Ataulfo, whereas SW-480 and MOLT-4 were the most sensitive cell lines to Haden, as determined by cell counting. The efficacy of phenolic extracts from all mango varieties in inhibiting cell growth was tested on SW-480 colon carcinoma cells. Ataulfo and Haden demonstrated superior efficacy, followed by Kent, Francis and Tommy Atkins. At 5 mg GAE/L, Ataulfo inhibited the growth of colon SW-480 cancer cells by ~79% while the growth of non-cancer colonic myofibroblasts CCD-18Co cells was not inhibited. The growth inhibition exerted by Ataulfo and Haden polyphenols on SW-480 cells was associated with an increased mRNA expression of pro-apoptotic biomarkers (caspase 8, Bax and Bim) and cell cycle regulators (PKMYT1), cell cycle arrest and a decrease in the generation of reactive oxygen species. Overall, polyphenols from several mango varieties exerted anti-cancer effects.

Keywords: Mango, *Mangifera indica*, cancer, cell cycle regulation, polyphenols, gene expression regulation, cell proliferation.

INTRODUCTION

Consumer interest in mango fruits (*Mangifera indica*) and derived products has been increasing in recent years based on fruit olfactory properties, and on potential in preventing chronic diseases (1). Moreover, mango fruits have found their way into the unofficial classification as so-called superfruit. Cancer is one of the leading chronic diseases and causes of death worldwide (2). Major types of cancer include lung, colorectal, breast, and prostate cancer (2). Colon cancer is the third most common cancer in USA for both men and women, with 108,070 estimated cases of colon and 40,740 cases of rectal cancer diagnosed in 2008 (3). Overall, mortality associated with colon cancer might be decreased by controlling risk factors. Among these risk factors, diet is one of the modifiable factors which may be modified through an increased intake of fruits and vegetables (3).

Plant polyphenols are increasingly being considered as sources of natural colon cancer chemopreventive compounds based on safety and efficacy assessments (4). The edible portion of mango contains polyphenols and carotenoids that may protect against cancer. Polyphenols 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, hydrolysable tannins, within a wide range of molecular weight and galloylation, and the xanthone mangiferin (5-6). Bioavailability of mango polyphenols seems to be limited by high molecular weight and galloylation degree. However, limited bioavailability of some polyphenols such as gallotannins might not represent a limitation in the exertion of possible health benefits since they likely reach the large intestine (7-8), where they may act protecting colon carcinogenesis.

Few studies have shown the anticancer activity of mango extracts, and they suggest cell line specificity; e.g. mango extracts have been shown to protect against prostate cancer in *in vitro* and *in vivo* models (9) and to inhibit the cell cycle in the G(0)/G(1) phase of HL-60 cells (10), although their lack of effectiveness against MCF-7 breast cancer cells has also been reported (11). In addition, some of the phytochemicals found in mango, i.e. the triterpene lupeol and the xanthone mangiferin have shown to exhibit anti-cancer activity. Mangiferin showed chemopreventive action against lung

carcinogenesis induced by benzo(a)pyrene in Swiss albino mice (12). The chemopreventive action of mangiferin might be related to induction of permeability in mitochondria (13). Lupeol and related terpenoids exerted varied cytotoxicity against several cancer cell types (B16 2F2 mouse melanoma, leukemia HL60, U937, K562 melanoma G36; human lung carcinoma (A-549), human colon adenocarcinoma (DLD-1) (14).

The overall anti-cancer potential of mango polyphenols might be in part associated to their antioxidant properties, which contribute to enhance cell defense capacity and modulate molecular pathways in target cells (15). These compounds may inhibit promotion and progression stages of cancer by interfering in cell cycle regulation, signal transduction pathways, transcription and activating apoptosis (activation of pro-apoptotic genes and pro-apoptotic proteins) in neoplastic cells (16).

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

MATERIAL AND METHODS

Chemicals

All solvents and chemicals used were of analytical grade. Standards obtained from Sigma (St. Louis, MO, USA) used for identification purposes by HPLC were as follows: gallic acid, *p*-OH-benzoic acid, *p*-coumaric acid, *m*-coumaric acid, caffeic acid, ferulic acid, vanillic acid, mangiferin, quercetin, (+) catechin, ellagic acid and protocatechuic acid.

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 firm mid-ripe 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. Pulp was homogenized and storage at -80 °C until needed.

Extraction of polyphenols

Frozen pulp (500 g) 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 rotavapor (Büchi, Switzerland). The aqueous extract was mixed with methanol (1:1) and centrifuged at 2000RPM for 10 min at 7°C to remove insoluble precipitates. The methanol was evaporated in rotavapor at 40 °C and polyphenols were concentrated on a C18 cartridge (Waters Corporation, Milford, MA) previously conditioned with acidified methanol (0.01% HCl) and nanopure water. The aqueous extract was loaded on the C18 cartridge. Polyphenols bound to the matrix were eluted with 50 mL 100% methanol (F1). The water from the wash (unbound fraction) was partitioned into ethyl acetate (EtAc) (1:4,v/v) using a separatory funnel in order to recover C18 non-adsorbed polyphenols, such as phenolic acids. Thus, some hydrophilic non-phenolic compounds such as organic acids, reducing sugars and soluble proteins were excluded (17). The ethyl acetate phase (F2) was combined with the methanol eluate (F1). The solvents were evaporated under vacuum at 40 °C and the volume made up to 50mL with nanopure water (Figure 4).

The phenolic aqueous solution was frozen at -80°C and freeze-dried (Labconco Corporation, Kansas City, MO) at -50 °C and at 0.01mBar of pressure. These extracts were further used for the cell culture assays by applying different doses based on total phenolic content measured spectrophotometrically by the Folin-Ciocalteau assay against an external standard of gallic acid (mg GAE/L) (18). The optical density (OD) values were recorded on a FLUOstar Omega plate reader (BMG Labtech Inc, Durhan, NC) at 726 nm. Dried extracts were reconstituted in culture medium and sterile filtered prior to use in cell culture experiments.

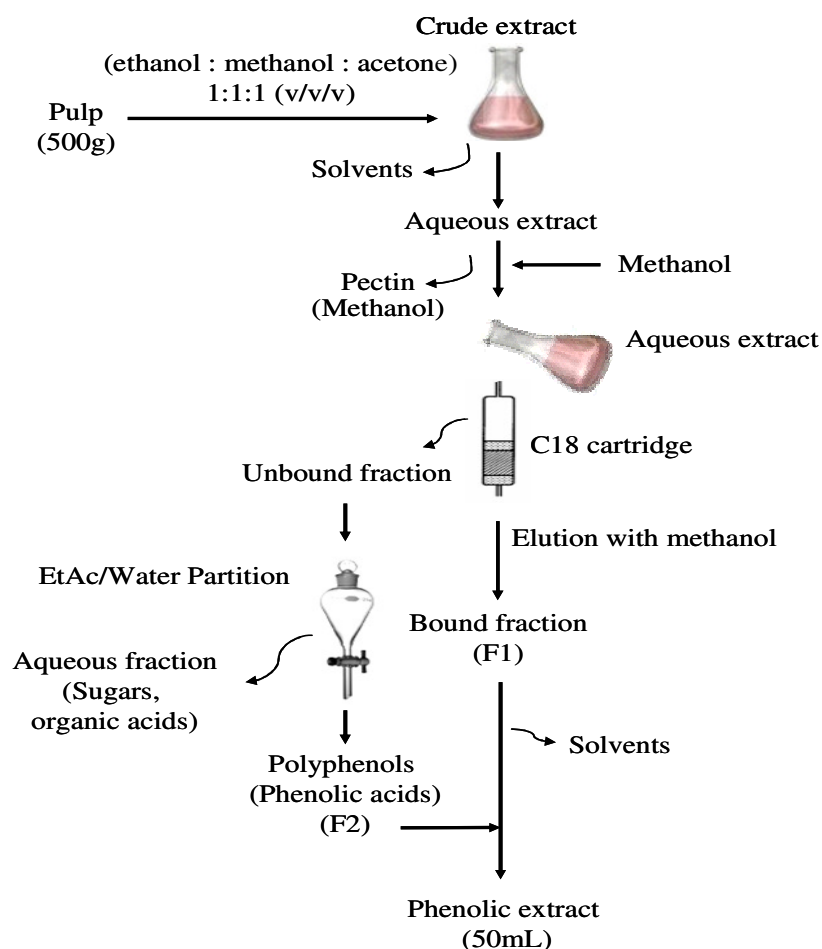


Figure 4. Procedure used for extraction of mango polyphenols

Antioxidant capacity (ORAC assay)

The antioxidant capacity of polyphenols from the different mango varieties was measured using the oxygen radical absorbance capacity (ORAC) assay as described by Ou et al, 2001 (19), 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 520 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 3.1 to 50 μ M Trolox. Results were quantified in μ mol Trolox equivalents/100g pulp.

HPLC-DAD and HPLC-ESI/MSⁿ analysis

Individual polyphenols were characterized using a Waters Alliance 2690 (Milford, MA, USA) HPLC system using a 4.6 x 250 mm Dionex Acclaim 120 C₁₈ column (5 μ m) and a 4.6 mm x 20 mm guard column. A gradient elution program run 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 run 100% Phase A for 3 min and changed from 100-70% in 17 min, 70-50% in 10 min, 50-30% in 20 min, 30-0% in 10 min, and then isocratic at 0% Phase A for 5 min prior to column re-equilibration (20).

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). Polyphenolic extracts were analyzed using a Waters Alliance 2690 (Milford, MA, USA) HPLC system equipped with a Dionex Acclaim ® 120 C₁₈ 5 μ m column, 4.6 mm x 250 mm column and a 4.6 mm x 20 mm guard column. Phenolic compounds were separated using 0.5% formic acid in water (solvent A) and 0.5% formic acid in acetonitrile (solvent B) as mobile phases, at a flow rate of 0.4 mL/min. Gradient program ran phase A for 5 min, from 100-70% in 25 min; 70-50% in 15 min; 50-0% in 25 min; and returning to original composition in 8 min. 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.

Identification of phenolic compounds was performed by comparison of their retention times and UV-visible spectral characteristics with those from standards obtained from Sigma-Aldrich (St Louis, MO) as well as their mass spectrometric properties.

Cell culture

Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). 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 and the androgen receptor positive LnCap prostate cancer cells were cultured in RPMI-1640 medium. 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 media used for cell proliferation experiments. Culture media were supplied by Invitrogen (Gibco™, Invitrogen Corp., Grand Island, NY). Cells were maintained at 37 °C with a humidified 5% CO₂ atmosphere. For cell proliferation assay, cells were seeded in 24 well-plates (15,000 cells/well), for ROS assay in 96-well plates (10,000 cells/well), for cell cycle kinetic assay 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 polyphenols (mg GAE/L) reconstituted in culture medium, and sterile-filtered before use.

Cell proliferation

Cell proliferation was assessed by using an electronic counter (Z2™ Series, Beckman Coulter, Inc, Fullerton, CA). The difference in cell-number between final incubation time (72 h) and incubation start (0 h) represents net growth. The cell growth inhibition was expressed as IC₅₀, the concentration of total polyphenols which inhibits cell growth by 50%.

Cell cycle kinetics

Cells were treated with mango polyphenols (5 and 10 mg GAE/L) for 24 h and harvested according to Mertens-Talcott *et al.* (2007) (21). Briefly, cell pellets were obtained by trypsination and centrifugation. Pellets were fixed with 90% ethanol at -20 °C for 4 h. Cells were resuspended 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. DPBS (Dulbecco's phosphate buffered saline 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 Immunocytometry Systems) acquisition software as previously described (21).

Quantitative RT-PCR

Human SW-480 colon adenocarcinoma cells were pretreated with Ataulfo and Haden polyphenols (2.5-10 mg GAE/L) for 24 h. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA), and 470 ng of RNA were used to synthesize cDNA using a Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY). Real time-PCR reactions were performed using 2µL of cDNA as previously described (22). Optimal semi-quantitative conditions were set to fall in the linear PCR product range 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'- TCCTGTAGAGGCTGAGGTGTCA -3',
Caspase 8:	F:	5'- GGCTCCCCCTGCATCAC-3',
Caspase 8:	R:	5'- CCTGCTAGATAAGGGCATGAATCT-3',
Bax:	F:	5'- CCAAGGTGCCGGAAGTGA -3',
Bax:	R:	5'- CCCGGAGGAAGTCCAATGT -3'
Bim:	F:	5'- TGCCAGGCCTTCAACCA -3',
Bim:	R:	5'- GTTCAGCCTGCCTCATGGA-3'

PKMYT1:	F:	5'-CCTCTGCACTTTTAAACCTTTTATCCT-3',
PKMYT1:	R:	5'- GCAGAGAAGACCATGGGAGTTC -3',
p21:	F:	5'-GAG CTC TGG GTG GTC ATG GA -3',
p21:	R:	5'- ATC CTG GTG TGG GTG ACG AT -3',
TBP:	F:	5'-TGCACAGGAGCCAAGAGTGAA-3',
TBP:	R:	5'-CACATCACAGCTCCCCACCA-3',

Reactive oxygen species (ROS)

The production of ROS was analyzed according to Meng *et al.* (2008) (23), with some modifications. Briefly, cells were pretreated with mango polyphenols (1.5-10mg GAE/L) over 24 h followed by 200µM H₂O₂ for 2 h. Upon removal of culture medium with phenolic extracts, cells were rinsed with DPBS (Dulbecco's phosphate buffered saline solution, Ca-Mg free), and exposed to 200µM H₂O₂ for 2h. After washing the H₂O₂ with DPBS, cells were exposed to 10µM of 2', 7' -dichlorofluorescein diacetate (DCFH-DA) at 37 °C and incubated for 30min. The fluorescence signal was monitored at 520 nm emission and 480 nm excitation using a FLUOstar Omega plate reader (BMG Labtech Inc, Durhan, NC). Fluorescence intensity was used as an indicator of ROS level.

Statistical analysis

Quantitative data represent mean values of three or more repetitions with the respective standard deviation (SD) or standard error of the mean (SE). Data were analyzed by one-way analysis of variance (ANOVA) using post-hoc 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 polyphenols and antioxidant activity of mango varieties

Total phenolic content obtained from mango pulps assessed by Folin-Ciocalteu assay (Table 1) demonstrated that Ataulfo extracts contained the highest amount of total polyphenolis normalized to gallic acid equivalent (GAE) (56.7 ± 0.3 mg GAE/100g).

Table 1. Total polyphenols and antioxidant activity (ORAC) of different mango varieties

Mango Cultivar	Total phenolic content ¹ (mg GAE/100g pulp)	Antioxidant activity ¹ (μ molTE/100g pulp)
Ataulfo	$56.7^a \pm 0.3$	$326.6^a \pm 4.8$
Haden	$19.1^b \pm 1.8$	$225.8^b \pm 0.6$
Francis	$17.8^{b,c} \pm 0.3$	$219.0^b \pm 1.0$
Kent	$16.4^c \pm 0.9$	$150.0^c \pm 0.9$
Tommy Atkins	$15.3^c \pm 1.6$	$156.6^c \pm 1.1$

¹Average of three independent determinations \pm SD. Different letters indicates significance ($p < 0.05$).

Previous studies showed that the total phenolic content from mango pulp may range from 48.4 to 294 mg GAE/ 100g (24-25), which may be influenced by several factors including ripening stage, cultivar, geographic production, harvesting, processing and storage conditions, microbial infection, and so on (5, 25-26). Determination of total polyphenols might be relevant when analyzing the antioxidant and anticarcinogenic activities from fruit, since both are in part attributable to the polyphenols content. As natural antioxidant compounds, polyphenols may interrupt radical chain reactions by scavenging reactive oxygen species and free radicals (27). Ataulfo phenolic extract was the highest in antioxidant activity (326.6 ± 4.8 μ mol Trolox equivalents/100g mango pulp), which may be explained by its higher phenolic content as compared to the other varieties (Table 1). The antioxidant activity of polyphenols from all mango varieties correlated to their total phenolic content ($r = 0.91$). In this study, phenolic extracts do not contain additional antioxidants commonly found in mango including ascorbic acid, soluble proteins, and reducing sugars. This might explain why previous studies have been reported higher ORAC values for mango pulp extracts (326-1002 μ mol TE/100g),

with a slightly higher correlation between ORAC value and total phenolic content ($r = 0.98$) (24, 28).

HPLC-DAD and HPLC-ESI-MSⁿ analysis of Ataulfo and Haden polyphenols

The predominant polyphenols in mango pulp were previously identified as gallic acid and gallotannins, with lower concentrations of numerous other phenolic acids, flavonol and xanthone glycosides (5-6). Individually, most of the polyphenols present in mangos contribute to their total antioxidant capacity. The health-related claims of polyphenols found in mango pulp (29) have been supported by *in vitro* and *in vivo* studies and have been ascribed to their anti-cancer and antioxidant properties (16, 24, 28).

HPLC analysis of Ataulfo and Haden polyphenols confirmed the presence of gallic acid and mangiferin (Figure 5). Both compounds were found in all cultivars, with exception of Francis, which did not contain mangiferin (Figure 5). Previous studies also confirmed our findings (25). Moreover, hydrolyzable tannins composed of gallic acid units esterified to a glucose moiety (6) were structurally characterized by HPLC-ESI-MSⁿ analysis (Table 2, Apendice). Galloyl glucosides identified in Haden presented chemical structure ranging from one (mono-*O*-galloyl-glucoside) to nine (nona-*O*-galloyl-glucoside) units of gallic acid esterified in a glucose core; whereas the characterization of Ataulfo phenolic extract revealed the presence of higher molecular weight gallotannins, varying from mono-*O*-galloyl-glucoside to undeca-*O*-galloyl-glucose. Similarly, gallotannins with 4-7 galloyl residues were previously identified in mango pulp from the cultivar Tommy Atkins (29). According to Figure 5, polyphenols with elution pattern after 58 min comprise gallotannins with molecular weight ranging from 939 (penta-*O*-galloyl-glucoside) to 1851 Da (undeca-*O*-galloyl-glucose).

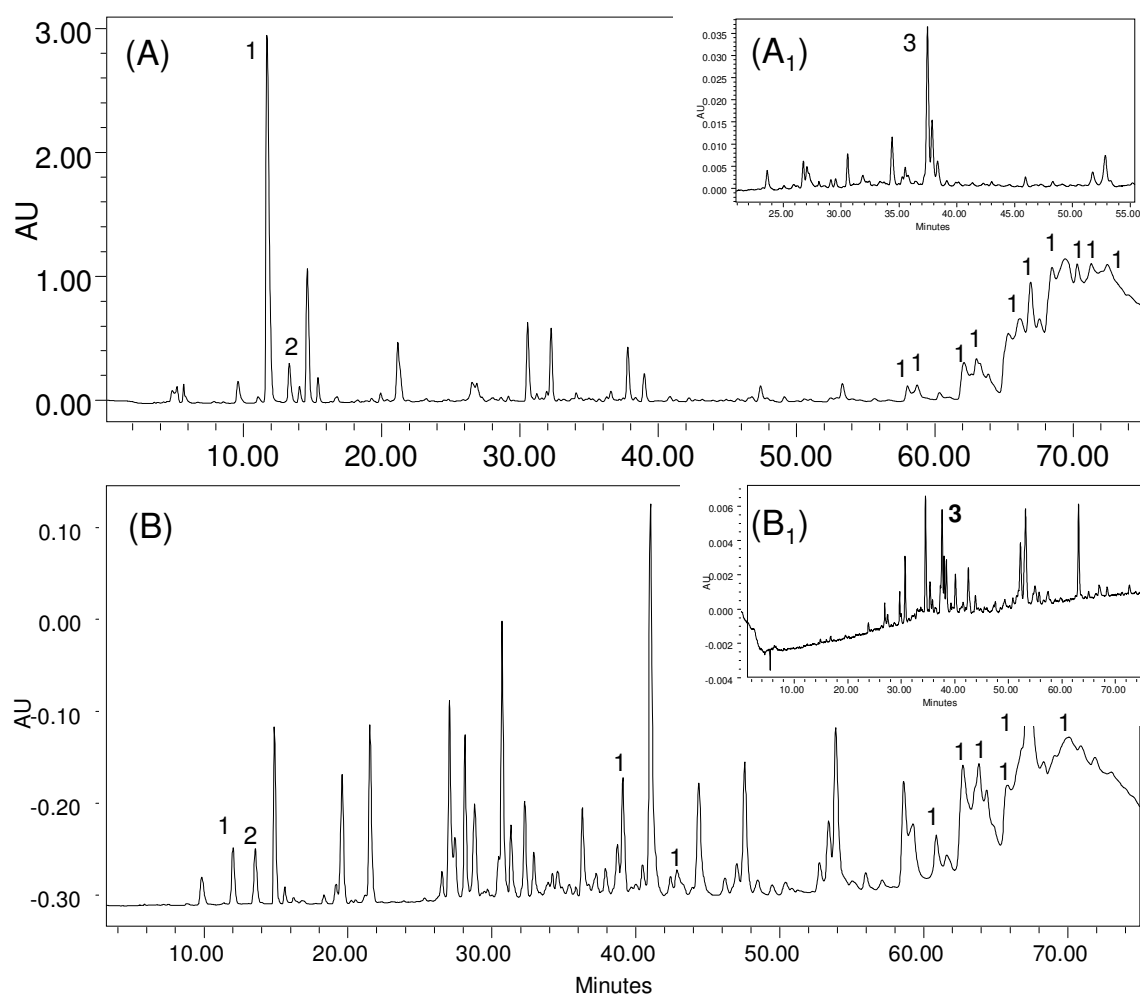


Figure 5. Representative chromatograms at 280 nm of Ataulfo (A) and Haden polyphenols (B). (1) gallotannins; (2) gallic acid. Representative chromatograms at 366 nm of Ataulfo (A₁) and Haden (B₁) (3) mangiferin.

Table 2. Polyphenols profile from the mango varieties Ataulfo, Haden, Kent, Francis, and Tommy Atkins determined by HPLC-DAD and HPLC-ESI-MSⁿ analysis.

Class	Polyphenols	Ataulfo	Haden	Kent	Francis	Tommy Atkins
Phenolic acid	Gallic acid	✓	✓	✓	✓	✓
Xanthone	mangiferin	✓	✓	✓	nd	✓
Gallotannins	mono- <i>O</i> -galloyl- glucose	✓	✓	✓	✓	✓
	di- <i>O</i> -galloyl- glucose	nd	✓	nd	nd	nd
	tetra- <i>O</i> -galloyl- glucose	nd	✓	✓	✓	nd
	penta- <i>O</i> -galloyl- glucose	✓	✓	✓	✓	✓
	hexa- <i>O</i> -galloyl- glucose	✓	✓	✓	✓	✓
	hepta- <i>O</i> -galloyl- glucose	✓	✓	✓	✓	✓
	octa- <i>O</i> -galloyl- glucose	✓	✓	✓	✓	✓
	nona- <i>O</i> -galloyl- glucose	✓	✓	✓	✓	✓
	deca- <i>O</i> -galloyl- glucose	✓	nd	nd	nd	nd
	undeca- <i>O</i> -galloyl-glucose	✓	nd	nd	nd	nd

nd= not determined or not detected.

Cell-growth suppressive activity of Haden and Ataulfo mango polyphenols on different cancer cell lines

Based on their superior antioxidant capacity, Ataulfo and Haden were selected to assess the growth inhibition activity of their polyphenols in different cancer cell lines (Figure 6).

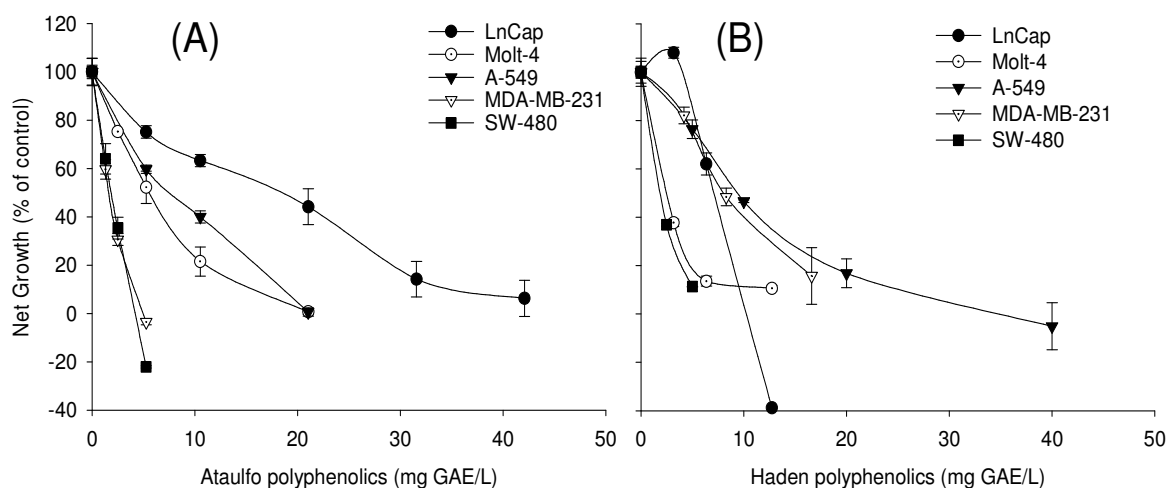


Figure 6. Cell proliferation of prostate LnCap, leukemia Molt-4, lung A-549, breast MDA-MB-231 and colon SW-480 cancer cells treated with Ataulfo (A) and Haden (B) polyphenols. Cells were treated with different concentrations of extracts and cell growth was assessed after 72 h incubation. Values are means \pm SD, $n=3$.

Ataulfo and Haden polyphenols inhibited the cell proliferation of all cancer cell lines in a concentration-dependent manner. The IC_{50} values (concentration that inhibits the growth by 50%) show that Ataulfo polyphenols suppressed the cancer cell growth in the following order of efficacy: SW-480 = Molt-4 = MDA-MB-231 > A-549 = LnCap within a range of 0-42 mg GAE/L (Table 3). Likewise, IC_{50} values indicate that human colorectal SW-480 carcinoma cells were inhibited by both Haden and Ataulfo polyphenols to a similar extent (Table 3).

Table 3. IC_{50} values of polyphenols extracted from Ataulfo and Haden mango varieties for growth suppression of different human cancer cell lines

Mango Cultivar	IC_{50} (mg GAE/L)					
	SW-480	MDA-MB-231	LnCap	A-549	Molt-4	CCD-18Co
Ataulfo	$1.6^1 \pm 0.2^b$	$1.3^1 \pm 0.1^b$	$18.0^2 \pm 0.0^a$	$13.2^1 \pm 5.5^a$	$5.0^1 \pm 1.0^b$	$> 10^2$
Haden	$2.3^1 \pm 0.2^b$	$8.3^1 \pm 0.0^a$	$7.0^1 \pm 0.0^a$	$8.3^2 \pm 1.8^a$	$2.0^1 \pm 0.5^b$	ND

¹Average of three independent determinations \pm SD. ²Average of two independent determinations. Different letters indicate statistical significance at $p < 0.05$. ND, not determined.

Overall, mango polyphenols have low bioavailability, in part, due to the presence of higher molecular weight polyphenols such as gallotannins (8, 30-31). Thus, the chemopreventive protection of mango polyphenols, specifically those with larger molecular weight, may be relevant to the prevention of colon cancer *in vivo*. However, limited bioavailability of high molecular weight polyphenols might not represent a limitation in the exertion of possible health benefits since they likely reach the large intestine (7-8), where they may act protecting colon carcinogenesis.

Overall, these results suggest that polyphenols from Ataulfo and Haden varieties inhibit the proliferation of all tested types of cancer cell lines, and SW-480 colon cancer cell line had high susceptibility to the treatment.

Growth suppressive activity of mango polyphenols from different varieties on colon cancer cells

Based on their high sensitivity to mango polyphenols and the likely *in vivo* effects of mango polyphenols in modulating human gut microflora, SW-480 cells were selected for the following analyses. Polyphenols extracted from Ataulfo, Haden, Kent, Francis and Tommy Atkins induced a concentration dependent growth-suppression in human SW-480 colon cancer cells (Figure 7).

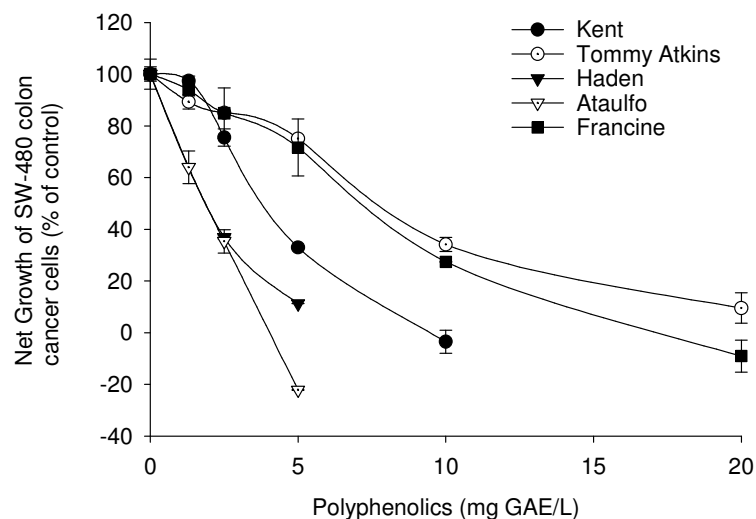


Figure 7. Cell-growth suppressive effects of polyphenols from Kent, Tommy Atkins, Haden, Ataulfo and Francis mango varieties on colon SW-480 human cancer cells. Cells were incubated with extracts and net growth was measured after 72 h incubation. Values are mean \pm SD, n=3.

Among the five cultivars, Ataulfo and Haden polyphenols showed the highest cell growth-inhibition in SW-480. At 1.3 and 2.5 mg GAE/L, Ataulfo polyphenols suppressed the growth of SW-480 cells by 36% and 65%, respectively, at 72 h of incubation. When incubated with 5 mg GAE/L of Ataulfo polyphenols, net cell growth was decreased by 22% indicating that the number of cells at 72h was lower than at the baseline (0 h), suggesting net cell killing (Figure 7). Polyphenols from Haden reduced cell growth in a concentration-dependent manner, with 63% and 89% of inhibition achieved at 2.5 and 5 mg GAE/L respectively. The order of efficacy for the tested mango extracts was: Ataulfo = Haden \geq Kent \geq Francis > Tommy Atkins based on their IC₅₀ values (Table 4).

Table 4. IC₅₀ values of polyphenols extracted from mango varieties for growth suppression of human SW-480 colon cancer cells

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

¹Average of three independent determinations ± SD. ²Average of two independent determinations. Different letters indicate statistical significance at the level p< 0.05.

A previous study reported low cytotoxic efficacy of mango aqueous extracts in MCF-7 breast cancer cells when compared to other plant-based foods consumed in Mexico (11). Polyphenols in that study were extracted with water and did not contain compounds with lower solubility in water such as higher molecular weight gallotannins, as demonstrated by Percival *et al.*, 2006 (10).

Apparently, the degree of galloylation may play a role in the cytotoxic effects in cancer cells (32-33), which may indicate that some of the anti-cancer effects may be increased by the high degree of galloylation of mango-polyphenols. Tannin structure-activity relationship studies indicate that tannins which exhibited potent inhibitory activity on the tumor cell motility have common characteristics such as galloyl groups substituting all the hydroxyl groups of β-D-glucose and some of them cross-linked to form hexahydroxydiphenoyl. Ellagitannins including the most potent 1,2,4-tri-O-galloyl-3,6-hexahydroxydiphenoyl-β-D-glucose (punicafolin) showed to be more potent than gallotannins (mono, di and penta-O-galloyl-β-D-glucose) (34). In similar study, Coriariin A, which has four galloyl groups, showed higher antitumor effects than agrimoniin, that has two hexahydroxydiphenol groups (35). This results are in agreement with Lizarra *et al.* (2008) (33) findings. The more galloylated Witch hazel fractions showed better effectiveness at inhibiting proliferation of HT-29 and HCT-116 human colon cancer cells, at arresting the cell cycle at the S phase as well as at inducing apoptosis and necrosis. Interestingly, the apoptosis and cell cycle arrest effects were proportional to their galloylation level. Moreover, witch hazel fractions with a high degree of galloylation were also the most effective as scavengers of both hydroxyl and superoxide radicals and in protecting DNA damage triggered by the hydroxyl radical system. Moreover, tannin-rich fraction (85.1- 93.2% of the TPC) extracted from four

cultivars of muscadine grapes showed a 2-4 greater inhibitory activity against HT-29 and Caco-2 colon cancer cells, when compared to phenolic acid fraction (36), likely as a result of its higher capacity in preventing oxidative stress (37-38).

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

Growth-suppressive activity of Ataulfo polyphenols on cancer cells as compared to normal cells

The growth inhibition effects of mango polyphenols were investigated in non-cancerous colonic myofibroblasts (CCD-18Co) cells compared to SW-480 cells. At 5 mg GAE/L, Ataulfo polyphenols suppressed the growth of SW-480 cells by ~ 79 %, whereas the growth of CCD-18Co cells was not inhibited at the same concentration. Even at the highest concentration of Ataulfo polyphenols (10 mg GAE/L), the growth inhibition of CCD-18Co cells was only $13 \pm 6\%$ (Figure 8). Previous studies have reported that growth-inhibitory effects of polyphenols were greater in colon cancer as compared to non-tumorigenic colon cells (39-40). In general, bioactive natural compounds which has growth-suppressive effects in cancer cells but do not affect normal cells, may have a potential in chemoprevention, which is suggested by these data.

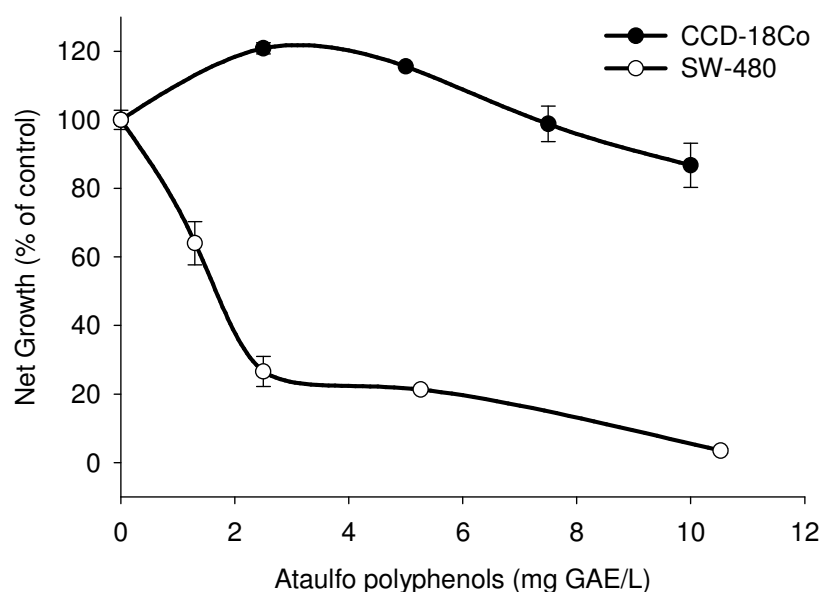


Figure 8. Cell-growth suppressive effects of Ataulfo polyphenols on human SW-480 colon cancer cells and colonic myofibroblasts CCD-18Co cells. Cells were incubated with phenolic extract and net growth was measured after 48 h. Values are mean \pm SD, n=3. Asterisk indicates a significant difference compared to untreated control (*) $p \leq 0.01$.

Cell Cycle Regulation

Ataulfo and Haden polyphenols induced G₂/M cycle arrest (Figure 9). Haden polyphenols at 10 mg GAE/L were more effective than Ataulfo in increasing the number of cells in G₂/M phase by 1.9-fold. Haden polyphenols also induced cell cycle arrest in the S phase in a dose-dependent manner, whereas Ataulfo polyphenols did not show significant effect at this phase. Previous studies demonstrated that polyphenols from mango influenced cell cycle transition in Caco-2 colon cancer cells with an increased percentage of cells in the S and G₂/M phase (41). Similarly, grape polyphenols 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 (32). Gallotannins induced S-phase arrest in HCT-116 human colon cancer cells, which was associated with an increase in p53 protein levels and p21 mRNA and protein levels (42). Overall, the demonstrated cell cycle modulating properties of gallic acid and

galloylated derivatives as well as other polyphenols present in mango pulp have likely contributed to the anti-cancer properties in SW-480 cancer cells.

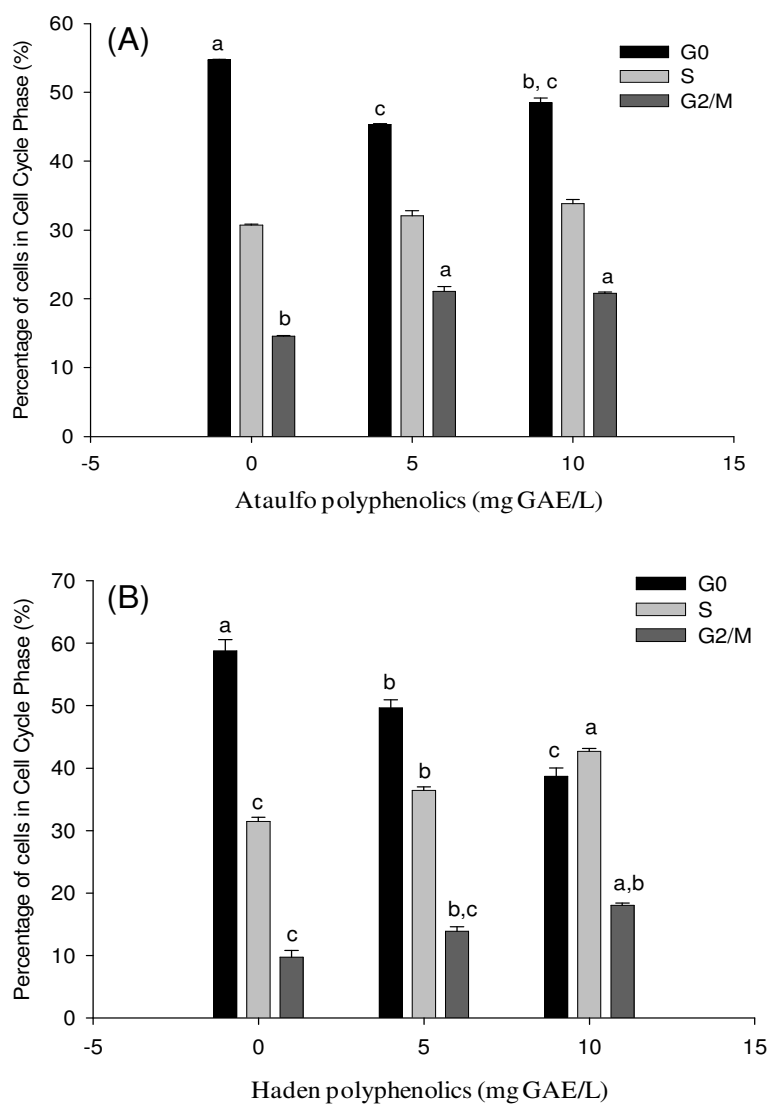


Figure 9. Cell cycle analysis of SW-480 colon cancer cells treated with Ataulfo and Haden polyphenols for 24 h. Values are means \pm SE (n=3), different letters indicate significance at $p < 0.05$.

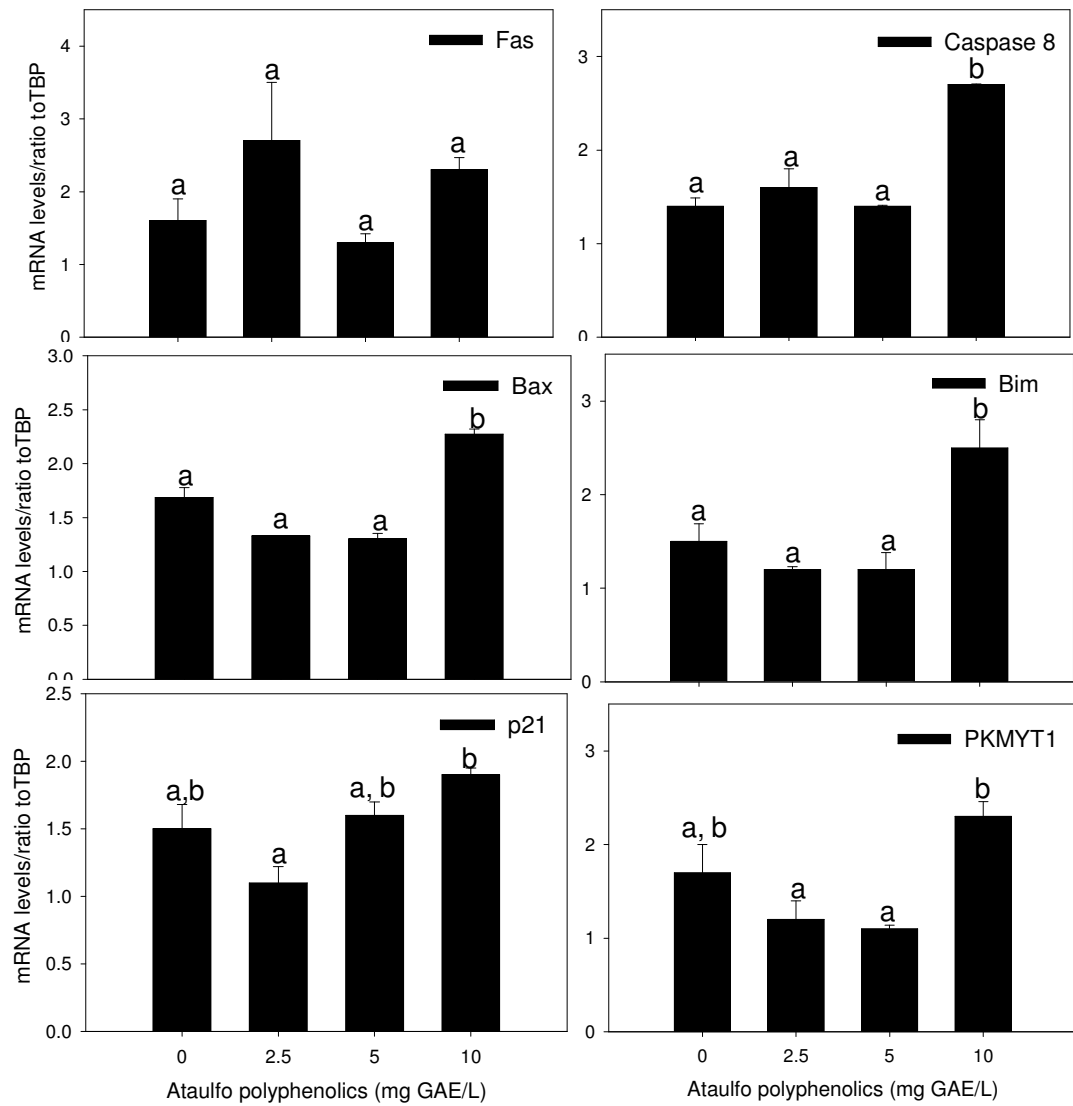
Gene Transcriptional Regulation

The expression of genes involved in the regulation of apoptosis, referred to as “programmed cell death” were investigated. Two pathways involved in the activation of caspases have been studied: the extrinsic death receptor pathway induced by ligation of the Fas-receptors, or Fas-mediated apoptosis, and the intrinsic or mitochondrial pathway (43). The former leads to activation of caspase-8, an initiator of the caspase cascade resulting in proteolysis of cellular proteins and death by apoptosis (43), whereas the latter is mainly regulated by the Bcl-2 family members, which include pro-apoptotic and pro-survival proteins (44). Some pro-apoptotic proteins, i.e. Bax form channels and contribute to regulate preexisting channels that permeabilize the outer mitochondrial membrane, which releases caspase-activating proteins into the cytosol (45), whereas others i.e. Bim neutralize certain pro-survival Bcl-2 sub-family of proteins (46).

The effects of Haden and Ataulfo polyphenols on the expression of genes involved in apoptosis were investigated within a concentration-range 0-10 mg GAE/L in SW-480 cells. Results indicate that both the extrinsic death receptor and the intrinsic mitochondria pro-apoptotic pathways were targeted at the transcriptional level by Ataulfo and Haden polyphenols, after 24 h of treatment (Figure 10).

Caspase 8 gene expression was up-regulated by Ataulfo at the highest dose (10mg GAE/L) by 2-fold (Figure 10.A), suggesting the contribution of the extrinsic pathway to the induction of apoptosis. However, the expression of Fas did not seem to be regulated within the time frame and concentration range used and/or might be being targeted at post-translational level (43). When used at the highest concentrations, Ataulfo polyphenols up-regulated the transcription of mitochondria-related pro-apoptotic Bax and Bim by 1.3 and 1.7 fold respectively (Figure 10.A), whereas Haden polyphenols only increased Bax mRNA (2.6 fold), without any effect on Bim gene expression (Figure 10.B).

(A)



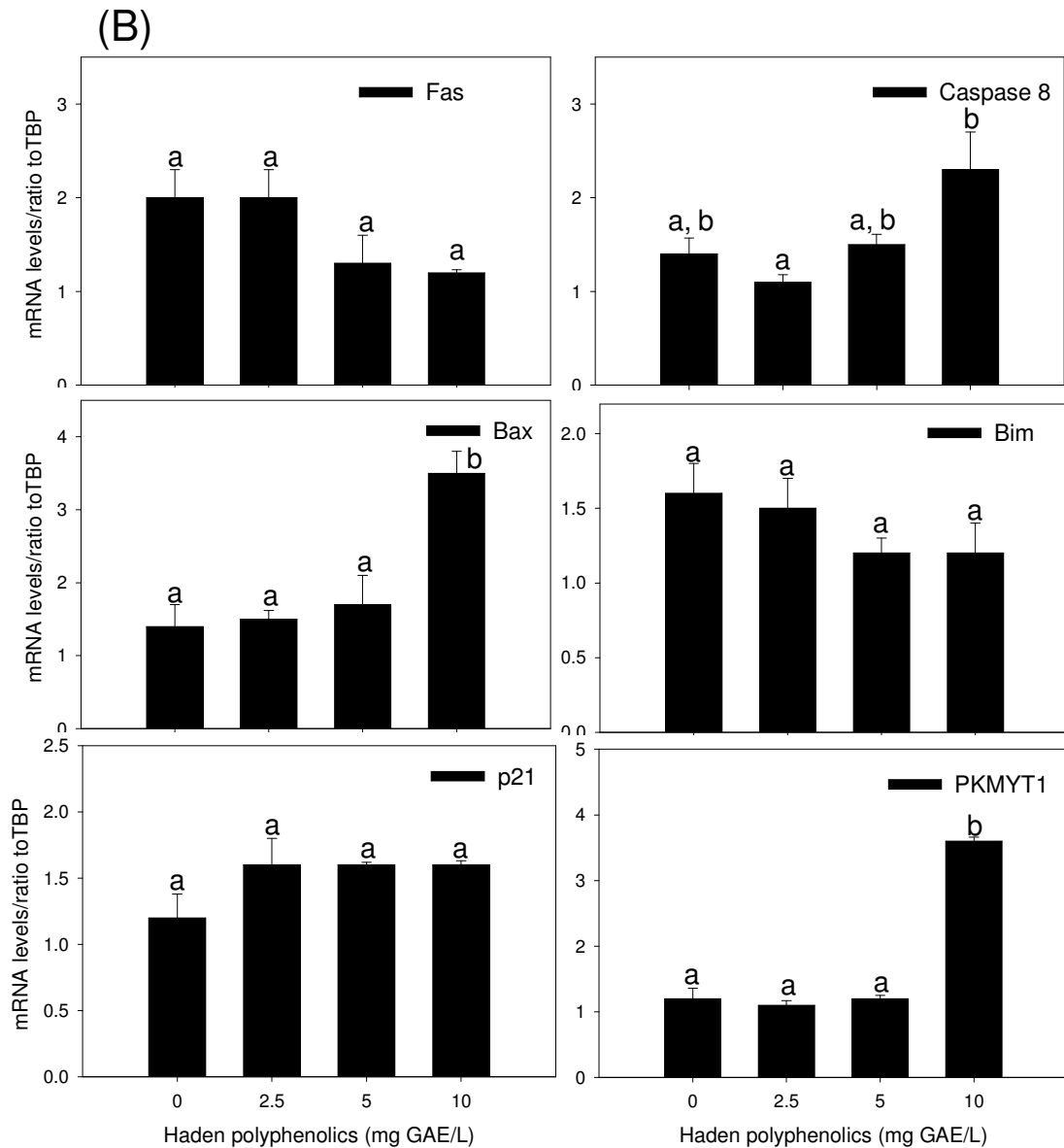


Figure 10. mRNA expression of SW-480 colon cancer cells treated with Ataulfo (A) and Haden (B) polyphenols after 24 h and analyzed by real time PCR as ratio to TATA-binding protein (TBP) mRNA. Values are means \pm SE (n=3). Different letters indicate significance at $p < 0.05$.

Natural plant extracts and phytochemicals appear to target the intrinsic mitochondrial pathway (13, 47) and a crosstalk between the intrinsic and extrinsic pathways occurs (45). This is the first study reporting gene expression regulation of mango polyphenols on colon SW-480 cancer cells. However the main polyphenols

identified in mango, i.e. gallotannins have shown to regulate the pro-apoptotic and cell cycle related proteins on colon (42) and breast cancer cells (48). The structurally related ellagitannins also regulated the expression of cell cycle and genes of the Bcl-2 family members in MDA-MB-231 breast cancer cells (49).

In this study, the effects of mango polyphenols on the expression of genes encoding the cell cycle regulator proteins p21 (Cip1/WAF1) (p21) and the protein kinase-membrane associated tyrosine/threonine 1 (PKMYT1), involved were analyzed. Results showed that Ataulfo and Haden polyphenols do not affect the expression of p21 (Cip1/WAF1) mRNA (Figure 10), which was consistent with no regulation of G₀/G₁ phase for Ataulfo treated-cells, but not consistent with the observed inhibition in G₀/G₁ phase for cells treated with Haden polyphenols (Figure 9.A). Both Ataulfo and Haden polyphenols upregulated PKMYT1 gene expression by 1.4 and 3 fold, respectively. The protein encoded by 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 suppress G2/M transition, which result in G2/M arrest (50).

Individual phytochemicals found in mango have been reported to modulate genes involved in cell cycle regulation, i.e. quercetin down-regulated cyclin D and survivin at both the transcription and protein expression levels in SW480 colon cancer cells (51); penta-O-galloyl- β -D-glucose induced cell cycle arrests in the S and G₁ phases on prostate cancer cells (52), and gallic acid decreased the expression of Cdks *in vivo* (53). As observed with many individual polyphenols, the influence on gene expression by mango polyphenols as a whole 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.

Protective effects against reactive oxygen species (ROS)

Oxidative damage by reactive oxygen species seems to be a crucial event for the initiation of cancer (15), and a chemopreventive agent might interfere with this step and prevent cancer by reducing the ROS production. Therefore the potential of mango polyphenols in protecting the colon cells against reactive oxygen species (ROS) was investigated. Pre-treatment of SW-480 cells with 1.5, 2.5 and 5 mg GAE/L Ataulfo

polyphenols for 24h significantly reduced the 200 μ M H₂O₂ induced-ROS production by ~ 14%, 28% and 70%. Haden polyphenols were less effective in protecting against H₂O₂-induced oxidative damage at 2.5 and 5 mg GAE/L polyphenols, with 14% and 17% reduction of ROS production compared to the control (Figure 11.A). However, the concentration-dependent protection of SW-480 against ROS production was reversed at the highest dose (10mg GAE/L) by both Ataulfo and Haden polyphenols.

Previous findings suggest that cancer cells use ROS signals to drive proliferation and other events required for tumor progression (54). Therefore the ROS generation inhibition may contribute to slow down the tumor progression. The observed increase of ROS in cancer cells caused by mango polyphenols 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 (54).

In contrast, when the non-cancer colonic myofibroblasts CCD-18Co cells were pre-treated with Ataulfo and Haden polyphenols at the same concentration-range (1.5-10 mg GAE/L), a protection against ROS production was achieved in a dose-dependent manner, including the highest concentration of 10mg/L. Within 1.5-10 mg GAE/L, Ataulfo and Haden reduced the ROS generation by 14-25% and 6-16%, respectively (Figure 11B). These results demonstrate that at concentrations which induce oxidative damage in cancer cells (10mg GAE/L), mango polyphenols still protect the non-cancer cells against ROS. Overall, Ataulfo polyphenols were more potent compared to Haden in the protection of non-cancer colon CCD-18Co cells against ROS. These results corroborate previous findings which demonstrated how natural compounds may target the production of ROS in their cytotoxic effects in cancer cells but not in normal cells (55).

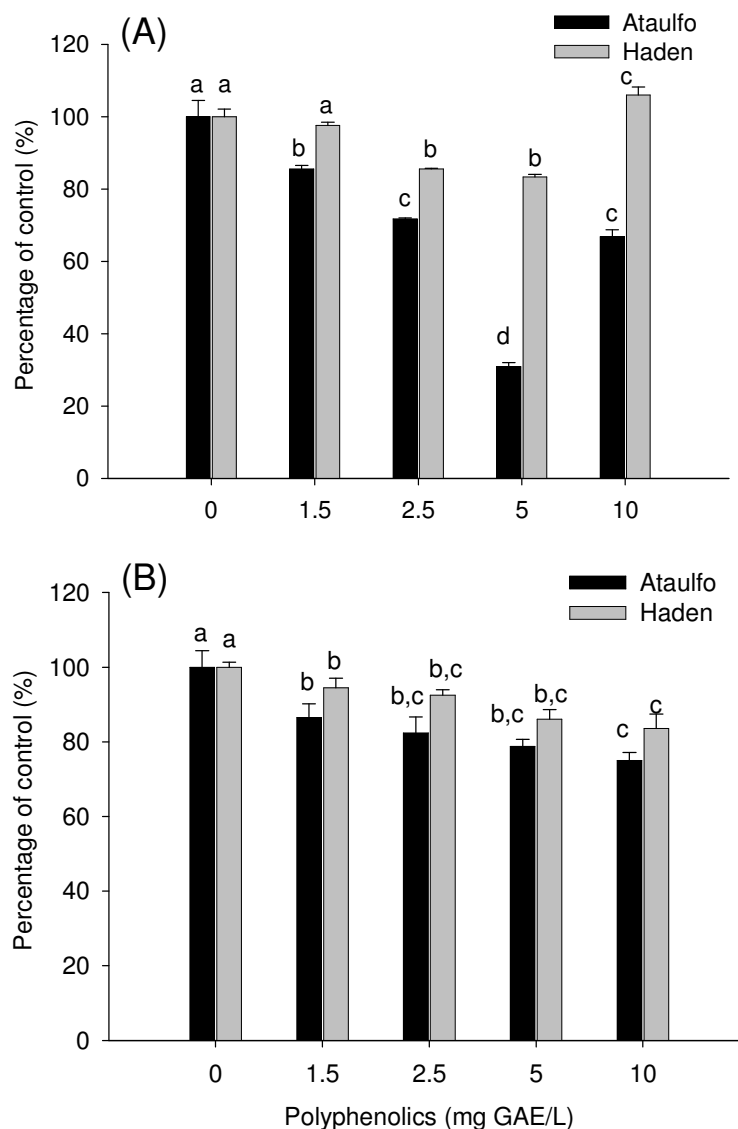


Figure 11: Protective effects of Ataulfo and Haden polyphenols against H₂O₂- induced ROS production on SW-480 cancer cells (A) and CCD-18Co non-cancer cells (B). Cells were pretreated with extract for 24h and exposed to 200μM H₂O₂ for 2h. Values are means ± SD (n=6), different letters indicate significance at p < 0.05.

Conclusion

Polyphenols identified in the edible part of these mango varieties comprise gallic acid, mangiferin and a wide range of high molecular weight hydrolysable tannins. Ataulfo and Haden polyphenols inhibited the growth of human SW-480 colon cancer

cells which were showed high sensitivity to the treatment. SW-480 gene regulation influenced by Ataulfo and Haden polyphenols 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 polyphenols exerted protection of non-cancer CCD-18Co colon cells by lowering the ROS generation in a dose dependent manner. Based on their anti-cancer effects and protective effects in normal cells, but not in cancer cells, mango polyphenols may have a high potential as chemopreventive agents.

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ABSORPTION AND BIOLOGICAL ACTIVITIES OF POLYPHENOLS FROM DIFFERENT MANGO (*MANGIFERA INDICA* L.) VARIETIES AS AFFECTED BY *B*-GLUCOSIDASE HYDROLYSIS

ABSTRACT

Biological activities of polyphenols have been related to their bioavailability. Deglycosylation by β -glucosidases is a critical step in the absorption and metabolism of dietary polyphenol glycosides in humans. This study aimed to evaluate the effect of the hydrolysis of mango polyphenols with β -glucosidase on their antioxidant activity, cancer cell-growth suppression activity and *in vitro* intestinal absorption through human colon adenocarcinoma Caco-2 cell monolayers was evaluated. In addition, the antiproliferative effect of high and low molecular weight polyphenols rich-fractions on colon (SW-480) and breast (MDA-MB-231) cancer cells was studied. Phenolic extracts from mango pulp contained gallic acid, mangiferin, phenolic acid derivatives and gallotannins, which were characterized by HPLC-DAD and HPLC-ESI-MSⁿ analysis before and after enzymatic hydrolysis (0.17 mg β -glucosidase 1000 KU/g mango pulp/ 4 h / 35°C). Phenolic acids including gallic, caffeic, ferulic, *p*-coumaric and *p*-hydroxybenzoic acids consisted the main compounds derived from enzymatic hydrolysis. Caco-2 cell monolayers were incubated for 2h on the apical side with hydrolyzed and non-hydrolyzed mango extracts. Gallic, caffeic, ferulic, *p*-coumaric, vanillic and *p*-hydroxybenzoic acids were detected on the basolateral side for hydrolyzed extract but only gallic acid was detected for the non-hydrolyzed extract. High molecular weight polyphenols, mangiferin and gallotannins, were not transported. Mango pulp polyphenols (control) from all varieties inhibited the proliferation of HT-29 colon (0-27 μ g of gallic acid equiv/mL) and MDA-MB-231 breast (0-24 μ g GAE/ mL) human cancer cells by up to 99.8 and 89.9 %, respectively. Despite enhanced absorption facilitated by enzymatic hydrolysis, a significant increase in antioxidant activity, phenolic content and antiproliferative effects on breast and colon cancer cells was not observed. Additionally, both high (422; 788-1852 Da) and low (138-194 Da) molecular weight polyphenols rich-fractions equally inhibited cell proliferation of colon and breast cancer cells at the same extent (0-20 μ g of gallic acid equiv/mL), which may indicate

that the anti-cancer efficacy of mango polyphenolics is not dependent on enzymatic hydrolysis.

These results corroborate previous findings from *in vivo* studies, which suggest that the most of mango polyphenols are not absorbed intact through the small intestine, but may be hydrolyzed by intestinal enzymes into low molecular weight aromatic acids, which would be later absorbed; or when polyphenols are not absorbed, they likely reach the large intestine, modulating the gut microflora, and thus they contribute to reduce the risk of colon carcinogenesis.

Overall, polyphenols from several mango varieties exerted anti-cancer effects, and these effects may not require enzymatic hydrolysis by β -glucosidase.

Keywords: Polyphenols, *in vitro* absorption, Caco-2 cells, cell proliferation, mango, *Mangifera indica*, β -glucosidase, enzymatic hydrolysis.

INTRODUCTION

Mangoes (*Mangifera indica* L.) are one of the most important tropical fruits marked in the world, with a global production exceeding 33 million tons in 2007. Moreover, mangoes consist one of the most consumed fruits in Brazil due to sensorial characteristics and nutritional value, ranking the seventh position worldwide in terms of production in 2007 (1).

Furthermore, there has been increasing interest in understanding the mango phytochemicals biological properties due to their health-promoting characteristics such as antioxidant, antitumoral, anti-inflammatory, and immunomodulatory activities (2-7). Recently, mango fruit has been listed as a nutrient-rich fruit into the unofficial classification and so-called superfruit due to its considerable content of carotenoids, specially β -carotene, vitamin C and polyphenols, including gallic acid, gallotannins, quercetin and kaempferol glycosides as well as xanthone-C-glucosides (8-9).

In traditional medicine, the use of mango extracts as herbal drugs is widespread. *Vimang*, a mango stem bark extract, have been used at least 10 years in Cuba medicine with effectiveness against several diseases, like cancer (6). Moreover, studies *in vitro* and *in vivo* performed with individual polyphenols found in mangoes have been shown antitumor activities, such as induction of apoptosis, inhibition of tumor growth and angiogenesis (10-15). Recently, studies performed with mango pulp extracts have been demonstrated their chemopreventive potential (16-20), although conclusion regarding the biological activities of the mango phenolic fraction are still not well elucidated (17-18), since other bioactive compounds like L-ascorbic acid and carotenoids have been conjunctly evaluated (16-18).

Simple phenolic compounds like phenolic acids are known to be directly absorbed through the gut barrier by passive diffusion. However, more complex compounds like flavonoids and procyanidins are typically transformed in the colon by intestinal microflora into a wide array of bioactive low molecular weight metabolites, which are later absorbed through the gut barrier (21). In humans, it has been known that β -glucosidase activity has an important role in enhancing polyphenols absorption by catalyzing the hydrolysis of O-linked β -glycosidic in human body (22). Therefore, the

health benefits from phenolic consumption are also attributed to their bioactive metabolites and also to the modulation of the intestinal microflora (23).

Gallotannins represent the major compounds found in mango pulp, with size ranging from 332 (mono-*O*-galloyl-glucose) to over 1852 Da (undeca-*O*-galloyl-glucose) or large (9). Considering that punicalagin (1084 amu) was reported to be the highest molecular weight compound absorbed intact through the gut barrier after oral ingestion (21, 24), it is assumed that higher molecular weight polyphenols could not be absorbed intact in the body. However, limited bioavailability of these compounds might not limit their biological properties, since the highest local concentration of these compounds is found in the gut lumen, where polyphenols may act protecting colon carcinogenesis (23, 25-27). Therefore, it is relevant to measure the biological activity of polyphenols on cultured cells or isolated tissues in their form present in food, reflecting the potential health benefits associated with the consumption of the entire fruit. At the same time, it is also important to identify their metabolites and test their respective biological activities, since the hydrolysis of glycosides and further gut bacterial transformation of aglicones may lead to the production of more or less biologically active compounds (23).

This study aimed to evaluate the effect of hydrolysis of mango polyphenols with β -glucosidase on their *in vitro* intestinal absorption, antioxidant activity and cancer growth suppression activity. Results from these investigations are aimed at promoting efforts to understand the health-promoting properties of mango polyphenols.

MATERIALS AND METHODS

Plant material

Commercial varieties of mango (*Mangifera indica* L) Francis Kent, Ataulfo, Tommy Atkins and Haden were kindly donated by the National Mango Board, USA. Upon arrival at Texas A&M University, seeds and peels were removed from the fruit and the pulp was blended, homogenized and storage at -80°C until use.

Extraction of polyphenols

Frozen mango pulp (150g) was thawed and mixed with 500 mL of a 1:1:1 (v/v/v) ethanol: methanol: acetone solution, filtered with cheesecloth and whatman #1 filter paper . Solvents were evaporated at 40°C using a rotavapor (Büchi, Switzerland) and the aqueous extract was mixed with 200mL of methanol and partitioned into hexane (1:4, v/v) in order to remove carotenoids. The hexane phase was removed and the methanol phase centrifuged at 2000RPM for 10 min at 7 °C to remove insoluble precipitates. The methanol was completely evaporated using a rotavapor at 40 °C and polyphenols were concentrated on a C18 Waters Sep-Pak Vac 35cc 10g 20 cm³ minicolumns (Waters Corporation, Milford, MA) previously conditioned with acidified methanol (0.01%) and nanopure water. The aqueous extract was loaded onto the C18 cartridge and washed with distilled water. Polyphenols bound to the matrix were eluted with 100% methanol (bound fraction). The unbound fraction was partitioned three times into ethyl acetate (EtAc) (1:4, v/v) to recover non-adsorbed polyphenols, such as phenolic acids. Bound and unbound fraction were combined and completely evaporated under vacuum at 40 °C. In order to create optimal conditions to β -glucosidase activity, polyphenols were redissolved in 0.1M citric acid buffer (pH 5.0) up to a known volume (50mL) (Figure 12). The total phenolic content was measured spectrophotometrically by the Folin-Ciocalteu assay (28) against an external standard of gallic acid (mg/L).

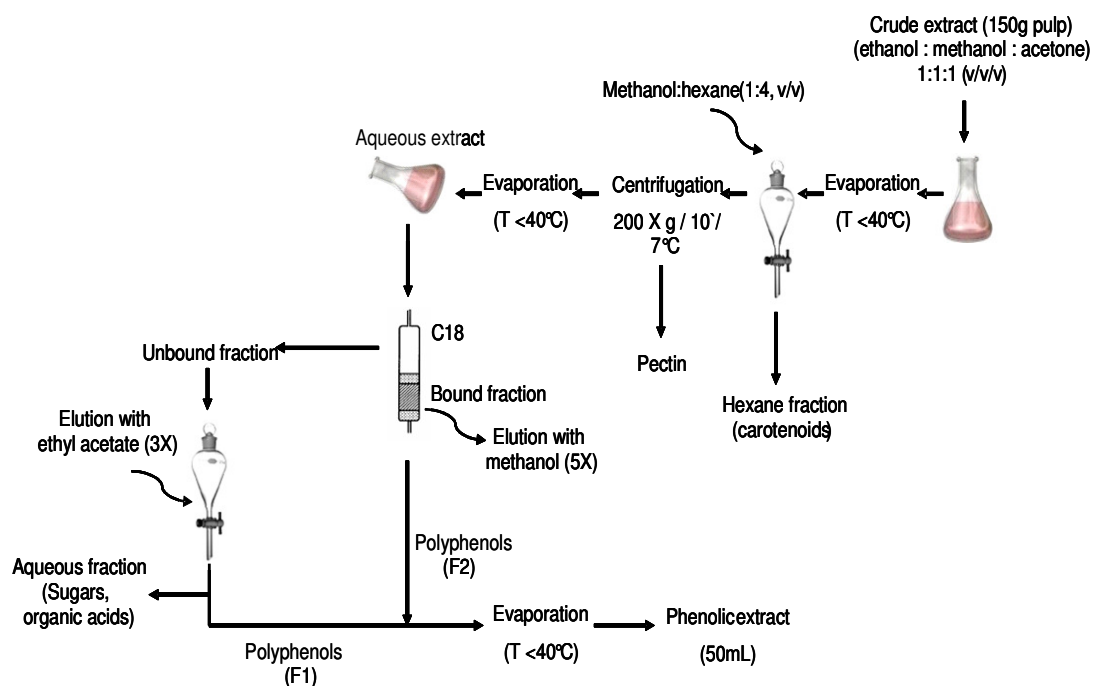


Figure 12. Procedure used for extraction of mango polyphenols.

Enzymatic hydrolysis

The bioavailability of dietary polyphenols is related to their absorption and metabolism in the body (29). Although enzymes such as tannase have been extensively used to hydrolyze tannins due to its higher efficacy in a complete degradation of the glycoside and simultaneously release of gallic acid (8), the enzymatic hydrolysis using β -glucosidase had the purpose of simulate the enzymatic activity in human gut, since small intestine requires exogenous (luminal) deglycosylation by several β -glucosidases, or cooperativity between epithelial transportes and intracellular β -glucosidases in order to facilitate the absorption of some phenolic compounds such as flavonoid glycosides (21, 30).

The phenolic extract (20mL, 60g pulp) was completed with 0.1M citric acid buffer (pH 5.0) up to a known volume, which corresponded to the volume equivalent in pulp (60mL) and incubated with β -glucosidase 1000KU (> 2500 units/mg enzyme)

(MP Biomedicals, Solon, OH) at the proportion of 0.17 mg enzyme/g mango pulp for 4 h at 35° C. The same volume of extract was incubated without enzyme (control) following the same procedures described above. In order to stop enzymatic activity, phenolic extract was boiled for 5 min and chilled on ice immediately. Extracts were sonicated and reloaded onto a C18 Waters Sep-Pak Vac 6cc 1g 2 cm³ minicolumns (Waters Corporation, Milford, MA) following the same procedure described in the previous item. The bound and unbound fractions were mixed, solvents completely evaporated under vacuum at 40 °C and polyphenols redissolved in 5mL of methanol (12g pulp/mL phenolic extract). The extracts were stored at -80°C until needed. Equal volumes of the control and hydrolyzed extract were evaporated in a SpeedVac concentrator (Thermo Fisher Scientific, San Jose, CA). Polyphenols (Control and Hydrolyzed) were reconstituted with the same volume of HBSS buffer pH=6.0 for the transepithelial transport model assay or in culture medium for cell proliferation assays.

Fractionation of mango phenolic extract

Polyphenols, the most abundant antioxidants in human diet, show considerable differences in their structure, which is directly associated to their bioavailability, antioxidant activity and anticancer-related properties (31-32). In order to compare the effect of the molecular weight range of mango polyphenols on the antioxidant and cancer cell growth suppression activities, Ataulfo hydrolyzed extract (5 mL) was fractionated into low and high molecular weight polyphenols-enriched fractions, as described in Figure 13. Polyphenols were bound onto a C18 Waters Sep-Pak Vac 6cc 1g 2 cm³ minicolumns (Waters Corporation, Milford, MA), previously conditioned with acidified methanol and water, and sequentially eluted with methanol 25% (v/v) and methanol 100%(v/v). The methanolic fraction (F1) was evaporated and the volume was made to 5 mL with methanol. The unbound fraction was partitioned three times into ethyl acetate (EtAc) (1:4, v/v). The MeOH 25% fraction was evaporated at < 40°C and the aqueous extract was reloaded again on C18 minicolumns. The polyphenols were washed with 100% methanol and mixed with the ethyl acetate fraction. The solvents were completely evaporated and the volume made to 5 mL with methanol (F2). F1 consisted mainly of high molecular weight (HMW) polyphenols, whereas F2 was

enriched with polyphenols within a lower molecular weight range (LMW). The extracts were storage at -80°C until needed. Equal volumes of F1 and F2 fractions were evaporated in a SpeedVac concentrator (Thermo Fisher Scientific, San Jose, CA). Polyphenols were reconstituted in culture medium for cell proliferation assays.

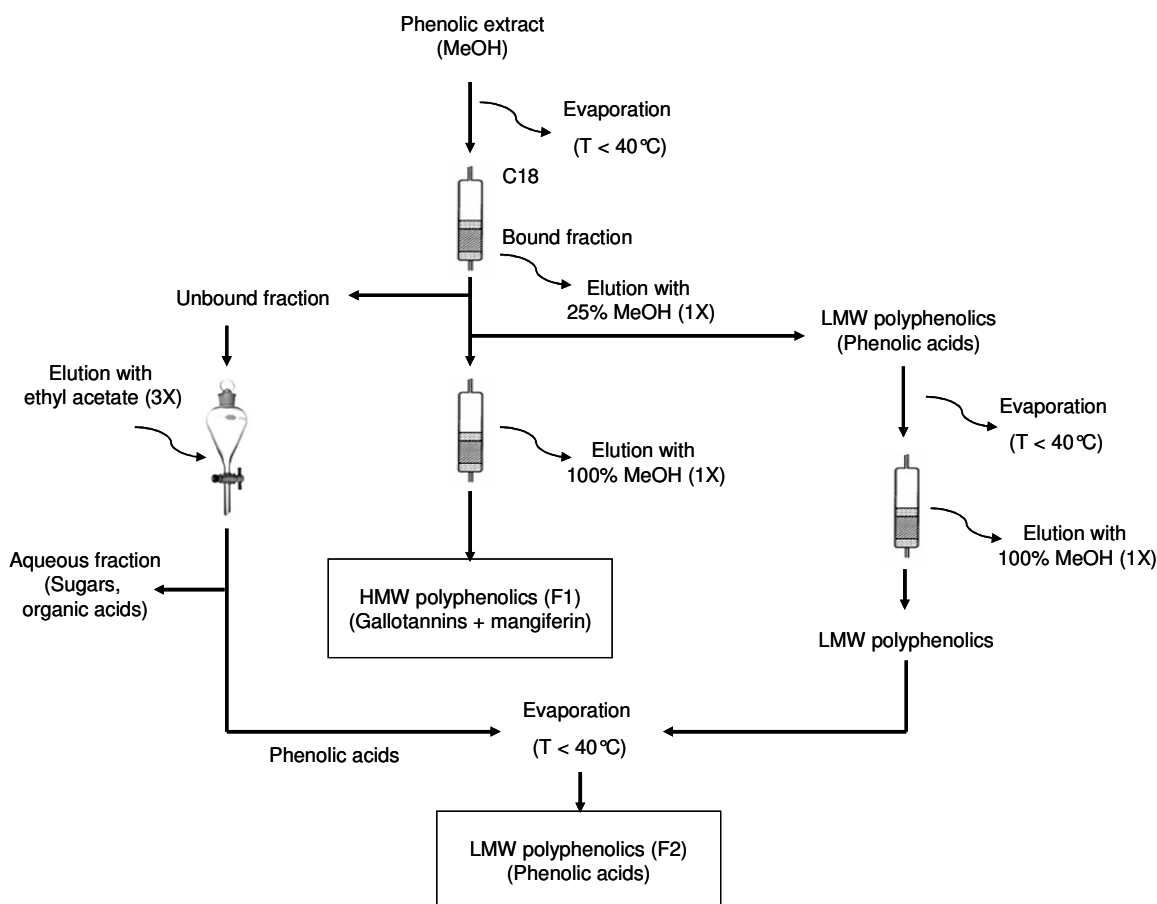


Figure 13. Procedure used for fractionation of mango phenolic extract.

HPLC-DAD and HPLC-ESI/MSⁿ Analysis

Polyphenols were analyzed using a Waters Alliance 2690 (Milford, MA, USA) HPLC system equipped with a Symmetry ® 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 mobile phases of 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 600 nm. Phenolic compounds were separated with a gradient program that ran phase A from 100-70% in 20 min; 70-50% in 10 min; 50-30% in 20 min; and 30-0% in 20 min, 100% phase B in 15min, and returning to the original composition in 3 min for column equilibration. Monitoring was performed at 250 nm (p-hydroxybenzoic acid and derivatives), 280nm (gallotannins) and 340nm (hydroxycinnamic acids, flavonols and xanthenes).

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). Polyphenolic extracts were analyzed using a Waters Alliance 2690 (Milford, MA, USA) HPLC system equipped with 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. Phenolic compounds were separated using 0.5% formic acid in water (solvent A) and 0.5% formic acid in acetonitrile (solvent B) as mobile phases, at a flow rate of 0.4 mL/min. Gradient program ran phase A for 5 min, from 100-70% in 25 min; 70-50% in 15 min; 50-0% in 25 min; and returning to original composition in 8 min. 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.

Identification of phenolic compounds was performed by their comparison of retention times and UV-visible spectral characteristics with those obtained from standards (Sigma-Aldrich, St Louis, MO) and mass spectrometric properties.

Antioxidant capacity (ORAC assay)

Hydrophilic antioxidant capacity of mango phenolic extracts, excluding further contribution of L-ascorbic acid, reducing sugars, and soluble proteins, was measured using the oxygen radical absorbance capacity (ORAC) assay, as described by Talcott and Lee (2002) (33). Peroxyl radicals were generated by 2,2'-azobis(2-amidinopropane) dihydrochloride, and fluorescence loss was monitored on a FLUOstar fluorescent microplate reader (BMG Labtech Inc, Durhan, NC) at 485 nm excitation and 538 nm emission. Phenolic extracts were equally diluted in pH 7.2 phosphate buffer in a 50, 100

and 200-fold. Data were expressed in μmol Trolox equivalent/100g mango pulp or μmol Trolox equiv/L phenolic extract.

Cell culture

Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured according ATCC recommendations. HT-29 human colon adenocarcinoma cells were cultured using McCoy's 5A modified medium with 1.5 mM L-glutamine (Gibco™, Invitrogen Corp., Grand Island, NY). 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. Culture media were supplied by Invitrogen (Gibco™, Invitrogen Corp., Grand Island, NY). Both media were supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% Penicillin-Streptomycin antibiotic mix. Cells maintained at 37 °C with a humidified 5% CO₂ atmosphere for propagation. A 2.5% FBS was used in culture medium for cell proliferation experiments. Cells were seeded overnight (1.5×10^4 cells/well) in a 24 well plates and treated with the same dilutions of hydrolyzed and control phenolic extracts from the varieties Haden, Francine, Kent, Ataulfo and Tommy Atkins, incubated with and without β -glucosidase.

Cell proliferation

Total cell numbers were indicative of the cell growth of cancer cells and the antiproliferative effects of mango polyphenols were determined following 48h of incubation, using a Beckman Coulter Particle Counter (Fullerton, CA). The difference in cell-number between final incubation time (48h) and the incubation start (0h) represents net growth. The polyphenols ability to inhibit the cell growth by 50% was expressed in IC₅₀, as mg gallic acid equivalent/L phenolic extract.

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% CO₂ in Dulbecco's modified Eagle's medium (1X) high glucose (DMEM) containing 20% fetal bovine serum (FBS), 1% 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 were seeded (100,000 cells/insert) 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. Fresh medium was replaced every 2 days. Cells (passages 5-15) were grown and differentiated to confluent monolayers for 21 days (34). Cells confluence and monolayers integrity were evaluated by the transepithelial electrical resistance values (TEER), measured with an EndOhm Volt ohmmeter equipped with a STX-2 electrode (World Precision Instruments Inc., Sarasota, FL) after 30 min of incubation with HBSS and 2h of incubation with the treatments. Monolayers with TEER values >450 Ω cm² at the beginning and >225 Ω cm² at the end were used for transport experiments. Additional control of the monolayers integrity was performed using lucifer yellow, a very hydrophilic probe which can permeate the cells only through the paracellular route. This probe (100 µg/ml) was added to the apical side, incubated for 1h 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.

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 polyphenols (control and hydrolyzed phenolic extracts) were equally diluted in HBSS adjusted to pH 6.0 (□□ 3,000 mg

gallic acid equivalent/L) to be used in cell culture. After incubation at 37°C for 30min of Caco-2 cells, 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 (-80°C), and analyzed within one week. Samples were filtered through 0.45µm PTFE membranes (Whatman, Florham Park, NJ) and injected directly into the HPLC-PDA system.

Statistical Analysis

Quantitative data represent means with the respective standard deviation (SD) of three or more replicates. Statistical analysis was performed using JMP 5 statistical software (SAS Institute, 2002). One-way analysis of variance (ANOVA) and Tukey-Kramer HSD test ($p < 0.05$) were used to compare the effect of different varieties on the analyzed variables and paired t-student to compare the effect of polyphenols hydrolysis on cancer cell growth suppression, total phenolic content and antioxidant activity. Pearson correlation coefficient was estimated to determine the correlation between phenolic content and antioxidant capacity.

RESULTS AND DISCUSSION

Mango pulp phenolic content and antioxidant activity

The total phenolic content (TPC), analysed by Folin-Ciocalteu assay, from the mango varieties Ataulfo (At), Haden (Ha), Kent (Ke), Francis (Fr) and Tommy Atkins (To) ranged from 15.1 to 55.5 mg GAE/100g pulp, according to Table 5, and At contained the highest TPC. Previous studies showed that mango TPC may range from 48.4 to 294 mg GAE/ 100g (35-37), as influenced by variety, ripening stage, geographic production, harvesting, processing and storage conditions. These factors might affect the mango phytochemical composition, and consequently, their antioxidant capacity (8, 37). Thus, determination of total polyphenols may be relevant when analyzing the antioxidant and anticarcinogenic activities from fruit, since both are in part attributable

to the polyphenols content. Our results showed that the antioxidant activity (AA) of mango pulp phenolic extract, determined by ORAC assay (33), correlated to the TPC ($r = 0.96$), and At polyphenols showed to be the most effective in protecting the fluorescent molecule from the peroxy radicals-induced oxidative degeneration ($288.7 \pm 10.2 \mu\text{mol Trolox equivalent}/100\text{g pulp}$ respectively) (Table 5). These results are in accordance with previous findings reported by Kim and co-workers (2007) (36). They showed that the decrease of mango AA positively correlated ($r = 0.98$) to the reduction of the TPC during ripening, even though the presence of other compounds in the mango clarified juice such as L-ascorbic acid, reducing sugars and soluble proteins have contributed to increase the ORAC value (36, 38). Therefore, in addition to the influence of ripening stage, variety and other factors as previously described in literature, the contribution of non-phenolic compounds for the AA of mango pulp extracts might also explain the higher ORAC values reported in literature, ranging from 326 to 1002 $\mu\text{mol TE}/100\text{g pulp}$ (36, 38).

Table 5. Total phenolic content and antioxidant activity of polyphenols extracted from different mango varieties.

Mango Variety	Phenolic content (mg GAE/L)	Phenolic content (mg GAE/100g pulp)	Antioxidant activity ($\mu\text{mol TE}/100\text{g pulp}$)
Ataulfo	1631.0 ± 48.4^a	55.5 ± 1.6^a	288.7 ± 10.2^a
Francis	577.3 ± 7.7^b	19.2 ± 0.3^b	198.2 ± 14.4^b
Tommy	454.0 ± 28.6^c	15.1 ± 0.9^c	180.8 ± 12.6^{bc}
Haden	498.1 ± 3.4^c	16.6 ± 0.1^c	164.0 ± 17.2^c
Kent	547.6 ± 22.3^{bc}	17.8 ± 0.0^{bc}	160.3 ± 4.0^c

Data are means of three or more independent determinations \pm SD. Means with different letters in the column are statistically different at $p < 0.05$ (Tukey-Kramer HSD test).

Enzymatic hydrolysis of mango pulp polyphenols

Phenolic content and antioxidant activity

The enzymatic hydrolysis of polyphenols extracted from the varieties Fr, Ha and To, following 4h of incubation with β -glucosidase at 35 °C, did not statistically affect the TPC and the AA from their phenolic extracts (Table 6). On the other hand, the hydrolyzed extracts of the varieties At and Ke showed a 28 % and 34.2 % lower than

those from control extracts, respectively (Table 6). This reduction may be associated to the formation of a precipitate, which could be visually observed following 4h of mango polyphenols incubation with β -glucosidase and was latter removed following elution of polyphenols onto C18 mini-column. However, even though the precipitation formation may occur likely related to tannin-protein interaction, in biological systems the same phenomena may not occur, since the stoichiometry of interacting species would tend to lead to soluble complexes only (39).

Table 6. Total phenolic content and antioxidant activity of hydrolyzed and control mango phenolic extracts.

Cultivar	Phenolic content (mg GAE/L)		Antioxidant activity (μ mol TE/L)	
	Hydrolysed	Control	Hydrolysed	Control
Ataulfo	2953.9 \pm 0.7 ^a	4102.0 \pm 8.2 ^b	15365.6 \pm 4.0 ^a	23337.5 \pm 42.7 ^b
Tommy Atkins	1345.8 \pm 8.5 ^a	1344.7 \pm 7.6 ^a	16113.5 \pm 101.3 ^a	15870.4 \pm 92.1 ^a
Francis	1732.1 \pm 27.7 ^a	1655.9 \pm 15.4 ^a	17980.0 \pm 286.0 ^a	17093.7 \pm 158.9 ^a
Kent	1193.6 \pm 69.8 ^a	1814.6 \pm 4.3 ^b	10749.6 \pm 429.0 ^a	15341.4 \pm 38.5 ^b
Haden	1159.8 \pm 51.9 ^a	1205.7 \pm 14.4 ^a	11457.9 \pm 412.4 ^a	11309.4 \pm 142.6 ^a

Data are means of three or more replicates \pm SD. Means with different letters in the line are statistically different at $p < 0.05$ (paired t-student test).

Hydrolyzable tannins consist of a polyol (typically β -D-glucose) partially or totally esterified with phenolic groups such as gallic acid (gallotannins) or ellagic acid (ellagitannins) (40-41). In gallotannins, galloyl groups are hydrophobic sites which can interact with aliphatic side chains of amino acids through hydrophobic association to form complexes (42). Interactions of tannins and proteins are essentially a dynamic surface phenomenon, generally reversible, which involves most frequently hydrophobic effects, which are reinforced by hydrogen bonds. Gallotannins bound more to proteins (histone, bovine serum albumin, casein and gelatin) and phospholipids (L- α -lecithin, L- α -cephalin and sphingomyelin) than to sugars (42). Relative affinities of tannins for different proteins may vary as much as 10000-fold (43-44). Tannins have lower affinity for compact globular proteins and preferential binding to proline-rich proteins, possibly due to more open and flexible conformations, which provides better access to polyphenol-binding sites (43, 45). Therefore, the polypeptide chain characteristics of some β -glucosidases, which contain highly conserved peptide motifs like Thr-Phe-Asn-

Glu-Pro (TFNEP) and Ile-Thr-Glu-Asn-Gly (ITENG) (46-48), suggest favorable tannin-interaction affinities. Binding affinities to protein, phospholipids and sugars are, in part, function of molecular size of gallotannins, which is enhanced by the addition of each galloyl group and reaches a maximum in the flexible disk-like structure of penta-*O*-galloyl-*D*-glucose (42). These evidences led to the inference that high molecular weight gallotannins should precipitate proteins more effectively. However, some studies suggest that this rule is an oversimplification and does not apply to all tannins because the biochemical properties of gallotannins isomers with the same molecular weight, including susceptibility to hydrolysis as well as ability to precipitate proteins, are structure-dependent (49-50). Studies have been shown that conformational mobility and flexibility of either the tannin or the protein are as significant as molecular size. Conformational freedom is necessary for strong binding, and therefore, gallotannins should precipitate certain proteins more efficiently than ellagitannins, which are conformationally rigid. Based on these evidences, the presence of gallotannins in At and Ke might explain the reduction on TPC after hydrolysis as a result of protein-tannin interaction-derived complex, although no significant reduction in TPC from the other varieties was found.

Likewise, the antioxidant capacity of Fr, Ha and To was not significantly affected by enzymatic hydrolysis (Table 6). In contrast, as a consequence of the reduction in the amount of hydroxyl groups present in the phenolic extract likely associated to tannin-protein interaction, a significant decrease ($p < 0.05$) in the antioxidant activity of At and Ke hydrolyzed phenolic extracts was observed when compared to AA from control extracts (Table 6). These results suggest considerable contribution of hydrolyzable tannins to antioxidant capacity of mango pulp.

HPLC-DAD and HPLC-ESI-MSⁿ analysis

Mango pulp polyphenols from phenolic extracts (\square 1000 ppm) from all varieties were characterized by HPLC-DAD and HPLC-ESI-MSⁿ analysis. Phenolic profile from control extracts varied depending on the variety (Figure 14). Phenolic acids and the xanthone mangiferin were characterized unambiguously following HPLC analysis. Mango pulp consisted of free forms and derivatives of hydroxycinnamic acids

caffeic, ferulic and *p*-coumaric acids, as well as free and esterified forms of gallic acid and *p*-OH-benzoic acid. Gallic and ferulic acids were detected in all varieties; caffeic acid was found in At, Ha and Ke; *p*-coumaric acid was detected in At, To, Fr; and Ke and *p*-OH-benzoic acid just detected in Ha. Mangiferin was identified in the most of the varieties, but not in Francis (Table 7). Gallotannins, which show UV spectral similarities to gallic acid at 280 nm comprised the predominant polyphenols in mango pulp, which chemical structure varied from one (mono-*O*-galloyl-glucoside) to eleven (undeca-*O*-galloyl-glucoside) units of gallic acid esterified in a glucose core, as characterized by HPLC-ESI-MSⁿ analysis (Table 7, Apendice). Similarly, gallotannins with 4-7 galloyl groups surrounding a glucose core were previously identified in mango pulp from the variety Tommy Atkins (9). Gallotannins with molecular weight higher than 939 showed elution pattern after 40 min (Figure 14). Even though flavonol and xanthone glycosides were hypothesized by monitoring at 340 nm, flavonol-3-*O*-glycosides, represented by quercetin and kaempferol glycosides, as well as xanthone-C-glucosides, as previously found in mango pulp (8-9), were not identified in our extracts.

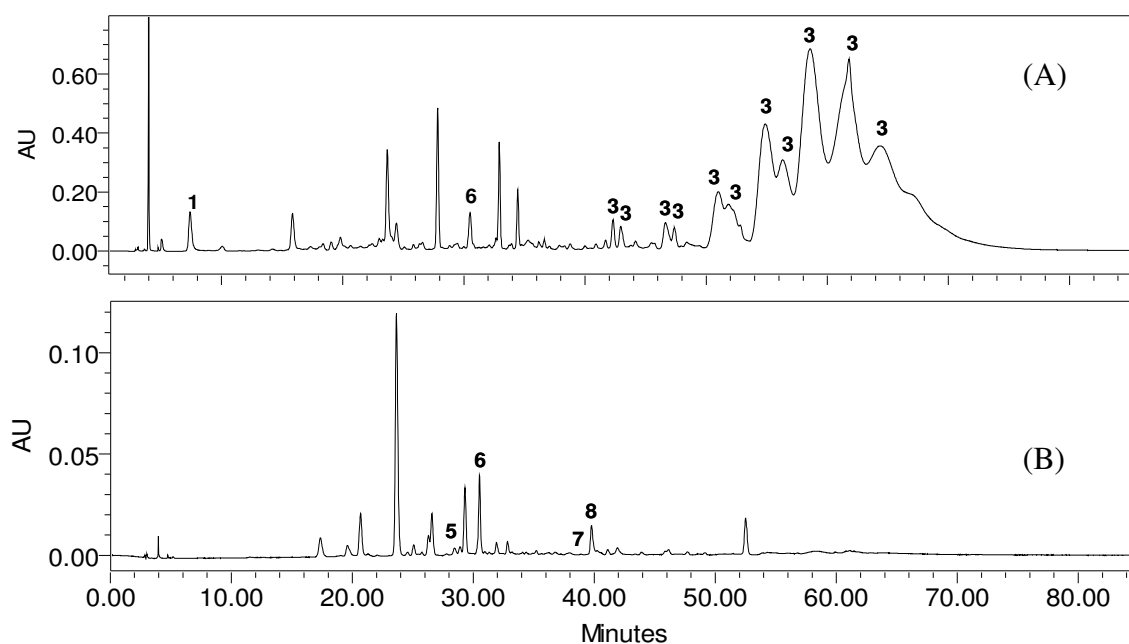


Figure 14. Representative chromatograms at 280 nm (A) and 340 nm (B) of Ataulfo mango pulp polyphenols from control phenolic extracts. Peak assignments: (1) gallic acid; (2) p-OH-benzoic acid; (3) gallotannins ; (4) vanillic acid; (5) caffeic acid; (6) mangiferin; (7) p-coumaric acid; (8) ferulic acid.

Table 7. HPLC-DAD and HPLC-ESI-MSⁿ of mango phenolic extracts (control) from the varieties Ataulfo, kent, Francis, Tommy Atkins and Haden.

Class	Polyphenols	Ataulfo	Haden	Kent	Francis	Tommy Atkins
Phenolic acids	Gallic acid	✓	✓	✓	✓	✓
	p-coumaric-acid	✓	nd	✓	✓	✓
	Ferulic acid	✓	✓	✓	✓	✓
	Caffeic acid	✓	✓	✓	nd	nd
	p-OH-benzoic acid	nd	✓	nd	nd	nd
	Vanillic acid	nd	nd	nd	nd	nd
Xanthones	mangiferin	✓	✓	✓	nd	✓
Gallotannins	mono- <i>O</i> -galloyl- glucose	✓	✓	✓	✓	✓
	di- <i>O</i> -galloyl- glucose	nd	✓	nd	nd	nd
	tetra- <i>O</i> -galloyl- glucose	nd	✓	✓	✓	nd
	penta- <i>O</i> -galloyl- glucose	✓	✓	✓	✓	✓
	hexa- <i>O</i> -galloyl- glucose	✓	✓	✓	✓	✓
	hepta- <i>O</i> -galloyl- glucose	✓	✓	✓	✓	✓
	octa- <i>O</i> -galloyl- glucose	✓	✓	✓	✓	✓
	nona- <i>O</i> -galloyl- glucose	✓	✓	✓	✓	✓
	deca- <i>O</i> -galloyl- glucose	✓	nd	nd	nd	nd
	undeca- <i>O</i> -galloyl-glucose	✓	nd	nd	nd	nd

nd= not identified.

Most of dietary polyphenols are found in the form of esters, glycosides, or polymers that cannot be directly absorbed in their intact form through small intestine. Thus, deglycosylation of polyphenols by several β -glucosidases are known to facilitate the absorption of some phenolic compounds as aglicones (21, 30). The human cytosolic β -glucosidase (hCBG), a group of nine enzymes with similarities in the amino-acid and structural features related to their substrate specificities, are present in the cytosol of liver, spleen, kidney, small intestine and lymphocytes of mammals (51), and β -glucosidase activity has been related to the intestinal absorption of glycosides *in vivo* and through Caco-2 cell monolayers (52-57).

As a result of *in vitro* enzymatic hydrolysis by β -glucosidase, changes in the chemical structure and concentration of individual polyphenols were observed, mainly regarding the profile of phenolic acids derivatives. On the other hand, no significant structural changes were observed in gallotannins, which remains as the major polyphenols in hydrolyzed extracts (Figure 15).

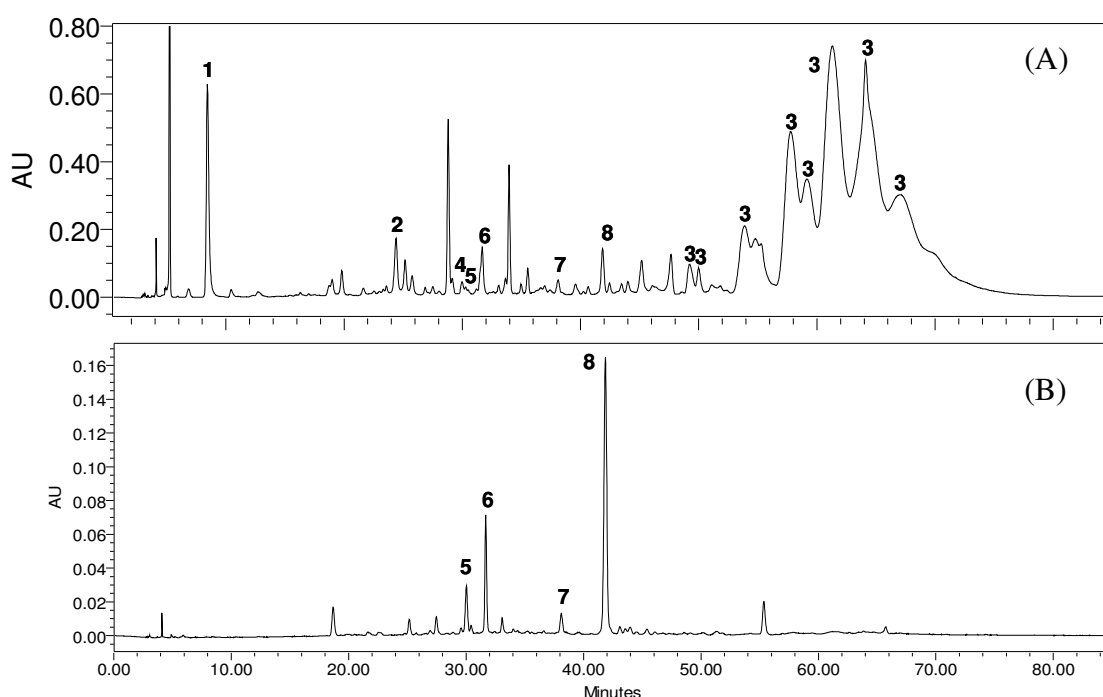


Figure 15. Representative chromatograms at 280 nm (A) and 340 nm (B) of Ataulfo mango pulp polyphenols from hydrolyzed phenolic extracts. Peak assignments: (1) gallic acid; (2) *p*-OH-benzoic acid; (3) gallotannin; (4) vanillic acid; (5) caffeic acid; (6) mangiferin; (7) *p*-coumaric acid; (8) ferulic acid.

Caffeic and *p*-coumaric acids, not previously detected in control extracts from Fr and Ha, respectively, were identified after hydrolysis. Indeed, derivatives of cinnamic acids, mainly represented by caffeic, ferulic, sinapic and *p*-coumaric acids, are rarely found in free form, but in general, they are esterified with quinic, tartaric acids or carbohydrates derivatives (29). Also, *p*-hydroxybenzoic acid was detected after hydrolysis in At and Fr extracts. Hydroxybenzoic acids are commonly found in a limited distribution in foods and have as the main representatives gallic, ellagic, protocatechuic and 4-hydrobenzoic acids (29, 58).

Vanillic acid, not previously identified in mango pulp, was identified in hydrolyzed extracts from At and Fr. This phenolic acid is a catabolic product of ferulic acid degradation, which was likely converted due to enzymatic treatment conditions like pH, or by enzyme, since some β -glycosidases have the ability to convert ferulic acid to

vallinic acid (58). Therefore, it is not hypothesized the presence of this compound in mango pulp.

Despite the considerable source of gallic acid due to the predominance of gallotannins with high level of galloylation in mango pulp, little increase in its concentration was observed (Table 8), suggesting that β -glucosidases was not able to completely hydrolyze gallotannins present in mango pulp. It might be related to the higher amount of gallic acid esterified in the glucose moiety or linked with another moiety of gallic acid via depsidic bond. Indeed, in contrast to tannases, which can hydrolyze ester and depsidic bonds between two galloyl residues in hydrolysable tannins (59-61), β -glucosidases catalyze only the hydrolysis of O-linked β -glycosidic bonds at the non-reducing end of carbohydrates with retention of anomeric configuration (51). Although these enzymes are capable of deglycosylate a broad variety of glycosides including aryl- and alkyl- β -D-glycosides, their physiological function may varies greatly depending upon their origin (plants, fungi, animals or bacteria) and substrate specificity (22). Regarding flavonoids, for instance, the human cytosolic β -glucosidase shows high specificity for 4- and 7-glucosides of isoflavones, flavonols, flavones and flavanones, but does not hydrolyze 3-linked flavonoids glucosides (22). However, the mechanisms related to the hydrolysis of hydrolyzable tannins by β -glucosidases are still unclear.

Table 8. Effect of enzymatic hydrolysis on the phenolic acids profile of mango phenolic extract.

Class	Polyphenols	Increase (fold) in polyphenols area after hydrolysis				
		Ataulfo	Haden	Kent	Tommy Atkins	Francis
Phenolic acids	Gallic acid	14.1	1.0	7.2	10.4	2.4
	<i>p</i> -hydroxybenzoic acid	ah	17.4	nd	nd	ah
	Vanillic acid	ah	Nd	nd	nd	ah
	Caffeic acid	19.6	7.2	16.7	nd	ah
	<i>p</i> -coumaric-acid	24.0	Ah	32.1	11.6	40.8
	Ferulic acid	123.2	79.9	1159.3	89.0	38.8

nd= not identified, ah = phenolic acids detected after enzymatic hydrolysis

Overall, the concentration of phenolic acids in all varieties, analysed by HPLC, following 4h of incubation with β -glucosidase, increased from around 2.4 (gallic acid) to 1159.3-fold (ferulic acid), which were the main phenolic compounds resulted from

β -glucosidase activity (Table 8). These results corroborate *in vivo* findings regarding the availability and metabolism of polyphenols in humans, which show that β -glycosidases activity in human gut may hydrolyze glycosides into aglicones with low molecular weight, including phenolic acids(62-63).

Transepithelial Transport Model

Transport of mango polyphenols, hydrolyzed or not, was studied using Caco-2 cell monolayers from the apical to the basolateral side. Caco-2 human colon cells are widely accepted as an *in vitro* system for predicting the bioavailability of drugs and others compounds and has been extensively used to predict the *in vivo* intestinal absorption of several polyphenols, including phenolic acids (64), flavonoids (65), and procyanidins (66). However, absorption studies using Caco-2 cells have been shown some limitations, which may not correctly predict the metabolism of polyphenols in human, since the deglycosylation step may not be involved (30). Nemeth *et al.* (2003) (30) showed lack or low level of β -glucosidase activity towards lactose and quercetin glucosides in fully differentiated Caco-2 cells incubated over 27 days, whereas high activity was observed for β -glucosidases (lactasephlorizin hydrolase and cytosolic β – glucosidase) isolated from samples of human small intestine and liver, leading to the conclusion of low activity of cytosolic β -glucosidase in Caco-2 cells. Therefore, previous hydrolysis of mango polyphenols shows to be a critical step to correctly predict their absorption *in vivo*. Hydrolyzed and control phenolic extracts at the same dilution (TPC \square 3000 mg GAE/L) were loaded into the apical side of the cell monolayers and individual polyphenols concentrations were analyzed over time by HPLC. Analytical HPLC chromatograms of mango polyphenols transported in the apical- basolateral direction through Caco-2 cell monolayers following 2h of incubation are present in Figure 15.

Phenolic acids were the mango polyphenols better transported through Caco-2 cells monolayers (Figure 16), whereas no absorption evidence was found to high molecular weigh compounds comprising gallotannins and mangiferin. Ferulic (FA) and *p*-coumaric acid (PC) were the most absorbed in cells treated with hydrolyzed phenolic extract, following *p*-OH-benzoic acid, caffeic (CA) and gallic acid (GA) (Table 9).

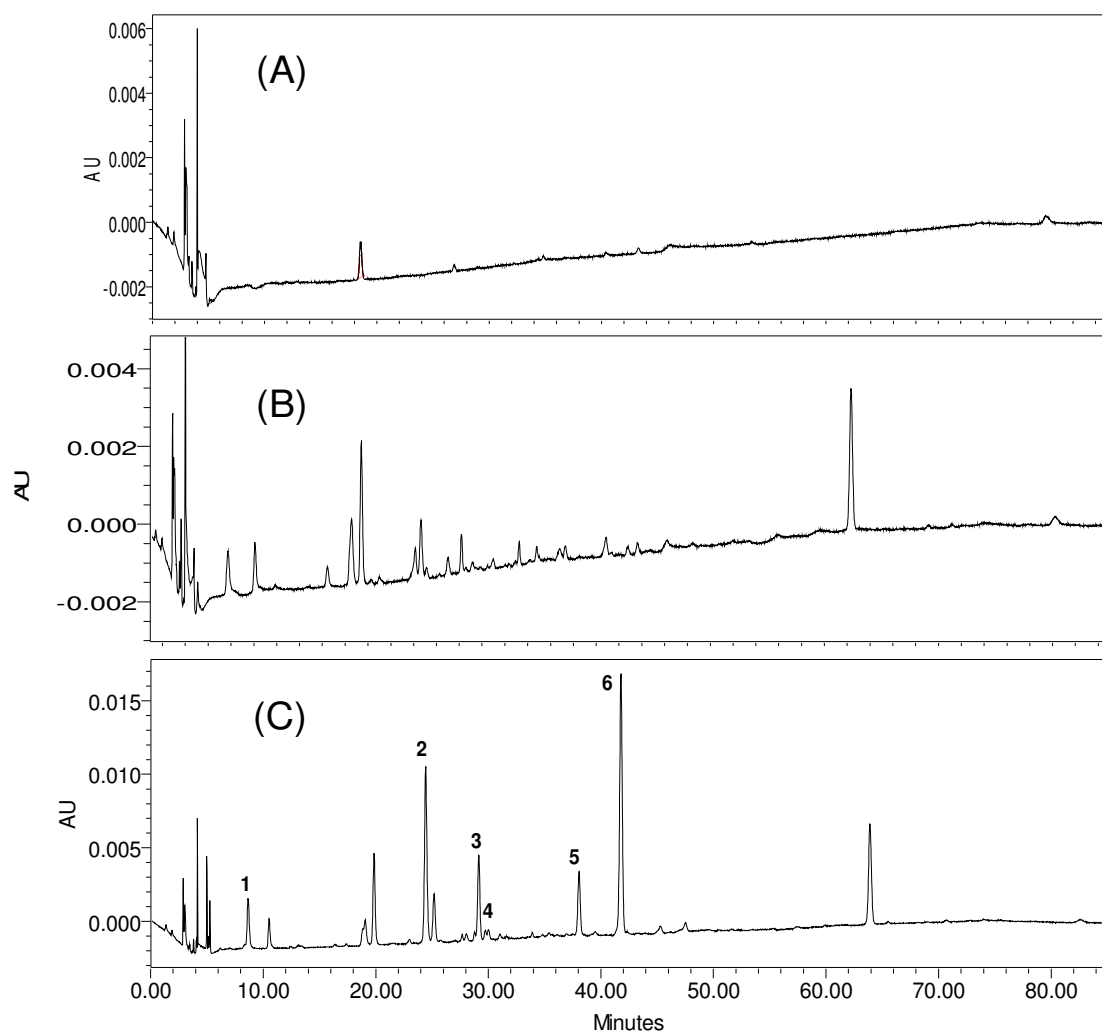


Figure 16. Chromatograms (280 nm) from samples taken from the basolateral compartment of Caco-2 cells following 2h of incubation with HBSS (pH 6.0) (A), control (B) and hydrolyzed (C) Ataulfo phenolic extracts. Peak assignments: (1) gallic acid; (2) p-OH-benzoic acid; (3) vanillic acid; (4) caffeic acid; (5) p-coumaric acid; (6) ferulic acid.

Table 9. Absorption (%) of phenolic acids through Caco-2 colon cancer cells following 2h incubation with control and hydrolyzed extracts.

Polyphenols	Control		Hydrolyzed extract	
	Concentration (mg GAE/L)	Absorption (%)	Concentration (mg GAE/L)	Absorption (%)
Gallic acid	8.8	12.7 ± 0.6	66.6	2.3 ± 0.2
<i>p</i> -OH-benzoic acid	nd	-	61.7	48.8 ± 4.4
Vanillic acid	nd	-	8.8	134.2 ± 13.6
Caffeic acid	nd	-	7.2	13.7 ± 1.4
<i>p</i> -coumaric-acid	nd	-	9.3	70.7 ± 7.5
Ferulic acid	nd	-	29.5	74.5 ± 8.8

nd= not detected

Our findings are in agreement with previous *in vivo* and *in vitro* studies using a Caco-2 cell monolayers (64-67) and evidence the influence of molecular weight and chemical structure on bioavailability of polyphenols. It has been demonstrated that GA, as well as *m*-hydroxybenzoic acid, are transported across human intestinal Caco-2 cells via a paracellular route, which permeability is characterized as a non-polarized, dose-dependent and pH independent (64, 68-70), whereas FA and PC are absorbed in Caco-2 cells by monocarboxylic acid transporter (MCT) (64, 68). CA, on the other hand, is absorbed by MCT and mainly by paracellular diffusion, in a non-saturable transport (69). As a result of different absorption mechanisms, studies have demonstrated higher *in vivo* absorption efficiency of PC as compared to GA, which showed permeation rates 70-100 fold lower than that of PC, when administrated in rats at the same doses (64, 71). Even though GA (61.7 mg GAE/L) was present in higher concentration in mango hydrolyzed phenolic extract, its absorption was the lowest detected when compared to the other phenolic acids permeability. Interestingly, despite GA concentration in control extract was 7.5- fold lower than in hydrolyzed extracts, gallic acid was 5.5 times more absorbed in control extracts-treated cells (table 9). In contrast to previous *in vivo* studies, that found similar concentrations of GA in urine of rats after consumption of the same amount of GA as a pure compound and in black tea (72-73), our results suggested that the presence of other phenolic acids might significantly influence the GA availability (Table 9). Even though some studies have been demonstrated the influence of food matrix on polyphenols availability (74-75), specific studies with GA taking into

account this issue have not been afforded yet. For instance, Konishi *et al.* (2004) (71) suggested that *p*-coumaric acid *in vivo* absorption was inhibited by benzoic acid, likely by inhibition of both MCT-mediated and the passive diffusion transport of PC.

It has been shown that the bioavailability of polyphenols is dependent to their physiochemical properties (27). For instance, the glycosylation influences chemical, physical and biological properties of a polyphenol and directly affect its polarity and partition coefficient, which is thought to govern the intestinal absorption of dietary polyphenols, because only passive diffusion seems to be involved (27). According to the Lipinski's Rule of 5, compounds with five or more hydrogen bond donors (OH and NH groups), ten or more hydrogen bond acceptors (N and O), molecular weight higher than 500, and Log P greater than 5 are usually poorly absorbed following oral administration (76-77). Indeed, several *in vivo* studies support this rule (71, 75, 78). In general, when hydroxycinnamic or hydroxybenzoic acids were ingested under free form, they were rapidly absorbed *in vivo* from the stomach or the small intestine and they were conjugated by the intestinal and/or hepatic detoxification enzymes. Studies carried by Konishi *et al.* (2004) (71) in rats suggested rapid absorption of GA in the upper part of the gut, from the stomach, from small intestine, or both. Investigations on hydroxycinnamic acids shown that when ingested under free form, they are rapidly absorbed from the stomach and small intestine. In rats, ferulic acid (FA) is well absorbed in the upper part of the rat gut (75, 79-80), whereas in humans, it has been rapidly detected in the plasma with urinary excretion reaching its maximum in 7 or 8 h after food ingestion (81-84). Similarly to FA, CA is well absorbed, and whatever the administrated dose in animals, most of the different metabolites disappear after 6h (85-86). Likewise, PC is also rapidly absorbed in the gastrointestinal tract when orally administrated in rats in its intact form (71). In addition, phenolic acids like FA, PC, CA and GA are better absorbed when administrated in humans and rats in free form as compared to esterified form (29).

A commonly accepted concept regarding polyphenols intestinal absorption is that glycosylated polyphenols need to be converted to the aglycone by glucosidases in the food or gastrointestinal mucosa, or from the colon microflora in order to be absorbed by passive diffusion (87). Overall, gallotannins upon hydrolysis yield glucose and gallic acid, whereas ellagitannins undergo lactonization to produce ellagic acid (23).

Punicalagin (PU), a water soluble ellagitannin found in pomegranates with molecular weight of 1084 (88), is known to be the highest molecular weight compound ever absorbed through the gut barrier after oral ingestion (21, 24), which was hydrolyzed into ellagic acid *in vivo*(89), and *in vitro* across the mitochondrial membrane (90).

Some studies have been shown bioconversion of ferulic acid into vanillic acid by β -glycosidases (58, 91-92), which may explain the absorption of vanillic acid over 100% (Table 9). This data suggest beta-glucosidase activity through intestinal monolayers.

Overall, our findings suggest that β -glucosidases may hydrolyze mango polyphenols *in vivo* into low molecular weight compounds, mainly phenolic acids, which are later absorbed through gut barrier. On the other hand, β -glucosidases activity did not contribute to enhance absorption of high molecular weight polyphenols, such as gallotannins. Because these polyphenols are not likely absorbed *in vivo* and reach the large intestine, they may modulate gut microflora and act protecting colon carcinogenesis.

Cell-growth suppressive activity of mango pulp polyphenols

Mechanisms associated to the chemopreventive effects of polyphenols comprise inhibition of carcinogen activation, induction of phase II enzymes, inhibition of arachidonic acid metabolism, modulation of different oncogenes, tumor suppressor genes, and signal transduction pathways, leading to cell proliferation inhibition, transformation, angiogenesis as well as induction of apoptosis (93).

Despite the biological activities of polyphenols has been related to their availability, studies indicate that health effects of polyphenols may not require their absorption through the gut barrier, since the highest local concentration of these compounds is found in the gut lumen (94). Therefore, it is relevant to measure the biological activity of polyphenols on cultured cells or isolated tissues in their form present in food. At the same time, polyphenols are extensively metabolized both in tissues and by the colonic microflora into a wide range of low molecular weight compounds, which may have lower or higher biological activities. Thus, it is important to identify their metabolites and test their respective biological activities. In this context,

cell-growth suppressive effects of mango polyphenols and their metabolites derived from hydrolysis with beta-glucosidase were studied using cancer cell culture models.

The antiproliferative activities of control and hydrolyzed mango phenolic extracts were evaluated in a cell culture model using MDA-MB-231 breast and HT-29 colon cancer cells. Polyphenols extracted from the pulp of all varieties induced a concentration-dependent cell proliferation inhibition on human HT-29 colon and estrogen independent MDA-MB-231 breast human cancer cells (Figure 17). Mango pulp polyphenols inhibited colon (0-27 μg of gallic acid equiv (GAE)/mL) and breast (0-24 μg GAE/mL) cancer cell proliferation by up to 99.8 and 89.9 %, respectively.

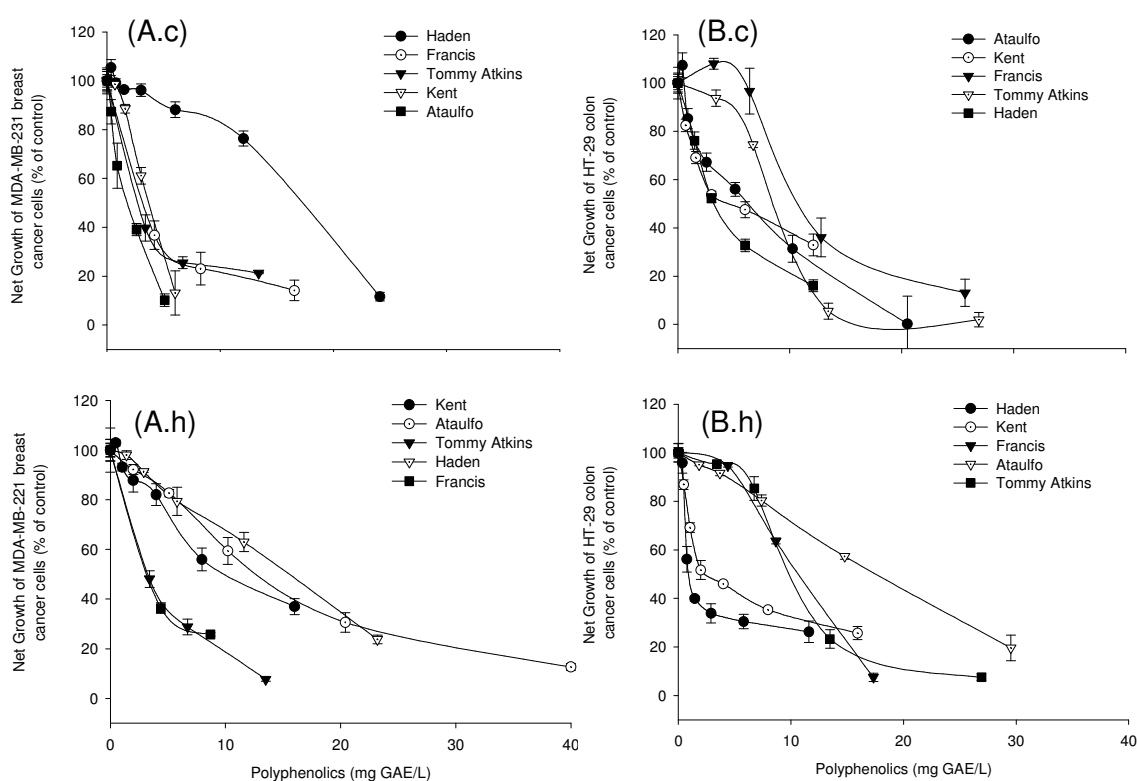


Figure 17. Cell-growth suppressive effects of mango polyphenols on estrogen independent MDA-MB-231 breast (A) and HT-29 colon (B) human cancer cells. Cells were incubated for 48h with control (c) and hydrolyzed (h) phenolic extracts. Values are mean \pm SE, n=3.

The 48h incubation-effect of mango polyphenols (control) from different mango varieties on the grown suppression of breast ($\text{IC}_{50} = 4.7 \pm 5.6 \mu\text{g GAE/mL}$) and colon ($\text{IC}_{50} = 6.3 \pm 4.5 \mu\text{g GAE/mL}$) cancer cells by 50% ranged from 1 to 15 μg of gallic

acid equiv/mL extract (Table 10), which was equivalent to 3-132 mg of mango pulp/mL culture medium (Table 11). Similar values were found to hydrolyzed extracts-treated cells (Figure 17). The hydrolysis of Fr, To and Ha mango polyphenols did not statically affect the cell proliferation of HT-29 colon and MDA-MB-231 breast cancer cells (Table 10).

Table 10. Effect of the treatment with control and hydrolyzed mango phenolic extracts on the growth suppression of MDA-MB-231 breast and HT-29 colon human cancer cell lines, expressed in terms of IC₅₀ values (mg GAE/L).

Variety	IC50 (mg GAE/L)			
	MDA-MB-231 (breast cancer)		HT-29 (colon cancer)	
	Control	Hydrolyzed extract	Control	Hydrolyzed extract
Tommy Atkins	2.3 ± 0.4 ^b	3.5 ± 0.4 ^c	8.5 ± 0.7 ^{a,b}	11.5 ± 2.1 ^a
Ataulfo	1.1 ± 0.1 ^b	1.2 ± 0.5 ^c	7.8 ± 1.8 ^{a,b,c*}	14.3 ± 3.1 ^{a*}
Francis	2.8 ± 0.4 ^b	4.1 ± 1.3 ^{b,c}	12.0 ± 2.0 ^a	10.3 ± 0.4 ^a
Haden	14.6 ± 4.9 ^a	13.3 ± 2.5 ^a	0.9 ± 0.1 ^c	0.9 ± 0.1 ^b
Kent	2.5 ± 1.4 ^{b*}	8.5 ± 0.4 ^{b*}	2.5 ± 2.1 ^{b,c}	1.3 ± 0.6 ^b

Data are means of three or more independent determinations ± SD. Means with different letters in the column are statistically different at $p < 0.05$ (Tukey-Kramer HSD test).

* Indicates statistical difference ($p < 0.05$) between the cancer cell growth inhibition activities of control and hydrolyzed phenolic extract, for the respective variety and cell line (line) (paired t-student).

Table 11. Cell-growth suppressive effects of mango polyphenols (control) on MDA-MB-231 breast and HT-29 colon human cancer cell lines, expressed in terms of IC₅₀ values (mg mango pulp/mL culture medium).

Variety	IC50 (mg mango pulp/mL culture medium)	
	MDA-MB-231 (breast cancer)	HT-29 (colon cancer)
Tommy Atkins	24 ± 4.0	84 ± 6.9
Ataulfo	3 ± 0.3	21.6 ± 4.9
Francis	21.9 ± 3.1	93.2 ± 15.5
Haden	132 ± 44.3	8.1 ± 0.9
Kent	20.7 ± 11.6	20.7 ± 17.4

Equivalent results for the cell-growth suppressive effects of mango polyphenols hydrolyzed or not on colon cancer cells suggest a possible contribution of β -glycosidases in polyphenols enzymatic cleavage followed liberation of free active cytotoxic aglycones that may act selectively on cancer cells, as demonstrated by Arafa

(2009) (52), who showed that the sensitivity degree in HT-29 and Caco-2 colon cancer cells towards glycoconjugates over 24-96h incubation was positively correlated with β -glucosidase activity assessed in these cell lines. At the same time, anticancer activity of mango polyphenols may be also attributed to the intact polyphenols, since a reduction in TPC of ataulfo after hydrolysis significantly decreased in 1.8-fold the effectiveness of polyphenols in suppressing HT-29 cell growth by 50% as compared to control extracts (Table 10), whereas Kent hydrolyzed extracts was 3.4-fold less effective in inhibiting cell proliferation of MDA-MB-231 cells. This difference is likely associated to the decrease in hydrolyzable tannins as a result of protein-tannin interaction, which may be negatively affected the mango biological activities. Considering that the chemopreventive properties of tannins have been related to their antioxidant, apoptosis-inducing, and enzyme inhibitory activities (94-97), these results together suggest that biological properties of mango polyphenols are ascribed to their bioactive metabolites derived from enzymatic hydrolysis as well as to the parental polyphenols.

Even though some studies evaluating the chemopreventive activity of mango pulp extracts and individual compounds have been performed (10, 19, 98), studies regarding the potential of mango pulp phenolic fraction on colon and breast cancer are still limited. Initially, Percival *et al.* (2006) (16) demonstrated that the whole mango juice (0.01-0.1%) was effective in inhibiting chemically induced neoplastic transformation of mammalian cell lines, BALB/3T3 cells, and also in reducing the number of foci by 50-70% in a dose-dependent manner. Later, Prasad and others (2008) confirmed the efficacy of aqueous mango pulp extract in inducing apoptosis in mouse prostate (19) and in combating oxidative stress-induced cellular injury of prostate in mice fed daily with 1mL extract (□ 0.2g fruit) for 15 consecutive days, by enhancing the level of antioxidant enzymes (20). Recently, Garcia-Solis *et al.* (2009) (17) demonstrated the efficiency of mango aqueous extracts against the viability of the breast cancer MCF-7 cell line. However, these studies failed to assess the anticancer activity of all polyphenols present in mango pulp because aqueous extraction is not efficient in recovering higher molecular weight tannins (99). Controversely, García-Solís, Yahia and Aceves (2008) (18) showed that short- and long-term whole mango consumption at physiological levels did not prevent mammary carcinogenesis in N-methyl-N-nitrosourea-treated rats, neither at the initiation nor at the promotion/progression steps

of carcinogenesis. In this study, the whole mango juice from the variety Ataulfo (0.02-0.06 g of mango/mL of water), containing carotenoids ($2.3 \pm 0.3 \mu\text{g}$ total carotenoids/mL) and polyphenols ($51.0 \pm 3.2 \mu\text{g}$ GAE/mL) was administrated in rats at 4.5, 8.5 and 12.7 g of mango/Kg b. wt., concentrations comparable to human intake of two (293 g of mango pulp per 70 Kg of b. wt.), four, and six mangoes per day, respectively. Although these studies have shown the contribution of mango pulp consumption for health benefits, biological activities of the mango phenolic fraction was not studied individually since other bioactive compounds like L-ascorbic acid and carotenoids were found in aqueous extracts or mango juice, respectively (17-18). These studies underscore the great chemopreventive potential of mango phytochemicals, especially polyphenols.

Many *in vitro* experiments with individual polyphenols found in mangoes have shown antitumor-related activities, mainly in colon and breast cancer, such as induction of apoptosis, inhibition of tumor growth and angiogenesis, and several have been confirmed *in vivo* (10-15). Mangiferin has been considered the main bioactive compound of *Vimang*, a industrial scale and commercially mango stem bark extract that have been used at least 10 years in Cuba medicine with effectiveness against several diseases, like cancer, with health benefits associated to its anti-inflammatory, immunomodulatory and antioxidant activities (2-7). Gallic acid (GA) has been shown significant cell growth inhibition in human cancer cell lines including esophageal, gastric, cervix, breast MCF-7, and colon human cancer cells HT-29, Colo201 and Colon 26 (mouse colon cancer), where the GA concentration necessary to inhibit breast and colon cancer cell growth by 50% were 0.15, 0.24, 0.18 and 0.26 mg/mL, respectively (98). Furthermore, several studies have been reported the anticancer-related activities of gallotannins, with potential in colon carcinogenesis (12-14, 100). 1,2,3,4,6-penta-O-galloyl- β -D-glucose (PGG), which is the precursor of gallotannins and is present in mango pulp, showed to induce cell cycle arrest, cell proliferation and apoptosis in a cell type-dependent manner on human MCF breast and jurkat T cancer cells (12-13). Additionally, treatment with gallotannins (20 $\mu\text{g}/\text{ml}$) for three days inhibited the growth of the colon cancer cells T-84 by 50% (100). In later study, the potential molecular mechanisms of action of gallotannins on colon cancer were evaluated *in vitro*. Al-Ayyoubi and Gali-Muhtasib (2007) (101) shown that gallotannins differentially

inhibited the growth of the isogenic human colon cancer cell lines HCT-116(p53 +/+) (IC50 value □ 45 µg/mL) and HCT-116 (p53 -/-) (IC50 value □ 30 µg/mL) versus normal human intestinal epithelial cells FHs 74Int (IC50 value > 60 µg/mL).

Indeed, previous studies have been shown that the major ability of tannins may have a positive impact on the absorption and metabolism of polyphenols and other constituents in human body, or may contribute to human body health benefits and cancer prevention *in vivo* by complexing enzymes involved in carcinogenesis process. On human physiology, beneficial effects of tannins may be explained by their ability to bind proteins. These interactions may result in enzyme activity inactivation by precipitation or by formation of soluble but inactive enzyme-inhibitor complexes and/or by complexation with the substrate which lead to a reduction in the enzyme reaction (102). However, mechanisms related to the effects of tannins on human food digestion are not fully understood. Recent studies have been shown that some physiological effects in the gut may counteract tannin antinutritional effects by increasing the production of endogenous (digestive proteases and lipases) (103) and salivary prolin-rich proteins (104) as well as biliary acids (105). In rats, pancreatic biliary juice neutralized the inhibitory effects of grape seed tannins on brush border hydrolase activities (105). Moreover, it has been suggested that the interaction between salivary prolin-rich proteins and tannins contribute to form complexes which may be stable throughout the digestion process, and this would allow the absorption and retention of nutritionally more useful food proteins (104). Additionally, as a result of tannin-protein interaction, tannins can act as antagonists of specific hormone receptors or inhibitors of particular enzymes involved in carcinogenesis process (106).

Considering that gallotannins show a great contribution to the mango pulp phenolic content, these evidences collectively suggest a potential role of mango polyphenols as chemopreventive agents against colon and breast cancer.

Cell-growth suppressive activity of low and high molecular weight polyphenols-rich fraction

Hydrolyzed phenolic extract was fractionated onto C18 in high (HMW fraction) and low molecular weight polyphenols (LMW fraction). The former represented the major mango polyphenols derived from mango pulp consumption in their intact form, whereas the latter correspond to low molecular weight polyphenols derived from hydrolysis with beta-glucosidase, which are potentially absorbed through gut barrier.

HMW fraction (F1) consisted of mangiferin (422.35) and hydrolyzed tannins with molecular weight ranged from 788 to 1851 Da, whereas LMW fraction (F2) comprise mainly phenolic acids with molecular weight ranging from 138.12 (*p*-hydroxybenzoic acid) to 194.18 Da (ferulic acid), as characterized by HPLC-DAD (Figure 18) and HPLC-ESI-MSⁿ analysis (Appendice).

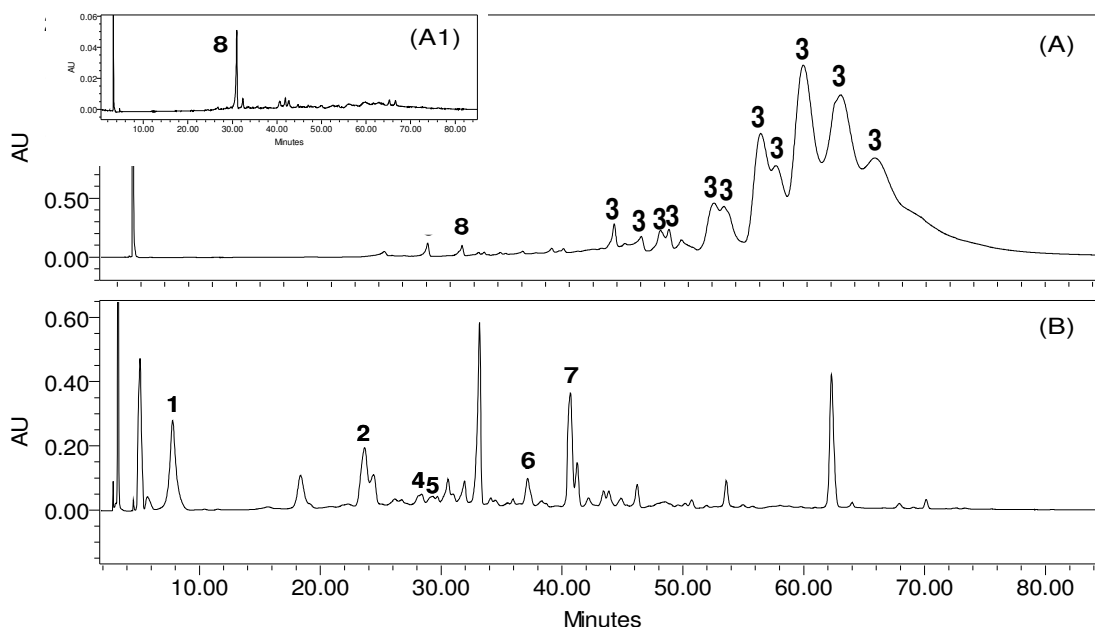


Figure 18. Chromatograms at 280 nm from HMW (A) and LMW (B) fractions (F2) from Ataulfo hydrolyzed extract. Peak assignments: (1) gallic acid; (2) *p*-OH-benzoic acid; (3) gallotannins; (4) vanillic acid; (5) caffeic acid; (6) *p*-coumaric acid; (7) ferulic acid. Representative chromatograms at 340 nm of HMW fraction (A₁): (8) mangiferin.

Although HMW fraction (4308.5 mg GAE/L) presented a TPC 15-fold greater than LMW fraction (283.4 mg GAE/L), its AA was □ 3-fold higher than AA from LMW fraction (Table 12), when fractions were analyzed at the same dilution. The higher amount of hydrolyzable tannins in HMW fraction may explain this difference. Indeed, tannins (condensed and hydrolyzable tannins) have been shown the strongest *in vitro* antioxidant activity as compared to other polyphenol structures (e.g., flavanols, flavonols, chalcones, flavones, flavanones, isoflavones, tannins, stilbenes, curcuminoids, phenolic acids, coumarins, lignans, and quinones) (31, 107). Because tannins has relatively many hydroxyl groups and are highly polymerized, these compounds have around 8-30 times better peroxy radical quenching capacity than simple phenols and Trolox (31, 99, 107), demonstrating significant scavenging action at lower concentrations.

Table 12. Total phenolic content (TPC) and antioxidant activity of the hydrolyzed phenolic extract and its fractions.

Fractions	Antioxidant capacity ($\mu\text{mol trolox eq./L}$)	TPC (mg GAE/L)
Hydrolyzed phenolic extract	25002.7 \pm 197.6	4592.0 \pm 22.8
HMW fraction	21157.4 \pm 590.7	4308.5 \pm 25.4
LMW fraction	7880.4 \pm 255.8	283.4 \pm 14.1

Data are means of three or more independent determinations \pm SD.

Although high molecular weight polyphenols showed higher contribution to the *in vitro* antioxidant capacity than the hydrolyzed phenolic extract (Table 12), the antioxidant potential *in vivo* may be distant from *in vitro* values. Several studies have shown that the antioxidant capacity *in vivo* may be related to low molecular weight metabolites derived from microbiota transformation or from antioxidant enzymatic system regulation and metabolism, which have antioxidant activities different from the parental compounds. Administration of grape seed proanthocyanidin-rich extract (250 mg/kg b.w.) in rats by intragastric intubation resulted in an increase of potential antioxidant of rat plasma, represented by the proanthocyanidins metabolites gallic acid, (+)-catechin, and (-)-epicatechin (108). Moreover, the supplementation of a meal with grape seed proanthocyanidins minimized the postprandial oxidative stress by decreasing the oxidants and increasing the antioxidant levels in plasma, which enhanced the resistance to oxidative modification of LDL (109). These results suggest that the intake

of tannins may increase the body resistance against oxidative stress and may contribute to physiological functions through their *in vivo* antioxidative ability.

Cells were incubated with the same dilutions of polyphenols-rich fractions and their antiproliferative activities were evaluated on MDA-MB-231 breast and HT-29 colon cancer cells (Figure 19).

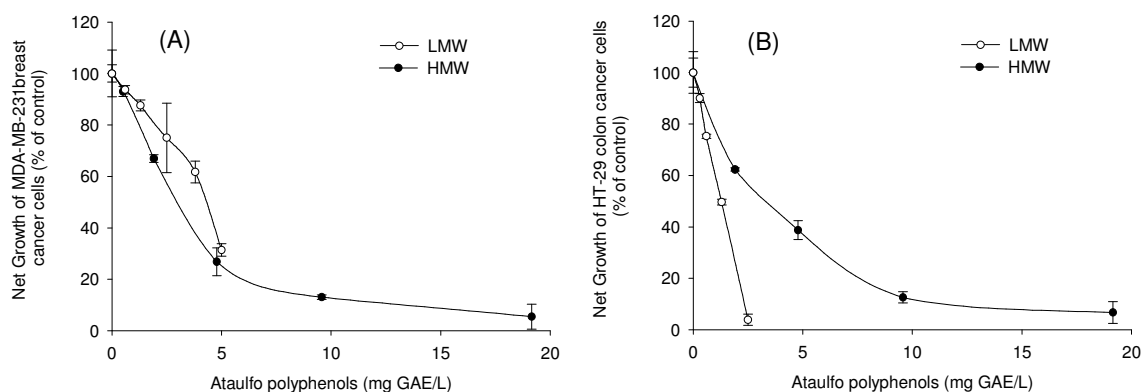


Figure 19. Cell-growth suppressive effects of mango polyphenols on estrogen independent MDA-MB-231 breast (A) and human HT-29 colon (B) cancer cells. Cells were incubated for 48h with HMW and LMW fractions from Ataulfo hydrolyzed extract. Error bars represent the standard error of the mean (n=3).

Considering equal dilutions, HMW fraction (F1) showed better effectiveness in inhibiting MDA-MB-231 breast and HT-29 colon cancer cell growth than LMW fraction (F2) (Figure 20). F1 and F2 fractions equally diluted at 1: 450 (v/v) inhibited HT-29 colon cell growth by 88.5 and 24.7 %, whereas MDA-MB-231 breast cancer cell proliferation was inhibited by 86.9 and 1.2 %, respectively (Figure 20). In terms of available amount of these polyphenols from the diet, these results suggest a higher contribution of high molecular weight to health benefits derived from mango pulp consumption, since free forms of phenolic acids are found in much lower concentrations than HMW compounds and will likely be available *in vivo* after deglycosylation process.

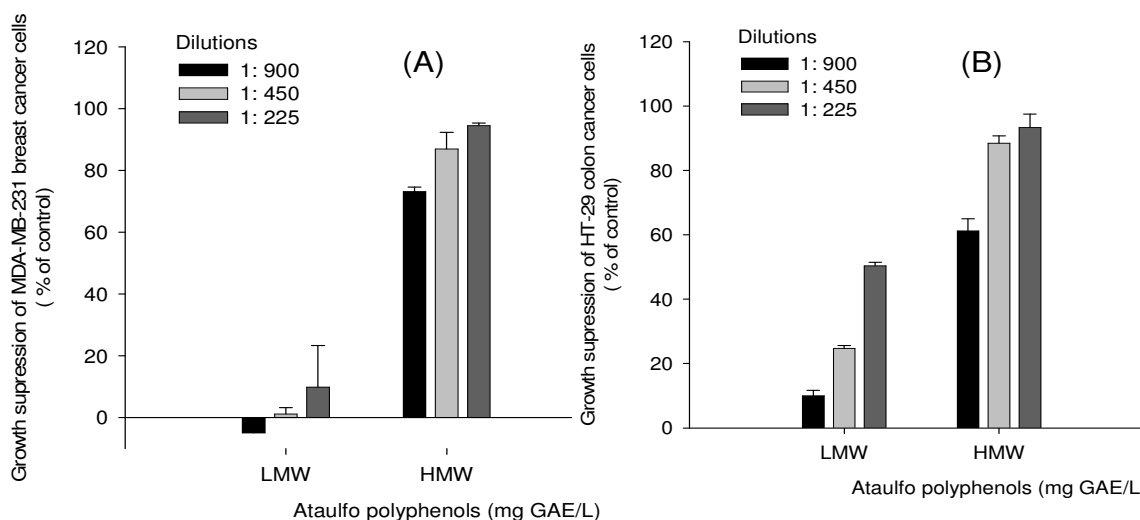


Figure 20. Effect of the treatment with equal dilutions of the HMW (F1) and LMW (F2) fractions from Ataulfo hydrolyzed extract on the growth suppression of MDA-MB-231 breast and HT-29 colon human cancer cell lines, expressed in percentage of control. Error bars represent the standard error of the mean (n=3).

The great difference in cell growth suppressive effects of F1 and F2 fractions is associated to their huge difference in TPC (15-fold) and, likely in tannin concentration, which contribute to the higher antioxidant capacity of F1 as compared to F2 (Table 19). On the other hand, when compared in TPC normalized to gallic acid equivalents (GAE), both fractions were equally efficient in inhibiting the cell proliferation of MDA-MB-231 breast and HT-29 colon cancer cells by 50% (Table 12). The proliferation of HT-29 colon cancer cells was inhibited in a dose-dependent manner by F1 (IC_{50} value = 3.0 μ g phenols /mL extract) and F2 fraction (IC_{50} value = 1.3 μ g phenols /mL extract). Similar efficacy was demonstrated in MDA-MB-231 cells treated with both fractions (Table 9).

Table 12. Effect of the treatment with HMW (F1) and LMW (F2) fractions on the growth suppression of MDA-MB-231 breast and HT-29 colon human cancer cell lines, expressed in terms of IC_{50} values (mg GAE/L).

Cell line	IC_{50} (mg GAE/L)	
	F2 (LMW)	F1 (HMW)
MDA-MB-231	4.2 \pm 0.1 ^a	2.5 \pm 0.0 ^a
HT-29	1.3 \pm 0.0 ^a	3.0 \pm 0.0 ^a

Data are means of three independent determinations \pm SD. Means with different letters in the line are statistically different at $p < 0.05$ (Tukey-Kramer HSD test).

Therefore, these results together suggest both contribution of intact polyphenols and their metabolites as chemopreventive compounds derived to the mango pulp consumption. Intestinal bacteria can transform dietary polyphenols into a wide range of low molecular weight compounds that are potentially more biologically active than the parent compounds (23, 25-27) (110). The bioactive ferulic acid, for example, may be released from its ethyl ester for the action of the bacteria found in human feces, including *Bifidobacterium lactis*, *Lactobacillus gasseri* and *Escherichia coli* (111). Urolithins, the metabolites derived from ellagitannins degradation by gut microflora, have been reported to exert biological activity (112-114), although the microbiota involved in their production was not identified so far. Additionally, proanthocyanidins from cranberries showed to be effective in reducing the risk of recurrent urinary tract infections by reducing adhesion of bladder cells (115-116). Russel *et al.* (2207) (117) demonstrated that cytokine-induced stimulation of the inflammatory pathways in colon cells was four-fold up-regulated in the presence of the free phenolic acid fraction from blueberries, suggesting that health benefits of blueberry phenolics as anti-inflammatory agents in the colon are likely resulted of microbial metabolism. Moreover, because of the limited absorption of high molecular weight polyphenols through intestinal gut barrier, substantial levels of unabsorbed dietary polyphenols in large intestine. These polyphenols may modulate the colonic microbiota by increasing e/or reducing the number of specific microbial strains and thus enhancing colon health. Tea polyphenols including epicatechin, catechin, gallic acid, caffeic acid and 3-O-methylgallic acid showed to repress the growth of *Clostridium perfringens*, *Clostridium difficile* and *Bacteroides spp.*, whereas the probiotics *Bifidobacterium spp.* and *Lactobacillus spp.* were less affected (110). Tannin-rich berries have been shown to exhibit antimicrobial properties against pathogenic bacteria, which may contribute to improve colon health (118). A lactobacilli-supplemented diet in rats treated with the colon carcinogen 1,2-dimethylhydrazine hydrochloride (DMH) induced a decrease in the extent of DMH-induced DNA damage, especially in rat colon cells. The chemopreventive effects associated to the supplementation with *Lactobacilli casei* were accompanied by changes in the activities of several xenobiotic metabolizing enzymes, where the concentrations of reduced glutathione and the activities of glutathione S-transferase, glutathione peroxidase and superoxide dismutase were down-regulated as a response to an

improved antioxidant status(119). According to these evidences, both parental polyphenols as well as their metabolites derived from gut microflora transformation may reduce oxidative stress, inflammation, pathogenic bacteria, and alter the balance between the bio-activation and detoxification metabolic pathways by inducing the production of enzymes. These beneficial effects derived from polyphenols consumption might conjunctly reduce the risk and/or protect against colon carcinogenesis by enhance colon health (120).

Indeed, even though the occurrence of hydrolyzable tannins (mango, pomegranate, berries) is much more limited that that of condensed tannins (tea, wine, muscadine grapes, berries) (121), several investigations have suggested that dietary administration of hydrolyzable tannins-rich foods inhibited events associated with both the initiation and promotion/progression of cancer *in vivo* (122-123). Blackberry, raspberry, and strawberry are known to contain predominantly hydrolyzable tannins (ellagitannins and gallotannins), whereas blueberries and cranberries are known to contain predominantly condensed tannins (proanthocyanidins) (124). Seeram *et al.* (2006) (125) showed that phenolic extracts from blackberry (IC₅₀ value = 64.60 µg/mL), blueberry (89.96 µg/mL) and black raspberry (89.11 µg/mL) shown to inhibit the growth of HT-29 colon cancer cells by 50% more efficiently than strawberry (114.20 µg/mL), cranberry (121.30 µg/mL), and red raspberry (187.60 µg/mL) extracts. In later study, polyphenol-rich berry extracts were screened for their antiproliferative effectiveness using Caco-2 human colon cancer cells. The viability of Caco-2 colon cancer cells was inhibited in a dose-dependent manner by strawberry (IC₅₀ value = 25.5 µg phenols /mL extract), arctic bramble (26.4 µg /mL), cloudberry (31.6 µg /mL) and lingonberry (28.7 µg /mL) phenolic extracts. Interestingly, the antiproliferative activity of procyanidins -rich fraction from lingonberry extracts was more effective than the original extract and anthocyanin-rich fraction (126). In similar study, Ross, McDougall and Stewart (2007) (127) shown that the antiproliferative activity of raspberry phenolic extracts is predominantly associated to ellagitannins instead of anthocyanins, because ellagitannins-rich fraction (IC₅₀ = 13.0 µg GAE/ml) was more effective in inhibiting human cervical cancer (HeLa) cell growth by 50% than whole raspberry phenolic extract (17.5 µg GAE/ml) and anthocyanin-enriched fraction (67.0 µg GAE/ml). Tannin-rich fractions (85.1- 93.2% of the TPC) from four varieties of muscadine grapes,

which contained predominantly hydrolyzable tannins (128), showed to inhibit the cell proliferation of HT-29 (□ 300 µg/mL) and Caco-2 (300-500 µg/mL) colon cancer cell lines, showing a 2-4 greater inhibitory activity as compared to phenolic acid fraction (129).

In addition, the degree of galloylation may play a role in the cytotoxic effects in colon cancer cells (130-131), which may indicate that some of the anti-cancer effects may be influenced by the high degree of galloylation of mango-polyphenols. Tannins which exhibited potent inhibitory activity on the tumor cell motility have common characteristics such as galloyl groups substituting all the hydroxyl groups of β-D-glucose and some of them cross-linked to form hexahydroxydiphenoyl. Ellagitannins including the most potent 1,2,4-tri-O-galloyl-3,6-hexahydroxydiphenoyl-β-D-glucose (punicafolin), showed to be more potent than gallotannins (mono, di and penta-O-galloyl- β-D-glucose) (132). In similar study, Coriariin A, which has four galloyl groups, showed higher antitumor than agrimoniin, that has two hexahydroxydiphenol groups (32). This results are in agreement with Lizarra *et al.* (2008) (131) findings. The more galloylated Witch hazel fractions showed better effectiveness at inhibiting proliferation of HT-29 and HCT-116 human colon cancer cells, at arresting the cell cycle at the S phase as well as at inducing apoptosis and necrosis. Interestingly, the apoptosis and cell cycle arrest effects were proportional to their galloylation level. Moreover, witch hazel fractions with a high degree of galloylation were also the most effective as scavengers of both hydroxyl and superoxide radicals and in protecting DNA damage triggered by the hydroxyl radical system. These evidences suggest chemopreventive effects of mango gallotannins, which present high level of galloyllation degree, varying from one to eleven galloyl moieties attached in a glucose core.

Conclusion

The absorption of mango polyphenols was predominantly characterized by low molecular weight compounds, mainly phenolic acids in their free forms like gallic, caffeic, ferulic, p-OH- coumaric and vanilic acids, with no evidence of absorption of high molecular weight polyphenols like mangiferin (422 Da) and hydrolyzable tannins

with molecular weight ranging from 788 Da (tetra-*O*-galloyl-glucose) to 1852 Da (undeca-*O*-galloyl-glucose). Despite the enzymatic hydrolysis had enhanced the mango polyphenols absorption through Caco-2 intestinal monolayers, in general, mango polyphenols hydrolysis with β -glucosidase activity did not increased total phenolic content, antioxidant activity and growth suppression of MDA-MB-231 breast and HT-29 colon cancer cells. Additionally, both high and low molecular weight polyphenols fractions equally inhibited cell proliferation of colon and breast cancer cells at the same extent (0-20 μ g of gallic acid equiv/mL). These results suggest that the most of mango polyphenols are not absorbed intact *in vivo* through the small intestine, but may be hydrolyzed by intestinal enzymes into low molecular weight aromatic acids, which are later absorbed, or likely reach the large intestine, modulating the gut microflora and protecting colon carcinogenesis. It suggests that the anti-cancer effects of mango polyphenols may not require their absorption *in vivo* through the gut barrier. Additional studies regarding the chemopreventive mechanisms are necessary to better elucidate the anti-cancer properties of mango polyphenols.

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SUMMARY AND GENERAL CONCLUSIONS

Polyphenols identified in the edible part of mango from different varieties (Haden, Ataulfo, Kent, Tommy Atkins and Francis) comprise gallic acid, mangiferin, phenolic acid derivatives and a wide range of high molecular weight hydrolysable tannins. Ataulfo and Haden polyphenols inhibited the growth of human SW-480 colon cancer cells, which showed high sensitivity to the treatment. Additionally, non-cancer CCD-18Co colon cells showed lower sensitivity to polyphenols treatment than human cancer colon cells. SW-480 gene regulation influenced by Ataulfo and Haden polyphenols suggested the induction of apoptosis through intrinsic and extrinsic mechanisms, in addition to cell cycle arrest. Cell cycle arrest in the G₂/M phase was related to the upregulation of PKMYT1 gene expression. Ataulfo and Haden polyphenols exerted protection of non-cancer CCD-18Co colon cells by lowering the ROS generation in a dose dependent manner. Based on their anti-cancer effects and protective effects in normal cells, but not in cancer cells, mango polyphenolic extracts may have a high potential as chemopreventive agent.

The absorption of mango polyphenols was predominantly characterized by low molecular weight compounds, mainly phenolic acids in their free forms like gallic, caffeic, ferulic, p-OH- coumaric and vanilic acids, with no evidence of absorption of high molecular weight polyphenols like mangiferin (422 Da) and gallotannins with molecular weight ranging from 788 Da (tetra-*O*-galloyl-glucose) to 1852 Da (undeca-*O*-galloyl-glucose). Despite enhanced absorption facilitated by enzymatic hydrolysis, a significant increase in antioxidant activity, phenolic content and antiproliferative effects on MDA-MB-231 breast and HT-29 colon cancer cells was not observed. Additionally, both high (422; 788-1852 Da) and low (138-194 Da) molecular weight polyphenols rich-fractions equally inhibited cell proliferation of colon and breast cancer cells at the same extent (0-20 µg of gallic acid equiv/mL), which may indicate that the anti-cancer efficacy of mango polyphenolics is not dependent on enzymatic hydrolysis.

These results corroborate previous findings from *in vivo* studies, which suggest that the most of mango polyphenols are not absorbed intact through the small intestine, but may be hydrolyzed by intestinal enzymes into low molecular weight aromatic acids, which would be later absorbed; or when polyphenols are not absorbed, they likely reach

the large intestine, modulating the gut microflora, and thus they contribute to reduce the risk of colon carcinogenesis.

Overall, polyphenols from several mango varieties exerted anti-cancer effects, and these effects may not require enzymatic hydrolysis by β -glucosidase.

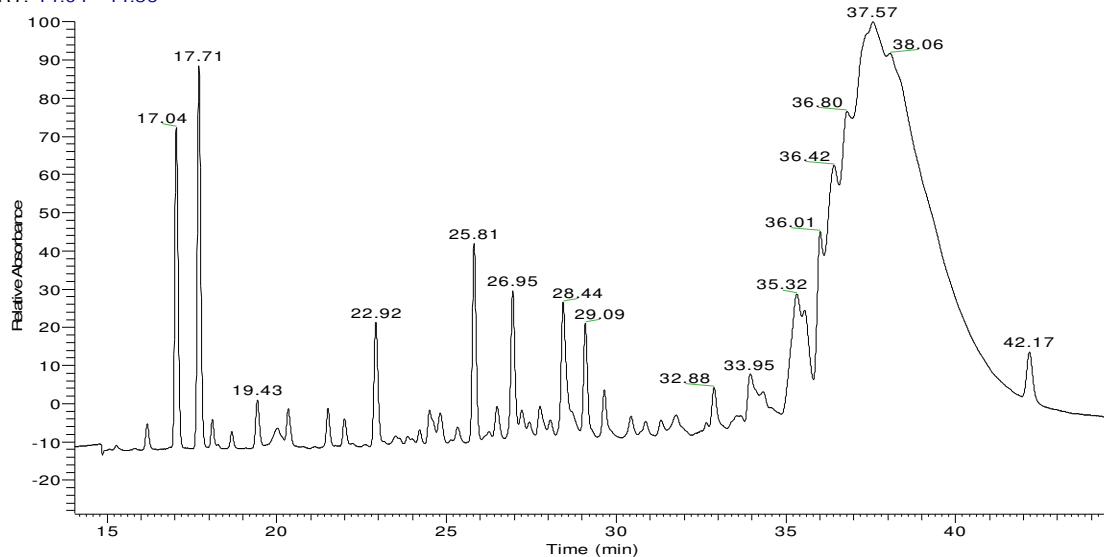
Although anti-cancer properties of mango pulp polyphenols was demonstrated, additional *in vivo* and *in vitro* studies regarding the chemopreventive mechanisms are necessary to better elucidate the anti-cancer properties of mango polyphenols.

APENDIX

Table 1. HPLC-ESI-MSⁿ of mango pulp polyphenols extracted from the varieties Ataulfo (A), Kent (B), Tommy Atkins (C), Francis (D) and Haden (E).

(A)

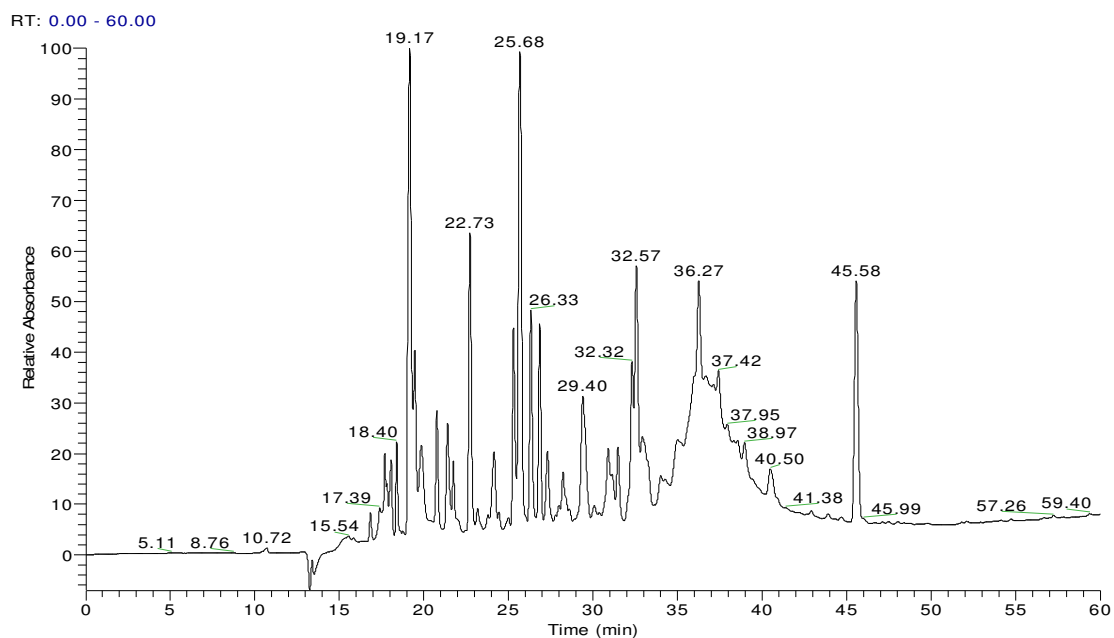
RT: 14.04 - 44.56



Peak	Identity	RT(min)	HPLC-DAD λ_{max} [nm]	HPLC-ESI(-)-MS ² (<i>m/z</i>) (% base peak)
1	mono- <i>O</i> -galloyl-glucose	17.71	237,278	MS ² [331.14]: 271.03 (100), 169.10(86.48), 223.05 (40.84)
2	mangiferin	28.46	209,237,259,318,365	MS ² [421.02]: 301.21 (100), 331.13(95.64), 403.06 (25.63)
3	penta- <i>O</i> -galloyl-glucose	33.95	238,277	MS ² [938.88]: 768.92 (100), 786.85 (21.23)
4	hexa- <i>O</i> -galloyl-glucose	35.32	238,277	MS ² [1090.66]: 1090.97(100), 1191.92(97), 958.61(84.31), 1198.45(69.63), 938.93(54)
5	hepta- <i>O</i> -galloyl-glucose	35.79	238,276	MS ² [1242.64]: 1016.47(100), 996.22(63.47), 902.56(61.88), 1220.62(57.16)

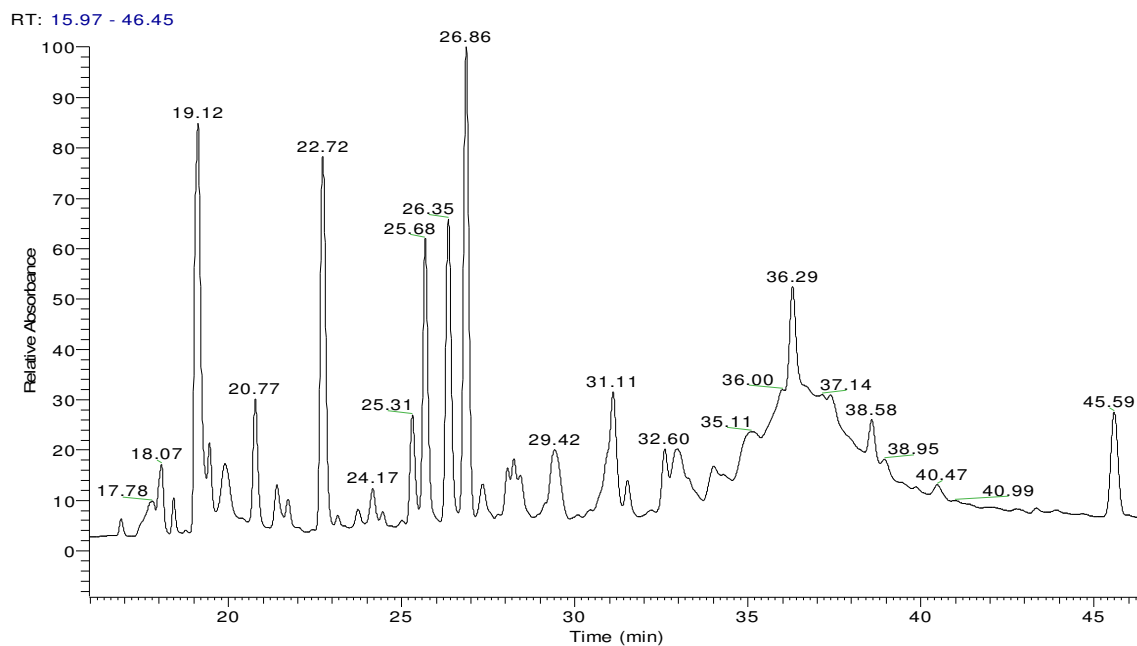
6	octa- <i>O</i> -galloyl- glucose	36.08	238,276	MS ² [1394.59]: 1090.73(100), 1242.54(77.07), 938.76(10.23)
7	nona- <i>O</i> -galloyl- glucose	37.51	239,274	MS ² [1546.56]: 1242.61(100), 1394.55(61.33), 1090.74(26.67)
8	deca- <i>O</i> -galloyl- glucose	38.17	239,274	MS ² [1698.46]: 1394.53(100), 1242.64(60.31), 1546.48(48.92)
9	undeca- <i>O</i> -galloyl- glucose	39.21	239,274	MS ² [1850.32]: 1546.49(100), 1394.56(84.88)

(B)



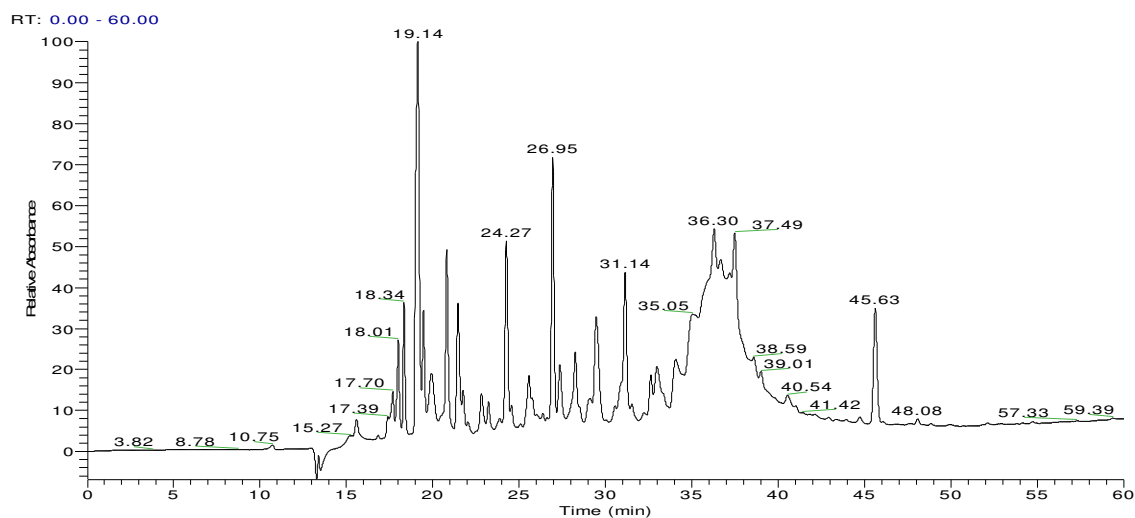
Peak	Identity	RT(min)	HPLC-DAD λ_{max} [nm]	HPLC-ESI(-)-MS ² (<i>m/z</i>) (% base peak)
1	mono- <i>O</i> -galloyl-glucose	19.25	232,271	MS ² [331.19]: 271.05 (100), 169 (84.3)
2	tetra- <i>O</i> -galloyl-glucose	31.40	232,275	MS ² [787.02]: 617.04 (100), 635.07 (42.04)
3	penta- <i>O</i> -galloyl-glucose	33.39	232,274	MS ² [939.05]: 769.04 (100), 787.08 (23.37)
4	hexa- <i>O</i> -galloyl-glucose	34.72	232,277	MS ² [1090.90]: 919.07(100), 920.28(52.32), 770.34(33.90)
5	hepta- <i>O</i> -galloyl-glucose	35.69	232,277	MS ² [1242.47]: 1090.17(100), 938.21(47.39), 1090(24.39), 769.70(17.34)
6	octa- <i>O</i> -galloyl- glucose	36.43	232,275	MS ² [1394.80]: 1241.41(100), 939.91(65.05), 938.03(33.67)
7	nona- <i>O</i> -galloyl-glucose	37.92	232,272	MS ² [1547.67]: 1242.78(100), 1090.86(65.14), 1394.76(39.79)

(C)



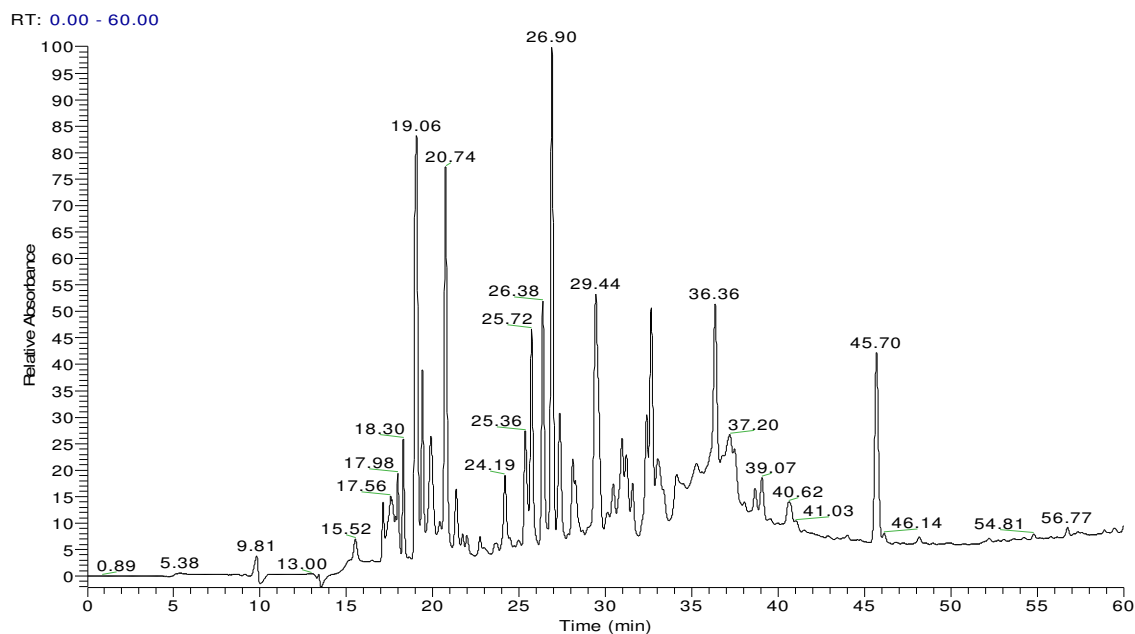
Peak	Identity	RT(min)	HPLC-DAD λ_{max} [nm]	HPLC-ESI(-)-MS ² (<i>m/z</i>) (% base peak)
1	mono- <i>O</i> -galloyl-glucose	19.25	232,279	MS ² [331.19]: 169 (100)
2	penta- <i>O</i> -galloyl-glucose	33.0	232,278	MS ² [939.11]: 769.08 (100), 787.05 (21.30)
3	hexa- <i>O</i> -galloyl-glucose	34.11	232,277	MS ² [1090.78]: 928.08 (100), 769.92(53.95)
4	hepta- <i>O</i> -galloyl-glucose	35.11	232,274	MS ² [1242.81]: 938.13(100),1014.16(88.90), 1088.93(26.34)
5	octa- <i>O</i> -galloyl-glucose	36.29	232,275	MS ² [1394.77]: 1241.89(100), 1089.90(51.81), 1241.10(39.06),1089.13(38.13), 939.96(30.40),
6	nona- <i>O</i> -galloyl-glucose	37.42	232,279	MS ² [1546.75]: 1240.77(100), 1392.84(64.45), 1393.70(53.19), 1241.79(92.42), 1242.72(39.50)

(D)



Peak	Identity	RT(min)	HPLC-DAD λ_{max} [nm]	HPLC-ESI(-)-MS ² (<i>m/z</i>) (% base peak)
1	mono- <i>O</i> -galloyl-glucose	19.25	232,279	MS ² [331.14]: 169 (100)
2	tetra- <i>O</i> -galloyl-glucose	29.44	232,272	MS ² [787.03]: 617.04 (100), 635.03 (17.23)
3	penta- <i>O</i> -galloyl-glucose	32.92	232,278	MS ² [939.05]: 769.06 (100), 787.00 (24.79)
4	hexa- <i>O</i> -galloyl-glucose	34.17	232,278	MS ² [1090.88]: 938.04 (100), 769.66(93.03), 768.23(50.27),752.40(47.72)
5	hepta- <i>O</i> -galloyl-glucose	35.17	232,277	MS ² [1242.81]: 1088.99(100) 949.98(74.94),1027.99(43.27),
6	octa- <i>O</i> -galloyl-glucose	36.08	232,274	MS ² [1394.88]: 1241.59(100), 1089.89(37.40), 1373.08(24.30), 937.99(17.01)
7	nona- <i>O</i> -galloyl-glucose	37.19	232,275	MS ² [1547.76]: 1242.78(100), 1394.78(41.77), 1090.79(55.46)

(E)



Peak	Identity	RT(min)	HPLC-DAD λ_{max} [nm]	HPLC-ESI(-)-MS ² (<i>m/z</i>) (% base peak)
1	mono- <i>O</i> -galloyl-glucose	19.06	232,279	MS ² [331.91]: 169 (100)
2	di- <i>O</i> -galloyl- glucose	25.58	232,281	MS ² [634.96]: 483.07 (100), 465.08 (21.26)
3	tetra- <i>O</i> -galloyl-glucose	29.23	232,272	MS ² [787.10]: 617.05 (100), 635.07 (51.85)
4	mangiferin	30.31	232,258,361	MS ² [421.18]: 301.24 (100), 331.14 (91.64)
5	penta- <i>O</i> -galloyl-glucose	33.01	232,278	MS ² [939.05]: 769.04 (100), 787.06 (14.73)
6	hexa- <i>O</i> -galloyl-glucose	35.16	232,278	MS ² [1090.81]: 928.16 (100), 769.06(79.53), 806.46(62.11),769.67(34.24)
7	hepta- <i>O</i> -galloyl-glucose	35.32	232,277	MS ² [1242.81]: 1088.99(100) 949.98(74.94),1027.99(43.27),
8	octa- <i>O</i> -galloyl-glucose	36.20	232,274	MS ² [1395.14]: 1242.86(100), 1241.87(24.26), 1090.83(77.86), 1373.08(24.30), 938.95(44.95)

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