Polyphenolic profile and antiproliferative activity of bioaccessible fractions of zinc-fortified fruit beverages in human colon cancer cell lines

A. Cilla1, M. J. Lagarda1, R. Barberá1 and F. Romero2


Abstract

Introduction: Colorectal cancer risks could be reduced by polyphenol-rich diets that inhibit tumour cell growth. Aims: To determine the polyphenolic profile of four fruit beverages (FbZn, FbZnFe, FbZnM and FbZnFeM) as affected by the presence of Zn with/without Fe and with/without skimmed milk, and the digestion conditions. To evaluate the antiproliferative activity of bioaccessible fractions against Caco-2 and HT-29 cells. To clarify whether cell cycle arrest and/or apoptosis is involved in their possible antiproliferative activity.

Methods: The polyphenolic profiles were analyzed by RP-HPLC-DAD before and after in vitro gastrointestinal digestion. Cell proliferation and viability were measured using Trypan blue test, mitochondrial enzyme activity by means MTT test, cell cycle distribution using flow cytometry and apoptosis by means Hoechst dye.

Results and discussion: The presence of zinc, iron and/or milk decreased the soluble extractable phenolic content before digestion probably by chelate formation, FbZn and FbZnFe being the samples with the highest soluble extractable phenolics. After digestion, a decrease in phenolics was observed in all zinc-fortified samples (up to 32% with respect to the original fruit beverages) - the FbZnFeM sample showing the lowest soluble extractable phenolic content, though with the lowest percentage decrease in phenolics (14%). FbZnM digest (~50 μM total soluble extractable phenolics) was the sample that most inhibited Caco-2 and HT-29 cell proliferation after 24 h of incubation, without cytotoxicity. The specific combination of phytochemicals in FbZnM digest proved cytostatic and significantly suppressed proliferation through cell cycle arrest in the S-phase in both cell lines, without apoptosis.

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PERFIL POLIFENÓLICO Y ACTIVIDAD ANTIPROLIFERATIVA DE LAS FRACCIONES BIOACCESIBLES DE BEBIDAS A BASE DE ZUMO DE FRUTAS SUPLEMENTADAS CON CINC EN LÍNEAS CELULARES HUMANAS DE CÁNCER DE COLON

Resumen

Introducción: Los riesgos de cáncer colorrectal podrían reducirse mediante dietas ricas en polifenoles, los cuales pueden inhibir el crecimiento de células tumorales.

Objetivos: Determinar cómo el perfil polifenólico de cuatro bebidas a base de zumo de frutas (FbZn, FbZnFe, FbZnM and FbZnFeM) puede verse afectado por la presencia de Zn con/sin Fe y con/sin leche desnatada, así como por las condiciones de digestión gastrointestinal. Evaluar la actividad antiproliferativa de las fracciones bioaccesibles en células Caco-2 y HT-29. Averiguar si un arresto en el ciclo celular y/o apoptosis están implicados en su posible actividad antiproliferativa.

Métodos: Los perfiles polifenólicos se analizaron mediante RP-HPLC-DAD antes y después de la digestión gastrointestinal in vitro. La proliferación y viabilidad celular se determinaron con el azul tripán, la actividad enzimática mitocondrial por medio del test MTT, la distribución del ciclo celular por citometría de flujo y la apoptosis mediante la sonda fluorescente Hoechst.

Resultados y discusión: La presencia de cinc, hierro y/o leche disminuyó el contenido de polifenoles solubles extraíbles antes de la digestión, probablemente por formación de quelatos. Tras la digestión, se observa un descenso en los polifenoles en todas las muestras suplementadas con cinc (hasta un 32% con respecto a las bebidas de frutas originales), siendo la muestra FbZnFeM aquella con menor contenido de polifenoles solubles extraíbles, aunque con el menor porcentaje de descenso de polifenoles (14%). La muestra digerida de FbZnM (~50 μM total soluble extractable phenolics) fue la que más inhibió la proliferación de las células Caco-2 y HT-29 tras 24 h de incubación, sin citotoxicidad. La combinación específica de compuestos fitoquímicos en la fracción bioaccesible de FbZnM actuó de forma citostática, disminuyendo la proliferación celular de forma significativa mediante arresto del ciclo celular en la fase S en ambas líneas celulares, no acompañado de apoptosis.

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Introduction

Colon cancer is one of the most common cancers in developed countries, and it is recognized to be an environmental disease influenced by diet and lifestyle practices. In fact, Western diets (typically dense in fat and energy and low in fiber) are associated with an increased risk. Thus, dietary modification through plant derived polyphenolic-rich foods could be a good and cost-effective strategy for reducing colorectal cancer incidence due to direct exposure of the intestinal epithelium to these dietary ingredients. Among the bioactive phytochemical compounds present in our diet, polyphenols are the most abundant antioxidants, and suggested mechanisms for their anticancer effects include antioxidant and antiproliferative activities, as well as subcellular signaling pathways such as the induction of cell-cycle arrest and apoptosis. Nevertheless, polyphenols must reach the target tissues in sufficient amounts to exert their biological effects.

From a physiological perspective, food after consumption undergoes a gastrointestinal digestion process that may affect the native antioxidant potential of the complex mixture of polyphenols present in the food matrix before reaching the proximal intestine. In this sense, a decrease in dietary polyphenols in fruit juices subjected to in vitro gastrointestinal digestion has been reported. However, the majority of studies related to the antiproliferative activity of phytochemicals have been conducted with isolated polyphenols or crude extracts, which is unrealistic and may overestimate the biological activity, since no account is taken of digestion. Thus, we maintain that the use of real foods, although subjected to in vitro digestion, is more physiologically relevant to the situation found after actual oral ingestion. In this context, antiproliferative activity of digested chokeberry juice has been reported in Caco-2 cells.

Zinc is second only to iron among those elements for which a human nutritional requirement has been established. The adverse health consequences of zinc deficiency vary with age and can affect from schoolchildren to elderly people, mainly in developing countries. However, it was not until 2002 that Zn deficiency was included as a major risk factor to global and regional burden of disease, along with Fe, vitamin A and I deficiency. Among the intervention strategies to combat mineral (iron and zinc) deficiency in developing countries, short-term measures such as supplementation and fortification can be used to alleviate this situation. In this sense, the development of functional foods has increased spectacularly in recent years, including the design of new fruit beverages supplemented with minerals (iron and zinc) and milk which could be helpful in the context of dietary food strategies to prevent mineral deficiency and chronic diseases, especially some types of cancer - including those of the digestive tract. The use of this kind of beverages for zinc supplementation alone or in combination with iron and/or milk could be of interest for two reasons: a) fruit beverages lack zinc absorption inhibitors such as phytates, and b) is increased the contribution of antioxidants from the diet that may act in concert with inner antioxidants (i.e. ascorbic acid and polyphenols) present in fruit beverages. Milk derived proteins and iron are well-known dietary factors which can form chelates with polyphenols in the lumen, reducing their bioaccessibility under conditions of in vitro digestion, as previously reported in green tea, red wine, and by our group in fruit beverages supplemented with iron and iron plus skimmed milk. However, to our knowledge, there are no reports on the effect of zinc or mixtures of zinc with iron and/or skimmed milk on polyphenol bioaccessibility in foods. Thus, since zinc belongs to the transition group in the same way as Fe, it could also modify polyphenol bioaccessibility in fruit beverages.

Bioaccessible fractions of these fruit beverages have previously demonstrated a cytoprotective effect on Caco-2 cells against H2O2-induced oxidative stress. In addition, fruit beverage digest (Fb digest) exerted antiproliferative activity in Caco-2 cells due to arresting of the cell cycle in the S-phase, accompanied by a decrease in both cyclins B1 and D1, without apoptosis.

Objectives

The main objectives of this study in four zinc-fortified fruit beverages are: i) to determine the polyphenolic profile as affected by the presence of Zn with/without Fe and with/without skimmed milk, and the digestion conditions; ii) to evaluate the antiproliferative activity of bioaccessible fractions against Caco-2 and HT-29 colon cancer cell lines; and iii) to clarify whether cell cycle arrest and/or apoptosis is involved in their possible antiproliferative activity.

Materials and methods

Samples

Four fruit beverages (Fb) made from juice concentrates of grape, orange and apricot, and fortified with zinc (1.6 mg/100 mL of fruit beverage) with/without iron (3 mg/100 mL of fruit beverage) and with/without skimmed milk (1.04 g skimmed milk powder/100 mL of fruit beverage) (FbZn, FbFeZn, FbZnM and FbZn-FeM) were manufactured and supplied by the Research and Development Department of Hero Spain, S.A. (Alcantarilla, Murcia, Spain). The composition of these fruit beverages is reported in table I.

In vitro gastrointestinal digestion

Fruit beverages (Fbs) were subjected to successive in vitro gastric and intestinal digestion, as previously described. Blank of digestion was run in parallel and consisted of an equivalent volume of cell culture-degree

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Antiproliferative activity of zinc-fortified fruit beverages


water subjected to the same in vitro digestion (mixture of enzymes + salts). After digestion, and to ensure inactivation of enzymes and stability of phenolic compounds, aliquots of the bioaccessible fractions (hereafter termed Fb digests) were acidified to pH 2.0 with formic acid (1.5%), filtered through a Millex-HV13 0.45 μm membrane filter (Millipore Corp., Bedford, USA) and analyzed using RP-HPLC-DAD to determine the composition of soluble extractable phenolics. It has been recognized that analysis of soluble compounds recovered after digestion of a food sample, is a good estimate of those compounds that have been released from the food matrix into the digestive juice and that become bioaccessible.20

Sample preparation

The protocol described by Cilla et al.18 was applied. Aliquots of 10 mL of fruit beverages and Fb digests were centrifuged at 3,890 g for 15 min at room temperature. The majority of concentrated juices are very rich in phenolic compounds. For this reason it was necessary to dilute supernatants of fruit beverages (sample-to-water ratio 1:2) in distilled-deionized water. Then, supernatants of fruit beverages and Fb digests were filtered through a Sep-Pak cartridge (a reverse-phase C-18 cartridge; Millipore, Maryland, USA) which retains phenolic compounds. The cartridges were previously activated with 10 mL of methanol (MeOH) and 10 mL of water. Every 10 mL of sample was eluted with 2 mL of MeOH, and all methanolic fractions were collected, filtered through a Millex-HV, 0.45 μm membrane filter (Millipore) and then analyzed by RP-HPLC-DAD. Fruit beverage pellets obtained after centrifugation were washed with acid methanol (MeOH/formic acid, 97:3) (sample-to-acid methanol ratio 1:2) and vortexed and sonicated for 5 min to extract the phenolic compounds. The solution was centrifuged as described above, and the supernatant obtained was then filtered (0.45 μm) and analyzed by HPLC.

Results of total soluble extractable phenolics shown in table II are the sum of phenolics from supernatants plus phenolics extracted from pellets.

Analysis of phenolic compounds by RP-HPLC-DAD

The analytical procedure is based on that previously described by Cilla et al.18 with slight modifications. The RP-HPLC system used for the analysis consisted of a UV/vis photodiode array detector (L-7455, Merck-Hitachi, Darmstadt, Germany), a binary pump (L-6200, Merck-Hitachi) and an autosampler (L-7200, Merck-Hitachi). Chromatographic separations of samples (35 μL of filtrates) were carried out on a 250 x 4 mm i.d., 5 μm, C18 Mediterranean Sea column (Teknokroma, Barcelona, Spain) using water:formic acid (99:1, v/v) (A) and acetonitrile (B) as the mobile phases at a flow rate of 1 mL/min. The linear gradient started with 5% solvent B in solvent A, reaching 25% B at 25 min, 60% B at 35 min, 90% B at 36 min and 90% B at 41 min. Soluble extractable phenolic compounds were identified by retention time and spectra matching of standard compounds. Chromatograms were recorded at 350 nm (flavones), 320 nm (hydrocinnamic acid derivatives), 290 nm (flavanones) and 280 nm (flavan-3-ols), and quantification was carried out using the standards apigenin, chlorogenic acid, hesperidin and (+)-catechin (all standards purchased from Sigma-Aldrich, Madrid, Spain), respectively. The amount of total soluble extractable phenolics was calculated as the sum of all the different phenolic compounds quantified by HPLC analysis, and the results were expressed as milligrams per liter of juice (mg/L).

Table I

Composition of the fruit beverages analyzed

<table>
<thead>
<tr>
<th>Component</th>
<th>g/100 g</th>
<th>FbZn</th>
<th>FbFeZn</th>
<th>FbZnM</th>
<th>FbFeZnM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmosis water</td>
<td>58.7</td>
<td>58.7</td>
<td>57.7</td>
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</tr>
<tr>
<td>Apricot puree</td>
<td>24.5</td>
<td>24.5</td>
<td>24.5</td>
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<td></td>
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<tr>
<td>Grape concentrate</td>
<td>7.2</td>
<td>7.2</td>
<td>7.2</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>Orange concentrate</td>
<td>4.2</td>
<td>4.2</td>
<td>4.2</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>Sugar</td>
<td>5.1</td>
<td>5.1</td>
<td>5.1</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>Skimmed milk powder</td>
<td>–</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pectin</td>
<td>0.354</td>
<td>0.354</td>
<td>0.354</td>
<td>0.354</td>
<td></td>
</tr>
<tr>
<td>L-ascorbic acid</td>
<td>0.054</td>
<td>0.054</td>
<td>0.054</td>
<td>0.054</td>
<td></td>
</tr>
<tr>
<td>Fe-sulphate</td>
<td>–</td>
<td>0.003</td>
<td></td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Zn-sulphate</td>
<td>0.0016</td>
<td>0.0016</td>
<td>0.0016</td>
<td>0.0016</td>
<td></td>
</tr>
</tbody>
</table>

Fb = fruit beverage (grape + orange + apricot).
Fe: supplemented with Fe sulphate (3 mg/100 mL of fruit beverage).
Zn: supplemented with Zn sulphate (1.6 mg/100 mL of fruit beverage).
M: skimmed milk (1.04 g skimmed milk powder/100 mL of fruit beverage).

Antiproliferative activity of zinc-fortified fruit beverages


563
Cell lines and culture conditions

Two human colon cancer cell lines (Caco-2 and HT-29) were used. Caco-2 cells (European Collection of Cell Cultures, ECACC 86010202, Salisbury, UK) used at passage 53-62 were grown as previously indicated. In turn, HT-29 cells (American Type Culture collection, HTB-38, Rockville, MD) used at passage 6-12 were grown in Dulbecco’s modified eagle medium (DMEM+GlutaMAX™-I) containing 1g/L glucose and pyruvate supplemented with 10% v/v fetal bovine serum, 1% v/v nonessential amino acids, 1% v/v HEPES, 1% v/v penicillin/streptomycin and 0.1% v/v fungizone at a final pH of 7.2-7.4 (all reagents were purchased from Gibco®, Invitrogen, Carlsbad, USA). Cells were maintained at 37 ºC in an incubator under a 5% CO2/95% air atmosphere at constant humidity. Both colon cancer cell lines were seeded at 10^4 cells cm^-2 density, allowed to adhere for 48 h, and used on day 3 after seeding (subconfluent homogeneously). Cells were also run in parallel and subjected to the same changes in medium.

Cell proliferation and viability tests

Viability of both cell lines treated with zinc-fortified Fb digests for 24 h was established using a Neubauer hemacytometer (Bad Mergentheim, Germany) and the Trypan blue dye exclusion test. Results of proliferation and viability in fruit beverages-treated cells are expressed as the percentage of the values obtained for control (blank of digestion) cells. All experiments were performed in triplicate.

Mitochondrial enzyme activity

To discard unspecific cytotoxicity in the antiproliferative activity of zinc-fortified Fb digest, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was used. Experiments were performed in triplicate, and the results are expressed as the percentage of viable cells with respect to the untreated control cells.

Morphological evaluation of apoptosis

Cells (1 x 10⁶) were treated for 24 h with FbZnM digest, fixed with MeOH:acetic acid (3:1), and stained with 50 mg/mL of Hoechst 33242 dye at 37 ºC for 20 min. Staurosporine 10 μM (Sigma), a well-known inducer of apoptosis, was used as positive control. Afterwards, the cells were examined under a Nikon Eclipse E800 epifluorescence microscope (Nikon, Tokyo, Japan). Morphological evaluation of apoptosis was carried out twice for each sample.

Flow cytometry analysis of cell cycle distribution

Cells (2 x 10⁶) were collected after treatment with FbZnM digest for 24 h and 48 h, fixed in ice-cold ethanol:PBS (70:30) for 30 min at 4 ºC, further resuspended in PBS with 100 μg/mL RNase and 40 μg/mL propidium iodide, and incubated at 37 ºC for 30 min. DNA content (10,000 cells) was analyzed by flow cytometry (Coulter, EPICS XL-MCL, Miami, FL) at λ_em = 536 nm and λ_exc = 617 nm. The analyses of cell cycle distribution were performed in triplicate for each treatment.

Resumption of cell cycling following treatment with FbZnM digest

Cells (2 x 10⁶) were treated with FbZnM digest for 24 h and 48 h. Then, treatment and control media were replaced with fresh growth medium, and the cells were grown for an additional 24 h and 48 h following media removal, after which cell cycle distribution was determined using the method mentioned above.

Statistical analysis

All values of each assay other than cell culture studies were based on independent triplicate samples and calculated as the mean ± standard deviation (SD). In cell culture studies, each experimental group consisted of n = 3, and data were expressed as the mean ± SD of three independent experiments. Significant differences between means of three or more data sets were determined by one-way analysis of variance (ANOVA) with post hoc Tukey testing. Differences between two data sets were determined by the Student t-test. Statistical significance was accepted for p < 0.05 (Statgraphics Plus v. 5.1, Maryland, USA).

Results and discussion

Effect of Zn with/without Fe and with/without skimmed milk presence and in vitro gastrointestinal digestion on the polyphenolic profile of fruit beverages

The polyphenolic profile and total soluble extractable phenolic content of zinc-fortified fruit beverages...
Antiproliferative activity of zinc-fortified fruit beverages

before and after in vitro simulated gastrointestinal digestion are shown in figure 1 and table II. Four main phenolic groups were identified in the beverages: hydroxycinnamic acid derivatives, flavones, flavan-3-ols and flavanones. Twenty-seven individual phenolics were identified by their UV spectra, though only the most significant ones are shown. Total soluble extractable phenolics of zinc-fortified fruit beverages before digestion ranged from 307 to 490 mg/L (table II), in agreement with values previously reported for different fruit juices, which ranged from 293 to 3,800 mg measured as gallic acid equivalents per liter of juice.

The effect of zinc alone or jointly with iron and/or skimmed milk supplementation upon the soluble extractable phenolic content of fruit beverages before and after digestion is shown in table II and figure 2. Prior to digestion, all zinc-fortified fruit beverages presented a significantly lower (p < 0.05) total soluble extractable phenolic content than Fb sample. Among the zinc-fortified fruit beverages, FbZn and FbZnFe showed the highest (p < 0.05) soluble extractable phenolic content, with losses of 14% and 20% in total phenolic content versus Fb sample - flavones and flavan-3-ols being the most affected compounds. On the other hand, milk-containing samples (FbZnM and FbZnFeM) presented the lowest amounts of soluble extractable phenolics as compared to Fb, with a decrease of 32% and 46%, respectively - hydroxycinnamic acid derivatives and flavan-3-ols being the most affected phenolic compounds compared to FbZn and FbZnFe. These findings could be attributed, on the one hand, to metal divalent-polyphenol formation of chelates as previously reported for polyphenolic-containing beverages supplemented with iron, and to the formation of hydrophobic complexes between zinc and tannic acid in beers, that could decrease soluble extractable polyphenols. Three possible metal-chelating domains in flavonoids have been reported: ortho-dihydroxyl groups, the presence of 5-OH and/or 3-OH in conjunction with a C4 keto group, and a large number of OH groups. These structural requirements are present in the phenolics occurring in the fruit beverages assayed in the present study. On the other hand, casein complexation with polyphenols related to hydrogen bonding and hydrophobic interactions could also diminish soluble extractable polyphenols, as recently reported in green tea and red wine supplemented with casein and iron. Both facts could explain the lowest soluble extractable polyphenol content in milk plus mineral containing samples.

After digestion, a decrease in total soluble extractable phenolic compounds was observed (fig. 2). The mild
### Tabla II

Main soluble extractable phenolic compound contents in Fbr, FbZn, FbZnFe, FbZnM and FbZnFeM (mg/L) before and after in vitro gastrointestinal digestion. Peak number is referred to Fig. 1. Compounds are grouped according to their chemical structures.

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Rt (min)</th>
<th>Compound</th>
<th>MS [M-H]</th>
<th>MS/MS</th>
<th>Fbr</th>
<th>FbZn</th>
<th>FbZnFe</th>
<th>FbZnM</th>
<th>FbZnFeM</th>
<th>Fb digest a</th>
<th>FbZn digest</th>
<th>FbZnFe digest</th>
<th>FbZnM digest</th>
<th>FbZnFeM digest</th>
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<tbody>
<tr>
<td>1</td>
<td>15.5</td>
<td>Neochlorogenic acid</td>
<td>353</td>
<td>191, 179</td>
<td>36.3 ± 0.5</td>
<td>21.0 ± 4.1</td>
<td>19.4 ± 0.7</td>
<td>12.2 ± 0.8</td>
<td>11.3 ± 0.8</td>
<td>11.5 ± 0.3</td>
<td>12.8 ± 0.4</td>
<td>12.7 ± 0.6</td>
<td>6.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>19.8</td>
<td>Chlorogenic acid</td>
<td>353</td>
<td>191, 179</td>
<td>76.7 ± 4.0</td>
<td>78.8 ± 5.7</td>
<td>71.6 ± 3.5</td>
<td>63.8 ± 0.8</td>
<td>38.9 ± 2.2</td>
<td>36.3 ± 2.8</td>
<td>55.5 ± 0.6</td>
<td>45.7 ± 2.0</td>
<td>47.8 ± 5.7</td>
<td>25.8 ± 1.7</td>
</tr>
<tr>
<td>6</td>
<td>21.2</td>
<td>Chlorogenic acid isomer</td>
<td>353</td>
<td>191, 179</td>
<td>9.9 ± 1.7</td>
<td>18.8 ± 1.2</td>
<td>16.7 ± 1.1</td>
<td>18.9 ± 0.2</td>
<td>12.4 ± 2.6</td>
<td>10.6 ± 3.4</td>
<td>15.3 ± 0.3</td>
<td>9.5 ± 0.1</td>
<td>7.1 ± 0.1</td>
<td>10.1 ± 3.6</td>
</tr>
<tr>
<td><strong>Total:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>144.4 ± 8.0</td>
<td>150.4 ± 6.7</td>
<td>133.3 ± 2.6</td>
<td>117.7 ± 2.2</td>
<td>75.1 ± 0.6</td>
<td>91.2 ± 8.0</td>
<td>94.7 ± 6.1</td>
<td>81.8 ± 3.7</td>
<td>80.1 ± 1.4</td>
<td>52.2 ± 0.8</td>
</tr>
<tr>
<td>7</td>
<td>22.0</td>
<td>Vicenin-2 (apigenin-di-C-hexoside)</td>
<td>593</td>
<td>473, 383, 353</td>
<td>10 ± 1.5</td>
<td>5.8 ± 0.7</td>
<td>5.7 ± 0.2</td>
<td>6.3 ± 1.2</td>
<td>5.0 ± 0.2</td>
<td>7.2 ± 1.6</td>
<td>4.7 ± 1.9</td>
<td>5.6 ± 0.6</td>
<td>3.7 ± 0.6</td>
<td>4.1 ± 0.1</td>
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<tr>
<td><strong>Total:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15.3 ± 3.6</td>
<td>7.7 ± 0.4</td>
<td>7.2 ± 0.1</td>
<td>6.9 ± 0.1</td>
<td>6.3 ± 0.2</td>
<td>9.5 ± 1.9</td>
<td>5.8 ± 1.9</td>
<td>6.8 ± 0.3</td>
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<tr>
<td>2</td>
<td>17.8</td>
<td>PCA dimer</td>
<td>577</td>
<td>289</td>
<td>56.0 ± 2.1</td>
<td>67.5 ± 1.7</td>
<td>59.8 ± 15.2</td>
<td>35.8 ± 0.2</td>
<td>28.7 ± 4.5</td>
<td>26.0 ± 3.3</td>
<td>44.4 ± 4.0</td>
<td>31.2 ± 8.0</td>
<td>36.6 ± 1.1</td>
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<td>3</td>
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<td>PCA trimer</td>
<td>865</td>
<td>577, 289</td>
<td>44.1 ± 0.6</td>
<td>67.1 ± 0.6</td>
<td>52.5 ± 16.2</td>
<td>54.2 ± 0.4</td>
<td>19.5 ± 0.1</td>
<td>19.5 ± 2.1</td>
<td>47.7 ± 4.6</td>
<td>53.3 ± 10.8</td>
<td>58.2 ± 7.1</td>
<td>85.7 ± 8.1</td>
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<td>4</td>
<td>19.4</td>
<td>PCA trimer</td>
<td>865</td>
<td>577, 289</td>
<td>47.2 ± 2.1</td>
<td>16.4 ± 0.6</td>
<td>3.1 ± 2.1</td>
<td>3.8 ± 2.6</td>
<td>2.5 ± 0.1</td>
<td>13.6 ± 0.5</td>
<td>11.0 ± 2.0</td>
<td>1.6 ± 0.1</td>
<td>3.3 ± 0.6</td>
<td>1.3 ± 0.2</td>
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<tr>
<td>10</td>
<td>31.1</td>
<td>PCA trimer</td>
<td>865</td>
<td>577, 289</td>
<td>14.7 ± 0.2</td>
<td>5.3 ± 0.4</td>
<td>9.1 ± 1.6</td>
<td>4.0 ± 1.1</td>
<td>7.7 ± 3.7</td>
<td>7.2 ± 0.3</td>
<td>6.2 ± 0.1</td>
<td>7.4 ± 1.4</td>
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<td>7.2 ± 1.5</td>
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<tr>
<td><strong>Total:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>194.0 ± 9.8</td>
<td>154.3 ± 5.3</td>
<td>124.5 ± 1.5</td>
<td>102.1 ± 7.1</td>
<td>57.2 ± 6.5</td>
<td>68.8 ± 10.0</td>
<td>109.4 ± 12.5</td>
<td>94.7 ± 0.3</td>
<td>103.7 ± 23.5</td>
<td>94.2 ± 9.9</td>
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<tr>
<td>8</td>
<td>22.3</td>
<td>Hexosyl-narirutin</td>
<td>777</td>
<td>580, 433, 271</td>
<td>17.8 ± 0.7</td>
<td>10.8 ± 0.5</td>
<td>16.1 ± 3.8</td>
<td>8.5 ± 1.5</td>
<td>8.3 ± 0.6</td>
<td>16.1 ± 1.6</td>
<td>10.5 ± 1.8</td>
<td>8.9 ± 0.9</td>
<td>8.3 ± 1.0</td>
<td>11.7 ± 6.8</td>
</tr>
<tr>
<td>9</td>
<td>30.1</td>
<td>Narirutin</td>
<td>579</td>
<td>271</td>
<td>41.6 ± 7.8</td>
<td>39.8 ± 0.8</td>
<td>45.3 ± 1.8</td>
<td>38.6 ± 1.3</td>
<td>40.8 ± 2.5</td>
<td>33.8 ± 1.9</td>
<td>39.5 ± 1.1</td>
<td>38.8 ± 1.6</td>
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<td>11</td>
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<td>Hesperetin</td>
<td>609</td>
<td>301</td>
<td>126.1 ± 28.9</td>
<td>108.9 ± 2.7</td>
<td>103.0 ± 4.8</td>
<td>86.9 ± 2.9</td>
<td>96.1 ± 6.0</td>
<td>62.7 ± 2.3</td>
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<td>57.6 ± 1.6</td>
<td>30.8 ± 5.8</td>
<td>48.6 ± 0.8</td>
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<td>12</td>
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<td>Didymin</td>
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<td>285</td>
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<td>9.5 ± 0.6</td>
<td>8.9 ± 0.2</td>
<td>9.5 ± 0.5</td>
<td>8.6 ± 0.3</td>
<td>7.3 ± 0.3</td>
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<td>7.6 ± 0.1</td>
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<td><strong>Total:</strong></td>
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<td>215.0 ± 37.0</td>
<td>177.6 ± 3.1</td>
<td>191.1 ± 6.4</td>
<td>157.4 ± 2.3</td>
<td>168.6 ± 5.4</td>
<td>134.1 ± 7.2</td>
<td>129.3 ± 6.1</td>
<td>127.5 ± 1.6</td>
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<td><strong>Total phenolics:</strong></td>
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<td></td>
<td></td>
<td></td>
<td>566.7 ± 62.1</td>
<td>489.7 ± 5.2</td>
<td>456.0 ± 5.5</td>
<td>384.2 ± 7.1</td>
<td>307.1 ± 11.4</td>
<td>303.6 ± 32.2</td>
<td>339.2 ± 1.6</td>
<td>310.7 ± 2.1</td>
<td>310.4 ± 17.6</td>
<td>263.8 ± 5.7</td>
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</table>

*aAs determined by Cilla et al. (2009)*

PCA, procyanidin; ND, not detected. Values are shown as means ± SD (n = 3). The corresponding total amount for each group includes other minor compounds not shown in the Table. Total soluble extractable phenolics are the result of the sum of each different phenolic group.
alkaline conditions reached during the digestion process, as well as possible interactions between polyphenols and other components in the in vitro digestion, such as enzymes, could explain this decrease.27 Losses of up to 32% (fig. 2) were found among zinc-fortified Fb digests compared to their non-digested counterparts, this reduction being smaller than in the case of Fb digest (47%) and the same fruit beverages with iron (54%) and iron with milk (34%) but not Zn supplemented, determined in our own previous work.18 A higher affinity of iron to polyphenols than in the case of Zn could at least explain the major decrease in soluble extractable polyphenols in the presence of iron.

These results compare well with those reported of losses in soluble phenolic compounds from 15.5% to 70% after digestion in chokeberry juice,7 orange juice28 and pomegranate juice.7 Following digestion, all digested samples offered similar total phenolic contents (table II), with the exception of FbZnFeM digest, which showed the lowest amount in total phenolics (p < 0.05) as well as in hydroxycinnamic acid derivatives and flavanones (p < 0.05). Despite the fact that FbZnFeM digest had the lowest content in total soluble extractable phenolics, it showed the lowest percentage decrease in phenolics after digestion (14.1%), among the digested samples tested. Digestion of milk-containing samples (FbZnM and FbFeZnM) produces casein phosphopeptides (CPPs) from enzymatic hydrolysis of native casein, which form complexes with iron and zinc.19 These Fe/Zn-CPPs complexes could diminish the formation of insoluble complexes between polyphenols and Fe/Zn, implying a lesser descent in total soluble extractable phenolics after digestion in milk-containing samples.

Flavones were the compounds most affected by the digestion process in all zinc-fortified fruit beverage digests compared to Fb digest, with a decrease of up to 53% in FbZnM digest. However, this decrease was only significant (p < 0.05) for milk-containing samples. Flavan-3-ols in turn showed no significant differences (p > 0.05) among sample digests, but in the case of the FbZnFeM digest high recovery of these compounds was shown compared to their non-digested counterparts, due to an increase in PCA trimer with retention time 19.2. However, in our previous study,18 flavan-3-ols virtually disappeared after

![Fig. 2.—Effect of zinc, iron and milk addition on the soluble extractable phenolic content of fruit beverages before (A) and after (B) digestion. Before digestion, values inside bars are losses of each phenolic group and values between parentheses above the bars are losses of total soluble extractable phenolics compared to Fb. After digestion, values inside bars are losses of each phenolic group and values between parentheses above the bars are losses of total soluble extractable phenolics compared to their non-digested counterpart.](image-url)
the digestion process in FbFeM digest. On comparing the results of the present study (table II) with those of the iron-fortified samples of our previous work, a positive effect can be seen derived from the presence of zinc related to flavan-3-ols content in Fb digests, probably due to higher solubility of zinc-flavan-3-ols chelates versus iron-flavan-3-ols chelates. It has been reported that flavan-3-ols have high iron-good chelation properties, and that the chelates formed are not soluble. In addition, it recently has been noted by Sreenivasulu et al. that specific structural polyphenols (tannic acid or polyphenolic beverages such as tea and red grape juice) could form chelates with zinc - thereby increasing its uptake in Caco-2 cells. This could be explained by a higher solubility of zinc-polyphenol chelates, since solubility is a pre-requisite before uptake in the intestinal tract, whereas these polyphenol sources decrease iron uptake.

Inhibition of Caco-2 and HT-29 cell proliferation by zinc-fortified fruit beverage digests

Subconfluent colon cancer cells (Caco-2 and HT-29) were preincubated during 24 h with FbZn, FbZnFe, FbZnM and FbZnFeM digests at a subtoxic dose of 7.5% v/v in culture medium (~ 50 μM total phenolics, ~ 25 mg/mL). The zinc content of zinc-fortified digests in the 7.5% v/v dose were: 6.2 μM (FbZn), 6.4 μM (FbZnFe), 9.1 μM (FbZnM) and 6.6 μM (FbZnFeM) calculated on the basis of a previous published work of our group. In a previous work it was checked that phenolic compounds were stable in culture medium during a 24 h incubation period.

In all cases cell viability was above 95%, though on considering blank of digestion, a small decrease (~6%) in cell proliferation compared to untreated cells was observed, possibly due to the remaining digestion enzymes in the digests. Therefore, to offer more correct results on the action of phytochemicals present in Fb digests of fruit beverages, values of inhibition of cell proliferation in fruit beverage-treated cells were referred to those obtained for blank of digestion-treated cells (fig. 3).

In Caco-2 cells, the highest antiproliferative activity (35% inhibition of proliferation) was achieved with FbZnM digest (p < 0.05), while in HT-29 cells FbZnFe digest (19%) and FbZnM digest (29%) were the samples that significantly (p < 0.05) inhibited cell proliferation referred to blank of digestion. Our group has recently reported a 53% inhibition of proliferation in Caco-2 cells upon continuous incubation during 24 h with Fb digest at ~50 μM total phenolics. Supplementation of these fruit beverages with Fe (FbFe) or Fe plus milk (FbFeM) instead of Zn (FbZn) or Zn plus milk (FbZnM) shows similar (9.8% vs 13.4%; FbFe vs FbZn) or lower antiproliferative activity (20.2% vs 35.0%; FbFeM vs FbZnM), respectively. In accordance, Bermudez-Soto et al.
reported an inhibition of Caco-2 cell proliferation of 40% and 70% when subjected to repeated exposure (2 h daily for 4 days) to digested chokeberry juice at 2% (~85 μM total phenolics) and 5%, respectively (~220 μM total phenolics).

The ideal anticancer agent would exert maximal capacity to kill tumor cells and/or inhibit tumor growth, without toxicity and side effects upon normal tissues.35 Therefore, to discard unspecific cytotoxicity in the antiproliferative action derived from zinc-fortified fruit beverage digests, we performed the MTT test. The MTT dye reduction assay is based on the catalytic activity of certain metabolic enzymes in intact mitochondria.36 Accordingly, this assay gave us an idea of the mitochondrial enzyme activities of metabolically active cells. No significant differences (p > 0.05) in mitochondrial enzyme activities were found in both colon cancer cell lines after treatment with FbZn, FbZnFe, FbZnM and FbZnFeM digests for 24 h at ~50 μM total phenolics compared to untreated control cells - indicating the absence of cytotoxicity in their antiproliferative activity (data not shown). Therefore, since FbZnM digest was the sample with the highest antiproliferative activity (significantly different from blank of digestion treated cells) in both cell lines without cytotoxicity, it was the only sample selected for subsequent assays to clarify the mechanism by which colon cancer cell growth was inhibited.

Mechanisms involved in the antiproliferative activity derived from FbZnM digest

Among the main events that dictate cancer evolution, uncontrolled cell proliferation and suppression of programmed cell death (apoptosis) provide the minimal environment necessary to support cancer progression.37 Targeting these pivotal events in cancers of the alimentary tract could be important for developing dietary preventing strategies. Thus, to unravel one of the possible mechanisms involved in the inhibition of cell proliferation after FbZnM digest treatment for 24 h at ~50 μM total phenolics, we carried out the morphological evaluation of apoptosis by monitoring changes in nuclear chromatin distribution that can be stained by the DNA-binding fluorochrome Hoechst 33242 dye. Incubation of Caco-2 and HT-29 cells with FbZnM digest mirrored the pattern followed by untreated and blank of digestion treated cells (data not shown), thus indicating the absence of apoptosis in our model cell system, as previously indicated by our group with Fb digest.18

Another mechanism involved in the antiproliferative action derived from FbZnM digest could be mediated by modulation of cell cycle progression. Cell cycle progression is regulated by the activity of cyclins, a family of proteins which activate the so-called cyclin-dependent-kinases (Cdks). In general, uncontrolled expression of cyclins and/or Cdks leads to either tumorigenesis or cell cycle arrest.38 Treatment of Caco-2 and HT-29 cells with FbZnM digest led to a significant (p < 0.05) increase in the proportion of cells in the S-phase in both cell lines, concomitant to a decrease (p < 0.05) in G0/G1-phase (only in HT-29 cells), and no differences in G2/M phase compared to blank of digestion-treated cells (fig. 4). These results suggest that the specific combination of phytochemicals present in FbZnM digest could modulate the proliferation of Caco-2 cells by blocking progression of the cell cycle in the S-phase, in agreement with our previous work in which Fb digest treatment for 24 h at ~50 μM total phenolics...

Fig. 4.—Analysis of cell cycle distribution of Caco-2 and HT-29 cells treated with FbZnM digest (~ 50 μM total soluble extractable phenolics) for 24 h and 48 h. Data are expressed as mean values ± SD (n = 3). *p < 0.05 and **p < 0.05 (two tailed t-test) indicate a significant difference compared to untreated cells and to blank of digestion, respectively.
exerted antiproliferative activity in Caco-2 cells due to arresting of the cell cycle in the S-phase, accompanied by the decrease in both cyclins B1 and D1.

Naturally occurring plant-derived phytochemicals of the human diet, such as polyphenols, are of particular interest as potential sources of compounds with cytostatic activity, due to the fact they are inherently low in toxicity. If FbZnM digest were to exert a cytostatic effect without cytotoxicity, the cells should regain normal proliferation following removal of FbZnM digest from treatment media in contact with the cells. To demonstrate this, treatment media was replaced by fresh growth medium, and cell cycle proliferation was determined 24 h and 48 h after media renewal. The results indicated that both Caco-2 and HT-29 cells can recover and resume normal proliferation after FbZnM digest was removed (fig. 5). In agreement with this, chokeberry juice digest-treated Caco-2 cells treated 2 h daily for 4 days at 80 μM total phenolics recovered normal proliferation after replacement of treatment media by fresh growth medium. This same observation was reported when human breast (MDA-MB-435 and MCF-7) and colon (HT-29) cancer cells recovered normal cell cycling within a day of tangeretin and nobiletin removal - supporting the hypothesis that both flavonoids only exert a cytostatic effect, without inducing damage likely to delay or suppress cell growth and/or survival.

Conclusion

Fortification of fruit beverages with zinc, with/without iron and with/without milk decreased the soluble extractable phenolic content before digestion, probably due to the formation of chelates. After digestion, a dramatic decrease in total soluble extractable phenolics was observed in all zinc-fortified samples (up to 32% with respect to the original fruit beverages) - the FbZn-FeM sample showing the lowest soluble extractable phenolic content, though with the least losses of soluble extractable phenolics after digestion (14%). FbZnM digest (~50 μM total soluble extractable phenolics) inhibited colon cancer cell proliferation (35% and 29% in Caco-2 and HT-29 cells, respectively), without causing cytotoxicity. FbZnM digest did not induce apoptosis but arrested the cell cycle in the S-phase in both cell lines. In addition, cells resumed normal cycling after FbZnM digest removal, indicating a cytostatic effect derived from the specific combination of phytochemicals present in this fruit beverage against colon cancer cells in vitro. Nevertheless, it is important to point out that in vitro studies are not always completely extrapolable to the in vivo situation, therefore, animal and human trials would be necessary to confirm the beneficial effects on these fruit beverages.

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