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The fermented non-digestible fraction of spent coffee grounds induces apoptosis in human colon cancer cells (SW480)

Nataly García-Gutiérrez [a], María Elena Maldonado-Celis [b], Mauricio Rojas-López [c], Guadalupe Flavia Loarca-Piña [a], Rocio Campos-Vega [a,*]

[a] Programa de Posgrado en Alimentos del Centro de la República (PROPAC), Research and Graduate Studies in Food Science, School of Chemistry, Universidad Autónoma de Querétaro, 76010 Santiago de Querétaro, Qro, Mexico
[b] Escuela de Nutrición y Dietética, Ciudadela de Robledo Crr 75 # 65-87, Universidad de Antioquia, Medellín AA 1226, Colombia
[c] Grupo de Inmunología Celular e Inmunogenética (GICIG), Facultad de Medicina, Carrera 51d No. 62-29, Universidad de Antioquia, Medellín AA 1226, Colombia

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ABSTRACT

Gut flora-mediated non-digestible/unabsorbed fraction of spent coffee grounds (hgf-NDSCG) was evaluated for its chemopreventive effect and molecular mechanisms involved on human colon adenocarcinoma SW480 cell survival. Small intestinal digestion released bioavailable ascorbic, chlorogenic and gallic acids from hgf-NDSCG reducing oxidative stress. The hgf-NDSCG inhibited SW480 growth (LC_{50}-19%) dose dependently by decreasing glutathione/oxidized glutathione (GSH/GSSG) ratio, indicating a cellular oxidative stress process. Flow cytometry analysis demonstrated the presence of apoptotic cells in a hypodiploid sub G_0/G_1-peak as a consequence of partial DNA loss. The pro-apoptotic mechanism of hgf-NDSCG was confirmed by SW480 cell toxicity induced by increased Caspase-3 activity and mitochondria dysfunction modulating oxidative stress using Annexin V and $DiOC_2(3)$ staining assays. The chemopreventive effects of spent coffee grounds (SCG) indicate that polyphenols present in hgf-NDSCG, digested and fermented by colon microbiota, can inhibit survival of human colon cancer cell and offer effective prophylactic value to prevent colon cancer.

Abbreviations: A, apical; ABTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); AA, antioxidant activity; B, basolateral; BSA, bovine serum albumin; bis-AAF-R110, bis-alanylalanyl-phenylalanyl- rhodamine 110; CRC, colorectal cancer; CB, coffee beans; DAD, diode array detection; DF, dietary fiber; DMEM, Dulbecco's modified eagle medium; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ECACC, European Collection of Animal Cell Culture; FBS, fetal bovine serum; FRAP, ferric reducing ability power; FSC, forward-scattered light; GF-AFC, glycylphenylalanyl-aminofluorocoumarin; GSH, reduced glutathione; GSSG, oxidized glutathione; hgf, human gut flora fermented; hgf-NDSCG, human gut flora fermented/non-digestible/unabsorbed-SCG; HPLC, high-performance liquid chromatography; ICP-AES, inductively coupled plasma atomic emission spectrometry; IDF, insoluble dietary fractions; LC_{50}, lethal concentration fifty; MMP, mitochondria membrane potential; NDSCG, non-digestible/unabsorbed spent coffee grounds; PBS, phosphate buffered saline; Papp, apparent permeability coefficient; PI, propidium iodide; ROS, reactive oxygen species; SDF, soluble dietary fraction; SCFAs, short-chain fatty acids; SCG, spent coffee grounds; TDF, total dietary fiber; 5-CQA, 5-caffeoylquinic acid.

* Corresponding author.
E-mail addresses: nat_gagu5487@hotmail.com (N. García-Gutiérrez), maria.maldonado@udea.edu.co (M.E. Maldonado-Celis), mrojaslop@hotmail.com (M. Rojas-López), loarca@uaq.mx (G.F. Loarca-Piña), chio_cve@yahoo.com.mx (R. Campos-Vega).

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1. Introduction

Colorectal cancer (CRC) remains the third most common cancer and the fourth leading cause of cancer-related mortality worldwide, despite advances in screening and diagnostic technology (Donovan, Selmin, Doetschman, & Romagnolo, 2016). The development of CRC is associated with several factors, including lifestyle (high alcohol consumption, high-fat low fiber diet, red meat, smoking, lack of physical exercise), aging, obesity, diabetes, inflammatory bowel diseases, family history and some genetic syndromes (Afrin et al., 2016). However, the incidence of CRC can be dramatically reduced by a healthy diet (Jia et al., 2013; Vainio & Weiderpass, 2006) rich in fruits, vegetables, grains and cereals containing many components, including dietary fiber, vitamins, and bioactive phytochemicals that affect cancer development (Derry, Raina, Agarwal, & Agarwal, 2013). In this regard, the identification of natural bioactive compounds that suppress cell proliferation and/or induce apoptosis could be a complementary and useful strategy to control the development and progression of colon cancer (Martín, Goya, & Ramos, 2016).

Earlier, we proposed that bean (*Phaseolus vulgaris* L.) and/or its constituents can potentially prevent colon cancer based on their anti-carcinogenic properties (Campos-Vega, Oomah, Loarca-Piña, & Vergara-Castañeda, 2013). Our studies demonstrated that fermented products metabolized by human gut microbiota of non-digestible fraction from cooked beans modulated survival, protein and gene expression associated with apoptosis, cell cycle arrest, and proliferation in human adenocarcinoma colon cancer cells (Campos-Vega, Guevara-Gonzalez, Guevara-Olvera, Oomah, & Loarca-Piña, 2010; Campos-Vega et al., 2012). The modulations in gene expression by the non-digestible bean fraction were confirmed in the colon tissue of AOM-induced rats, demonstrating the chemopreventive effect of common bean on early-stage colon cancer (Vergara-Castaneda et al., 2010, 2012). These effects were mainly attributed to the production of short-chain fatty acids (SCFAs), acetate, butyrate, and propionate by gut microbial fermentation of dietary fiber as well as the antioxidant compounds associated with dietary fiber. Butyrate inhibits histone deacetylase resulting in histone hyperacetylation and growth inhibition in the colonic epithelial cells, whereas propionate inhibits cell growth and activates apoptosis in colorectal carcinoma cells. This link between histone hyperacetylation, hyperacetylation-induced transcriptional regulation and growth inhibition has been considered the foremost factors in preventing colon cancer (Kasubuchi, Hasegawa, Hiramatsu, Ichimura, & Kimura, 2015). Furthermore, antioxidants that scavenge reactive oxygen species (ROS) can potentially inhibit the expression of aggressive cancer phenotypes. Although antioxidants exert chemoprevention by reducing genotoxicity and slowing cancer progression (Ibáñez et al., 2012; Stone, Krishnan, Campbell, & Palau, 2014), they can at high levels act as pro-oxidants inducing the selective killing of cancer cells (Martin-Cordero, Leon-Gonzalez, Calderon-Montano, Burgos-Moron, & Lopez-Lazaro, 2012).

Recently, we focused our attention on spent coffee grounds (SCG) as an excellent source of bioactive compounds, such as polyphenols and antioxidant dietary fiber, which support their use as functional food ingredient (Campos-Vega, Loarca-Piña, Vergara-Castañeda, & Oomah, 2015). SCG contains 43% total dietary fiber that exhibit antioxidant properties. Moreover, SCG are metabolized during simulated human gut colonic fermentation and its polyphenols are bio accessible during simulated gastrointestinal digestion (Campos-Vega, Loarca-Piña et al., 2015, Campos-Vega, Vázquez-Sánchez et al., 2015). SCG also exerts anti-inflammatory activity, mediated by SCFAs production from its dietary fiber, by reducing the release of inflammatory mediators (López-Barrera, Vázquez-Sánchez, Loarca-Piña, & Campos-Vega, 2016). The study also showed that human gut fermented-unabsorbed SCG fraction from medium roasted coffee produced elevated SCFAs and suppressed nitric oxide production in LPS-stimulated RAW 264.7 macrophages through cytokine modulation. We aimed our investigation on evaluating this human gut fermented-unabsorbed SCG on human colon adenocarcinoma SW480 cell survival and the potential mechanisms involved in chemoprevention. SW480 expresses a truncated form of adenomatous polyposis coli (APC) considered the typical "gatekeeper" tumor-suppressor gene, mutations of which are crucial initiating events in the development of human colorectal cancer (Lamprecht & Lipkin, 2003).

2. Materials and methods

Arabica coffee beans (CB) (medium-roasted) grown and harvested in 2014 (Chiapas, México) were purchased directly from the manufacturer. Chemicals were purchased from Sigma Chemical Co and J.T. Baker (México City, México).

2.1. Spent coffee ground preparation

Spent coffee ground was recovered from the filter of a coffeemaker (MOULINEX, heliora comfort, México) after 6 min brewing (7:100 w/v) at 90 °C. The spent coffee was freeze-dried, defatted (Soxhlet extraction, petroleum ether, 6 h), and then stored in the dark until use.

2.2. Proximate composition

AOAC procedures were used to determine moisture (method 925.10), lipid (method 920.39), ash (method 923.03), and nitrogen (method 920.87) contents of the CB and SCG samples (VA, 2002). Protein content was calculated as nitrogen × 6.25. Carbohydrate values were obtained by difference. The mineral content was determined by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (Mussatto, Carneiro, Silva, Roberto, & Teixeira, 2011).

2.3. Total dietary fiber (TDF) and resistant starch

Total dietary fiber, containing soluble dietary fraction (SDF), and insoluble dietary fraction (IDF) were determined following the method of Shiga, Lajolo, and Filisetti (2003). Resistant starch was quantified following the gravimetric method of Saura-Calixto, Goñi, Bravo, and Mañas (1993).

2.4. Analysis of polyphenols, caffeine and ascorbic acid by HPLC-DAD

Polyphenols and caffeine were analyzed by HPLC (High-performance liquid chromatography) on CB, SCG and on each digestion step (supernatants collected after centrifugation), following the method described by Ramírez-Jiménez, Reynoso-Camacho, Mendoza-Díaz, and Loarca-Piña (2014). Detection was accomplished with a diode array detector (DAD) and chromatograms were recorded at 280 nm. Quantification was performed using the external standard method with commercial standards of caffeic, chlorogenic, *p*-coumaric, ellagic, ferulic and gallic acids, (+)-catechin, quercetin, rutin as well as, caffeine and ascorbic acid. The values were expressed in mg of the respective standard.

2.5. Antioxidant activity

The antioxidant activities (AA) were evaluated using three antioxidant systems: 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Fukumoto & Mazza, 2000), and 2,2′-azinobis (3-ethylbenzothiazo line-6-sulfonic acid) (ABTS) (Nenadis, Wang, Tsimidou, & Zhang, 2004), and ferric reducing ability power test (FRAP) (Russo et al., 2015). The results were expressed as μmol Trolox equivalents per gram of dry sample (ABTS and DPPH), and μmol Fe^{2+} equivalents per gram of dry sample (FRAP).

2.6. In vitro gastrointestinal digestion and colonic fermentation

The adapted method of Campos-Vega, Loarca-Piña et al. (2015), Campos-Vega, Vázquez-Sánchez et al. (2015) was followed to mimic SCG gastric physiologic conditions (mouth-small intestine), characterized by coupling *in vivo* (the sample is chewed by healthy subjects), *in vitro* (stomach) and *ex vivo* (rat everted gut sac) assays. The human gut flora fermented (hgf) method (Campos-Vega et al., 2009) was used to estimate the effects of the sample from the small intestine mucosal side (outside)-referred as unabsorbed-SCG (NDSCG) fraction- in the colon (mixture of 0.5 mL gut bio accessible fraction and 0.5 mg of non-digested fraction). To perform the assay fecal inoculum, as a human colonic microbiota source, was prepared from stool supplied by two healthy subjects, who had not consumed antibiotics for at least 3 months and had no history of gastrointestinal diseases. The obtained extract was named human gut flora fermented-unabsorbed-SCG (hgf-NDSCG). This study approved by Universidad Autonoma de Queretaro Human Research Internal Committee complied with the National Institutes of Health Guide for Care and Use of Laboratory Animals. All participants signed informed consent.

2.7. Calculation of the apparent permeability coefficients

Apparent permeability coefficient (P_{app}) was determined according to Eq. (1) (Lassoued, Khemiss, & Sfar, 2011):

$$P_{app} = (dQ/dt)(1/AC_0) \quad (1)$$

where P_{app} (cm/s) is the apparent permeability coefficient, dQ/dt (mg/s) is the amount of drug transported across the membrane per unit time, A (cm^2) is the surface area available for permeation and C_0 (mg/mL) represents the initial concentration of the drug outside the everted gut sacs. The mean and the standard deviation values of P_{app} were calculated and expressed in 10^{-4} cm/s unit. The water flux, resulting from both water absorption and efflux in the jejunum segment was determined according to Khemiss et al. (2009).

2.8. Cell culture and treatments

SW480 cells were obtained from the European Collection of Animal Cell Culture (ECACC, Salisbury, UK). The cells were grown and maintained in Dubelcco's Modified Eagle Medium (DMEM, American Type Culture Collection) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY) and 1% antibiotic–antimycotic (Gibco, Grand Island, NY) at 37 °C under 5% CO_2- air atmosphere. Subculture of SW480 cell line was performed by trypsinization (trypsin/EDTA solution: 0.05/0.02%) (Sigma–Aldrich, Canada Ltd.). SW480 cells were cultured under the indicated growth conditions in 24-well plates at 3×10^4 cells/well. After 24 h, conditions were changed by adding different concentrations (5, 7.5, 10, 20 and 25%) of 100% hgf-NDSCG extract in DMEM containing 0.5% bovine serum albumin (BSA) (Sigma–Aldrich, Canada Ltd.). After the incubation, cells were harvested, hemocytometer counts performed and the growth inhibition rate was plotted to determine cell survival LC_{50} (lethal concentration fifty) value, and confirmed by ApoTox-Glo Tripex Assay (Promega, USA). DMEM medium containing 0.5% BSA was also added to control cell culture. The effect of hgf-NDSCG was normalized to the blank control (hgf-NDSCG without cells) and to the non-treated control (control; 0%/mL, 100%) cells. All data points were performed in duplicate and each experiment was repeated independently at least three times for statistical evaluation.

2.9. Cell cycle analysis

Cell cycle distribution was analyzed by labelling cells with propidium iodide (PI). Assays were carried out as described by Nicoletti, Migliorati, Pagliacci, Grignani, and Riccardi (1991). Briefly, 1×10^6 cells were seeded in 10 mm plates and harvested by trypsinization (0.5% trypsin/2.6 mM EDTA) 24 h after initial treatment with hgf-NDSCG (3.75, 7.5 and 15%). Then cells were centrifuged and fixed in 1 mL methanol:phosphate buffered saline (PBS) (9:1, v/v), washed twice in PBS and re-suspended in 200 μL PBS containing 0.25 mg/mL RNAse (Type I-A, Sigma-Aldrich, Germany) and 0.1 mg/mL PI. After incubation in the dark at 37 °C for 30 min, the PI fluorescence of 10,000 cells was analyzed using a FACSCanto II flow cytometer and the software BD FACSDiva 6.1.3. (BD Biosciences, San Jose). Cell clamps were excluded using the height and area signals on the forward scatter (FSC-H vs FSC-A at 488 nm). Cell cycle model was fixed using the software FlowJo 0.6.1 (Ashland, OR, USA) and applying the Watson model.

2.10. Mitochondrial membrane permeability changes

Changes in mitochondrial membrane permeability were assessed by using the MitoProbe™ $DiOC_2$ (3) (3,3′-diethyloxacarbo cyanine iodide) assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured with hgf-NDSCG (3.75, 7.5 and 15%), harvested by trypsinization at 24 h and stained with $DiOC_2$ (3) at 37 °C for 30 min in darkness. Cells were washed and re-suspended in PBS to analyze 10,000 events by flow cytometry with excitation at 488 nm and detection of the emission with the green (530/15 nm) and the red (780/60 nm) filters. This method allows quantifying cells with depolarized mitochondrial membrane by flow cytometry.

2.11. Caspase-3 activity

Attached and floating cells were harvested, washed twice in PBS and stored at −20 °C. Cell viability and Caspase-3 activity was detected by ApoTox-Glo Tripex Assay (Promega, USA). Cells 1×10^6 per well were seeded on solid white 96-well plates and treated with hgf-NDSCG (3.75, 7.5 and 15%). 20 mL of viability/cytotoxicity reagent containing both GF-AFC (glycylphenylalanyl-aminofluorocoumarin) substrate and bis-AAF-R110 (bis-alanylalanyl-phenylalanyl- rhodamine 110) substrate was added to all wells, and the content was mixed by orbital shaking for one minute. The cells were incubated for 1 h and the two fluorescence signals were recorded simultaneously. The fluorescence signal to determine cell viability was recorded with $400_{Ex}/505_{Em}$ filters, and cell cytotoxicity using $485_{Ex}/520_{Em}$ filters. After measurement of cell viability and cytotoxicity, 100 μL of substrate for Caspase-3 Z-DEVD attached to aminoluciferin was added to all wells, cleaved, and the luminescence signal from Caspase-3 activation was measured after 30 min incubation. Each plate had the cells without any treatment as negative controls. Each experiment was performed in duplicate.

2.12. Flow cytometric analysis of apoptosis

Phosphatidylserine exposure and membrane damage were detected via an Annexin-V-FLUOS and propidium iodide Staining Kit (Roche Diagnostics) according to manufacturer's protocol, by flow cytometric measurement of externalized phosphatidylserine. SW480 cells grown in 12 well plates were treated with hgf-NDSCG (3.75, 7.5 and 15%) for 24 h. Following treatment, cells were trypsinized and pelleted, washed in PBS and again pelleted. Cells were re-suspended in Annexin-V-Fluos binding buffer, then Annexin-V-Fluos and PI were added; cells were incubated in the labelling solution for 20 min at room temperature before analysis by flow cytometry. Cells were gated according to PI and Annexin-V staining. Cells positive for Annexin-V, PI or both were considered early apoptotic, necrotic or late apoptotic/dead, respectively. Assays were performed in duplicate.

2.13. GSH/GSSG measurement

Redox status of SW480 treated cells were measured by the GSH/GSSG-Glo assay kit from Promega (USA), according to the manufacturer's protocol.

2.14. Statistical analysis

The results are expressed as mean ± standard deviation of at least three independent experiments and analyzed by one-way ANOVA. Statistical significance was determined using Student's *t* test or Tukey's multiple comparison post-test ($p < 0.05$) according to Statistical Analytical System (JMP version 7.0, SAS Institute, Inc., Cary, NC, USA).

3. Results and discussion

3.1. Proximate composition

Carbohydrates were the main components of coffee beans (60.4%) and spent coffee grounds (57.4%), consisting primarily of total dietary fiber (mostly insoluble) in SCG (54%) and CB (49%) (Table 1). Resistant starch content was similar (6%) in both samples in accordance with our previous study (López-Barrera et al., 2016). SCG contained significantly higher lipid and protein contents than CB, due to concentration of the non-extracted components during coffee brew preparation. Many authors report lower protein contents (6.7 – 14%) in SCG, suggesting that our SCG protein values may be slightly overestimated due to the presence of other nitrogen-containing substances (caffeine, trigonelline, free amines and amino acids) (Delgado, Vignoli, Siika-aho, & Franco, 2008). SCG retained only 39% of minerals since most are easily extracted with hot water during instant coffee preparation. Potassium (P) and calcium (Ca) were the most abundant minerals followed by magnesium (Mg), while zinc (Zn) was the least abundant. These results are in agreement with earlier observations (Campos-Vega, Loarca-Piña et al., 2015, Campos-Vega, Vázquez-Sánchez et al., 2015). The trace minerals copper, iron, manganese, selenium (Se), and zinc (Zn), present in SCG (except Se), are integral constituents in the antioxidant defense system as metalloproteins. Globally, Ca, Fe, Se, iodine, and Zn deficiencies are the most widespread forms of mineral malnutrition (Lukaski, 2004; Welch & Graham, 2004), because of the high availability/accessibility of SCG in many countries worldwide, the inclusion of SCG in the diet could have a major impact on population health.

3.2. Content of selected bioactive compounds from coffee beans and spent coffee grounds and their bioaccessibility during gastrointestinal (mouth-colon) digestion

Three polyphenols were identified in CB and SCG, the latter retaining up to 35, 56 and 61% of chlorogenic acid, gallic acid and rutin, respectively present in CB (Table 2). The predominance of chlorogenic and gallic acids and trace amounts of rutin in roasted beans and SCG have been reported previously (Campos-Vega, Loarca-Piña et al., 2015, Campos-Vega, Vázquez-Sánchez et al., 2015; López-Barrera et al., 2016). However, our values for chlorogenic acid, gallic acid and rutin were higher (5.7, 3 and 80-fold for CB; 3, 2.4 and 162-fold for SCG, respectively) than those reported recently (López-Barrera et al., 2016). Rutin content of SCG and the amount of chlorogenic acid retained in SCG were similar to those reported for SC from medium roasted coffee (Campos-Vega, Loarca-Piña et al., 2015, Campos-Vega, Vázquez-Sánchez et al., 2015). The chlorogenic acid content of SCG is equivalent to those present in 35 mL of coffee assuming that a cup of coffee (200 mL) contains 95.8 mg of chlorogenic acid (Nardini, Cirillo, Natella, & Scaccini, 2002). Ascorbic acid was higher than individual phenolic acid content with SCG retaining 78% of ascorbic acid present in CB. High dose of ascorbic acid can act as an antioxidant to reduce cancer and/or selectively kill colorectal cancer cells with mutated KRAS or BRAF oncogenes, which are often refractory to approved targeted therapies (Reczek & Chandel, 2015), highlighting the importance of our results. Antioxidants may also engage with cellular signaling flow, regulating transcription factors and subsequently modulating gene expression involved in cellular metabolism and survival (Giampieri, Alvarez-Suarez, & Battino, 2014).

The salivary digest contained only ¼ of the SCG's chlorogenic acid content (Supplementary Table S1) suggesting that it was hydrolyzed to caffeic, *p*-coumaric and ferulic acids similar to those observed in coffee (Nardini et al., 2002). A significant proportion of chlorogenic acid was absorbed (29.8%) as indicated by its disappearance in the stomach, higher than the 16.3% reported after 30 min infusion in rat stomach (Lafay et al., 2006). Bioaccessibility of ascorbic acid, chlorogenic acid and rutin from SCG was signifi-

Table 1
Chemical and nutraceutical composition of medium-roasted coffee beans and spent coffee grounds.

Component	Coffee bean	Spent coffee grounds	*Component*	Coffee bean	Spent coffee grounds
*Moisture	3.60 ± 0.00[a]	5.40 ± 0.00[b]	*Minerals (mg/kg)*		
*Ash	6.90 ± 0.10[a]	2.70 ± 0.10[b]	Potassium	15.21 ± 0.03[a]	3.60 ± 0.60[b]
*Protein	14.50 ± 0.30[a]	16.20 ± 0.20[b]	Calcium	1.72 ± 0.02[a]	1.80 ± 0.10[b]
*Lipids	14.90 ± 0.10[a]	18.10 ± 0.10[b]	Iron	0.04 ± 0.02[a]	0.04 ± 0.16[a]
*Carbohydrates	60.40 ± 0.30[a]	57.40 ± 0.30[b]	Manganese	0.02 ± 0.02[a]	0.02 ± 0.01[b]
*Total fiber	48.60 ± 0.10[a]	53.90 ± 0.10[b]	Coper	0.01 ± 0.00[a]	0.13 ± 0.00[b]
*Soluble fiber	2.40 ± 0.10[a]	1.10 ± 0.10[b]	Zinc	0.004 ± 0.00[a]	0.004 ± 0.00[b]
*Insoluble fiber	46.20 ± 0.10[a]	52.80 ± 0.00[b]	Magnesium	2.24 ± 0.40[a]	1.56 ± 0.10[b]
*Resistant starch	6.00 ± 0.00[a]	6.10 ± 0.00[b]	Aluminum	0.02 ± 0.03[a]	0.01 ± 0.00[b]

Results are the average of 3 independent experiments ± SEM and are expressed as percentage (%) per *gram sample dry basis. Protein content was calculated as nitrogen × 6.25. Means in a row with different letters are significantly different ($p < 0.05$).

Table 2
Bioaccessibility of phenolic compounds and ascorbic acid from medium-roasted spent coffee during *in vitro* colonic fermentation.

Sample	Bioaccessibility					
	Acids				(+)-Catechin	Rutin
	Ascorbic	Chlorogenic	Gallic	Sinapic		
CB*	71.00[a]	47.62[a]	10.71[a]	nd	nd	15.91[a]
SCG*	55.01[b]	16.80[b]	6.01[b]	nd	nd	9.71[b]
Colonic fermentation						
6 h	2.40[e]	0.35[d]	0.48[d]	0.05[c]	nd	nd
12 h	4.90[d]	0.35[d]	0.81[c]	0.06[b]	0.51[b]	nd
24 h	6.20[c]	0.39[c]	0.38[e]	0.09[a]	1.31[a]	nd

Means in a column for each component with different letters are significantly different ($p < 0.05$). Concentrations are expressed as milligram equivalent of ascorbic acid, caffeic acid, chlorogenic acid, *p*-coumaric, ferulic acid, gallic acid, sinapic acid, (+)-catechin, quercetin or rutin per gram* or mg/mL sample, respectively. Results are the average of at least 3 independent experiments. nd, under detection limit.

cantly higher after gastric (stomach) than salivary (mouth) digestion (Supplementary Table S1). For example, the stomach digest contained almost 3 times (2.8–3.3×) the amount of ascorbic acid, chlorogenic acid and rutin than the salivary digest. Gallic acid was bioaccessible only minimally and 13% of SCG after salivary and gastric digestion, respectively indicating rapid absorption from the stomach (Lafay & Gil-Izquierdo, 2008). Similarly, the gastric digest contained significantly higher amounts (2.3–12.3×) of *p*-coumaric acid, ferulic acid, catechin and quercetin than salivary digest. Only caffeic acid was significantly higher (3.4×) in the salivary than in the stomach digest suggesting that it was susceptible to gastric low pH conditions unlike ascorbic and phenolic acids.

Small intestinal digestion of ascorbic acid increased ($Y = 0.0871x + 12.35$; $r^2 = 0.799$), whereas those of chlorogenic ($Y = -0.015x + 6.205$; $r^2 = 0.987$), caffeic ($Y = -0.008x + 0.955$; $r^2 = 0.987$) and gallic ($Y = -0.0308x + 3.72$; $r^2 = 0.928$) acids decreased linearly with increasing incubation time (15–60 min). Thereafter, concentration of ascorbic and gallic acids increased and chlorogenic acid concentration decreased after 2 h incubation. Coumaric acid, ferulic acid and quercetin concentrations were stable throughout the digestive tract. About 53% of SCG catechin disappeared at the end of 2 h of intestinal incubation (ileal digest) (relative to 15 min incubation), similar to grape catechin (44%) resulting from its interactions with pancreatic proteins (Xia, Deng, Guo, & Li, 2010) probably hydrolyzing flavonoids to less polar aglycones prior to gastrointestinal absorption. Small intestinal digestion of chlorogenic acid (30% of SCG after 2 h incubation) was similar to the absorption (25%) of coffee chlorogenic acid intake after coffee consumption (Crozier, Stalmach, Lean, & Crozier, 2012).

Fermentation time had minimal effect on chlorogenic acid release in the colon, whereas ascorbic acid levels increased linearly with time ($Y = 0.1964x + 1.75$; $r^2 = 0.869$) (Table 2). Gallic acid concentration increased from 6 to 12 h colonic fermentation and decreased thereafter. Catechin recovery increased significantly (2.5-fold) after 24 h colonic fermentation similar to those observed with SCG flavonoids (Campos-Vega, Loarca-Piña et al., 2015, Campos-Vega, Vázquez-Sánchez et al., 2015). Some phenolics (caffeic, *p*-coumaric and ferulic acids and quercetin) present during small intestinal digestion were absent/unavailable for colonic fermentation indicating rapid metabolism as reported previously (Anson et al., 2009). Other phenolics including rutin and sinapic acid were unidentified in the small intestinal and colon digest probably due to their interactions with other food constituents such as sugars, lipids and fiber, regulating their bioaccessibility (Toydemir et al., 2013). These results suggest that the highest proportion of ascorbic (≈30%), chlorogenic (≈33%) and gallic (50%) acids from SCG are available for absorption in the small intestine than during colon fermentation (2–13%). However, chlorogenic acid was also highly available (70% of SCG) during gastric digestion. Nevertheless, some of the colonic metabolites derived from phenolic acids may play a role in the protection of colon cancer and Alzheimer disease (Núñez-Sánchez et al., 2016; Wang et al., 2015).

Ascorbic, chlorogenic, gallic and *p*-coumaric acids exhibited high absorptive (apical to basolateral; A to B) transport across normal sacs of rat intestine according to the bilateral apparent permeability coefficients (P_{app}) values (Supplementary Table S2). P_{app} values increased linearly ($r^2 \geqslant 0.94$) with incubation time displaying higher transport throughout the intestine (A to B) for ascorbic, chlorogenic and gallic acids. This is in accordance with previous studies demonstrating active basolateral translocation of phenolic compounds (Campos-Vega, Loarca-Piña et al., 2015, Campos-Vega, Vázquez-Sánchez et al., 2015; Deuβer et al., 2013). The active efflux/uptake ratio for chlorogenic (15 and 30 min incubation) and gallic (30 min incubation) acids was greater than 2 indicating active efflux or uptake mechanism (Hubatsch, Ragnarsson, & Artursson, 2007). The absorption mechanism of ascorbic and *p*-coumaric acids was simply passive diffusion (ratio < 2).

3.3. Antioxidant activity of permeated spent coffee grounds (everted gut sac model)

Antioxidant activity (ABTS and DPPH) was unaffected by incubation time with higher ABTS than DPPH values. In contrast, the reducing potential of oxidative stress (FRAP) increased significantly ($p < 0.05$) with incubation time (Supplementary Table S2), doubling each time from 15 to 30 min and 60 to120 min. Moreover, the amount of absorbed compounds was highly correlated ($r \geqslant 0.996$; $p < 0.0001$) with antioxidant activities; chlorogenic acid exhibited the highest correlation with ABTS. The results of the absorbed phenolic compounds suggest that the antioxidant activity was primarily due to their biotransformation through the intestinal membrane.

3.4. Effect of hgf-NDSCG on SW480 survival and Caspase-3 activity

We investigated the role of hgf-NDSCG on human colon cancer cell survival; the cell viability was first analyzed by hemocytometer counts. The results (Fig. 1) showed that hgf-NDSCG treatment gradually reduced cell viability in a dose-dependent manner, (LC_{50} = 19%, equivalent to 2.85, 1.00 and 0.74 mmol/L of acetic, propionic, and butyric acids, respectively). Three LC_{50} (3.5, 7.5 and 15%) were selected to evaluate molecular changes, and confirm the dose-dependent effect of hgf-NDSCG on SW480 cells by ApoTox-Glo Triplex Assay. Cell viability was significantly reduced in all hgf-NDSCG treated cells groups compared to control, inducing toxicity and increasing Caspase-3 activation (Fig. 1); caspase-3 is an executioner caspase confirming that induced cell death resulted from an apoptotic process. This pro-apoptotic mechanism has been reported for natural bioactive compounds (açai berry

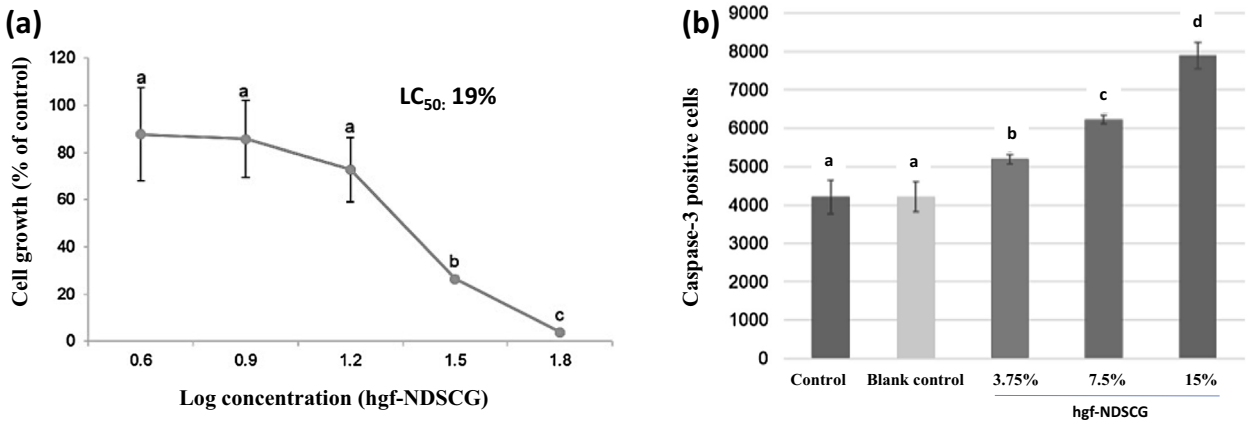

Fig. 1. (a) Concentration–response curve of hgf-NDSCG on SW480 colon carcinoma cells after 24 h incubation. The effect of hgf-NDSCG was normalized to the blank control (hgf-NDSCG without cells) and to the untreated control (control -; 0%/hgf-NDSCG mL, 100%) cells. LC_{50} was calculated from the antilog of the x-axis value at the inflection point of the sigmoid curve generated for the treatment (JMP V. 5.0). Values are the mean of three independent experiments with standard errors depicted by vertical bars. (b) Caspase-3 assays performed using the ApoTox-Glo Triplex Assay. Means in a bar with different letters are significantly different ($p < 0.05$).

Table 3
Different concentrations of hgf-NDSCG affected different phases of SW480 cell cycle and GSH/GSSG ratio.

Treatment group	Percent cells in each cell cycle phase				GSH/GSSG
% hgf-NDSCG	*SubG₀*	*G₀/G₁*	*S*	*G₂/M*	
Control (−)	07.5 ± 1.5[a]	29.9 ± 4.6[a]	1.5 ± 2.9[a]	26.3 ± 5.5[a]	1.9
Blank control	07.2 ± 1.2[a]	27.7 ± 4.1[a]	1.2 ± 1.5[a]	28.9 ± 3.7[a]	1.2
3.75	17.5 ± 2.1[b]	19.6 ± 1.2[c]	0.7 ± 1.9[a]	16.7 ± 1.6[b]	1.1
7.50	24.2 ± 2.9[c]	18.5 ± 4.0[c]	−2.9 ± 0.8[a]	13.2 ± 1.8[c]	0.6
15	28.8 ± 3.3[c]	11.4 ± 2.5[d]	−2.4 ± 0.3[a]	07.9 ± 0.2[d]	0.1

Cells were treated with vehicle or 3.75, 7.5 or 15% of hgf-NDSCG for 24 h. DNA was stained with propidium iodide and cell cycle distribution was detected using flow cytometry. Blank control: hgf-NDSCG without cells; non-treated control (control -): 0%/mL, 100% cells. GSH:GSSH represents the effect on reduced-oxidized glutathione ratio. Data are reported as the mean ± SEM of at least two independent experiments with triplicate. Means per column that do not share letters are significantly different from each other ($p < 0.05$).

extract) in SW480 (Afrin et al., 2016), and LoVo (grape seed procyanidins) in HT29, SW480 human colon cancer cells, wherein the compounds increased apoptosis levels through the activation of caspase-3 and PARP cleavage fragment (Forbes-Hernández et al., 2014). Ascorbic acid (1.64 mM), the major bio accessible colonic compound from SCG also inhibited SW480 proliferation and induced apoptosis partly due to ROS-dependent suppression of Sp transcription factors (Pathi et al., 2011). Our previous study (Campos-Vega et al., 2012) demonstrated that compounds other than polyphenolics could be contributing to reduced SW480 viability and Caspase-3 induction. For example, metabolism of the non-digested fraction from common beans by the human gut flora produced SCFAs which induced human colon cancer cell (HT29) growth inhibition accompanied by Caspase-3 activation and apoptosis induction (Campos-Vega et al., 2012).

3.5. Effect of hgf-NDSCG on GSH/GSSG ratio

Reduced glutathione is considered one of the most important ROS scavengers, and its ratio with oxidized glutathione may be used as a marker of oxidative stress (Townsend, Tew, & Tapiero, 2003). The hgf-NDSCG decreased GSH content and increased GSSG level, which resulted in a dose-dependent reduction in GSH/GSSG ratio (Table 3), suggesting that phytochemicals present in hgf-NDSCG can disrupt cellular redox balance. An enhanced reduced GSH/GSSG ratio were detected in rosemary polyphenols-treated (HT29) colon cancer cells (Ibáñez et al., 2012). However, the potential antioxidant and genoprotective properties of spent coffee have been demonstrated in human cells through ROS inhibition (Bravo, Arbillaga, de Peña, & Cid, 2013). Furthermore, 5-caffeoylquinic acid (5-CQA) and thermal degradation products from arabica and robusta filter coffee brews reduced ROS production in human colon cancer cells (HT29) (Bakuradze et al., 2010). However, extracts or compounds evaluated in those studies were not subjected to gastrointestinal digestion or colonic fermentation. Although agents with antioxidant activity may induce cancer preventive effects by reducing and/or preventing ROS increase at the cellular levels, some pro-oxidant agents in hgf-NDSCG could increase ROS levels to cytotoxic levels, mediated by the decreased GSH/GSSH ratio, thereby inducing a therapeutically useful selective killing of SW480 cancer cells. In fact, oxidative stress induction by pro-oxidant agents is an attractive anticancer strategy. The effects can be achieved by agents with both antioxidant and pro-oxidant properties (e.g. curcumin), which can act as cancer chemopreventive, carcinogenic, and chemotherapeutic agents mainly depending on concentration (Martin-Cordero et al., 2012). These results highlight the importance of gastrointestinal digestion and colonic fermentation of foods, and their effective health benefits.

3.6. Mitochondrial membrane permeability changes

Mitochondrial membrane permeability changes were evaluated by flow cytometry after staining cells with $DiOC_2$ (3) reagent because alterations of mitochondria membrane potential (MMP) and permeability cause mitochondria dysfunction, which is directly involved in the intrinsic apoptotic pathway, even involving Caspase-3. This cyanide dye accumulates in the mitochondrial matrix and is released in the cytosol after membrane depolarization (membrane with reduced $\Delta\Psi m$) (Maldonado-Celis et al., 2008).hgf-NDSCG treated SW480 cells exhibited high membrane

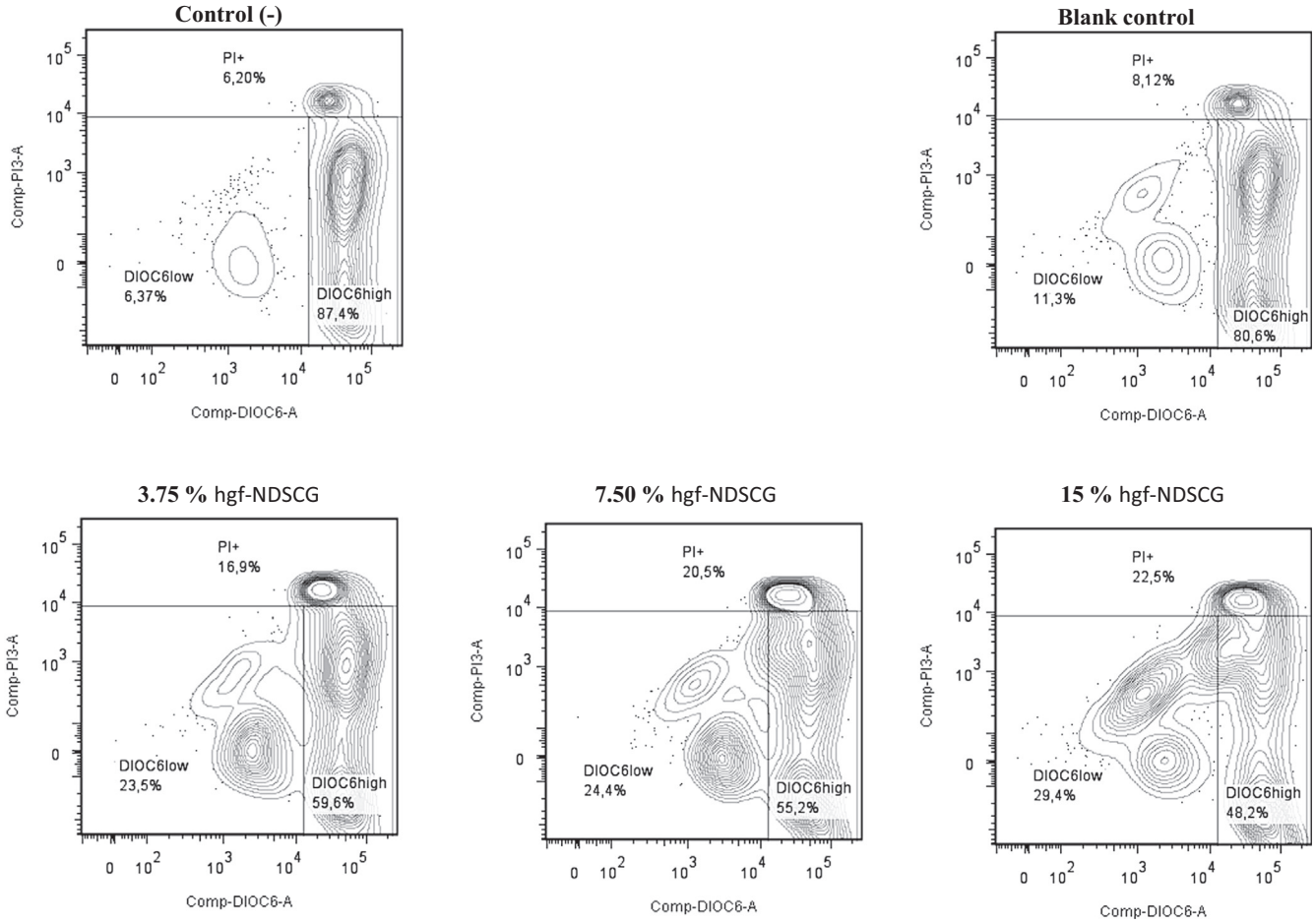

Fig. 2. Mitochondrial membrane potential ($\Delta\Psi M$) in SW480 cells treated with hgf-NDSCG. SW480 cells were stained using $DiOC_2(3)$, and then measured by flow cytometry. DiOC6 high: live cells showing high membrane polarization; DiOC6 low and PI negative: cells in latency, loose membrane polarization; DiOC6 positive and PI positive: cells starting to loose membrane polarization and to acquire PI; PI positive: dead cells. Blank control: hgf-NDSCG without cells; nontreated control (control -; 0%/hgf-NDSCG mL, 100%) cells.

polarization (DIOC6-high) to the area with cells in latency and loosing membrane polarization (DIOC6-low) compared to the control (Fig. 2); other cells were displaced to the area with dead cells with damaged membrane (PI+). ROS induction can depolarize the MMP, which eventually increases the expression of pro-apoptotic molecules, like Caspase-3, and decrease GSH levels (Yeh et al., 2012), which correspond with our results. Similarly, the present study demonstrated that hgf-NDSCG significantly decreased the MMP in SW480 cells in a dose-response manner, suggesting that hgf-NDSCG-induced mitochondria dysfunction may modulate the oxidative stress and lead to apoptosis. This modulation or intracellular ROS production by hgf-NDSCG needs to be studied.

3.7. Flow cytometric analysis of apoptosis and cell cycle

The ability of cancer cells to systematically avoid apoptotic mechanisms and signals is directly linked to their uncontrolled growth and proliferation subsequently allowing invasion and metastasis throughout the body (Hanahan & Weinberg, 2000). Annexin-V-Fluos labelling was performed on each SW480 cells (Fig. 3) to determine if cell death was caused by apoptosis. Labelling was detected via flow cytometry and gating was performed on both dual and single channel plots (Annexin-V and Propidium Iodide). Apoptotic cell death increased significantly in SW480 cells treated with hgf-NDSCG, in early apoptosis (Q3) (0.111, 0.091 and 0.335%; 3.75, 7.5 and 15% of hgf-NDSCG, respectively; $p = 0.05$) (Fig. 3). Significant increase was also observed in late apoptosis cells (Q1) (6.28, 11.2 and 19.5%; 3.75, 7.5 and 15% of hgf-NDSCG, respectively; $p = 0.05$). Cells showed increased necrosis in all treatment groups (Q2) although in low percentage (0.111, 0.228 and 0.840%; 3.75, 7.5 and 15% of hgf-NDSCG, respectively). We suggest that hgf-NDSCG induce apoptosis in SW480 cell line also through SCFAs since fermented hgf NDF products from common beans inhibited HT29 cell growth and modulated protein expression associated with apoptosis, as well as morphological changes linked to apoptosis, mediated mainly by SCFAs (Campos-Vega et al., 2010).

During the exponential growth period untreated SW480 cells were characterized by cell populations in the Sub-G_0 (7.5 ± 1.4), G_0/G_1 (29.9 ± 4.6), S (1.5 ± 0.9) and G_2/M (26.3 ± 5.5) phase of the cell cycle (Table 3). After 24 h incubation cell population at the Sub-G_0 phase increased significantly ($p < 0.05$) with increasing hgf-NDSCG levels, compared to control, indicating the presence of dead or dying cells with a DNA content lower than 2n resulting from induced cell death causing DNA degradation. In contrast, the proportion of cells in G_0/G_1, S and G_2/M phases significantly decreased ($p < 0.05$) in a dose-dependent manner compared to the control, without cell cycle arrest. A similar effect was exerted by a polyphenolic-rich methanolic extract of Pistacia pericarp on human adenocarcinoma cells (Rezaei et al., 2012). Although high amount of chlorogenic acids reach the colon (71%), the literature is lacking in investigations focused on the direct effects of coffee

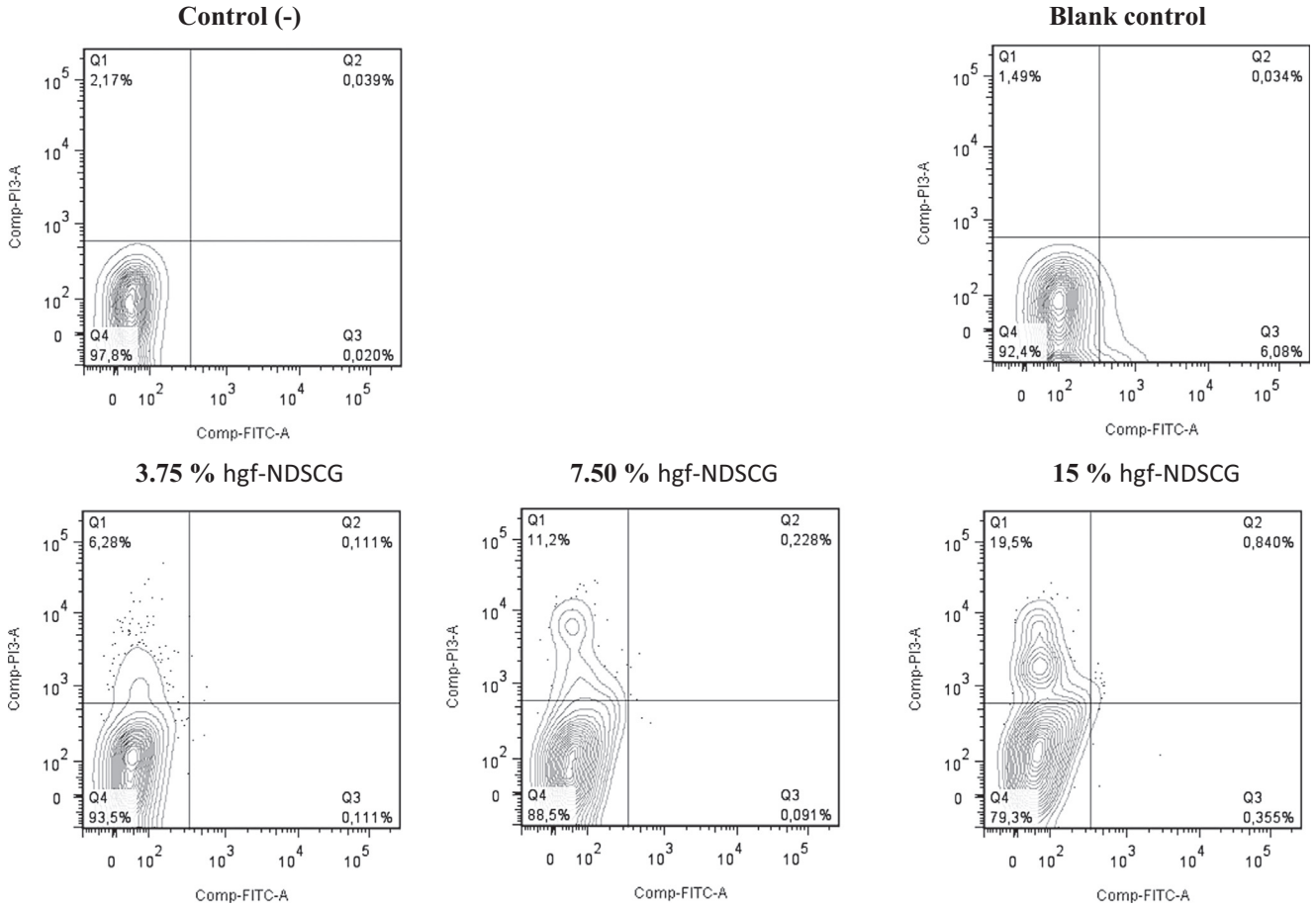

Fig. 3. Apoptosis analysis of SW480 cells in responses to hgf-NDSCG. The histograms represent one of at least three different experiments. Cells were harvested 24 h post treatment, stained with Annexin V-FITC/PI dye, and then analyzed using flow cytometry. Annexin V negative/PI negative presented as live non-apoptotic cells (Q4); annexin V positive/PI negative (Q3) indicated early phase of apoptosis; annexin V positive/PI positive presented as necrotic (Q2); annexin V negative/PI positive presented as late apoptosis (Q1). Blank control: hgf-NDSCG without cells; nontreated control (control -; 0%/hgf-NDSCG mL, 100%) cells.

polyphenols on this organ (Vitaglione, Fogliano, & Pellegrini, 2012). On the other hand, heterogeneous melanoidins presents in SCG reduce proliferation and induce cell cycle arrest in G_0 in LS180 colon cancer cells by modulating several key cell cycle regulators including cyclin D1, cdk4, cdk6, p21, p27, p53 and pRb (Langner et al., 2013). This information suggests that since hgf-NDSCG is a complex mixture of natural compounds, one or more compounds may be involved in SW480 cytotoxicity and possibly contribute to inhibit, delay or reverse carcinogenesis, as previously reported for dietary phytochemicals (Pratheeshkumar et al., 2012).

4. Conclusion

Our results revealed that the hgf-NDSCG SW480 survival inhibition was mediated by Caspase-3 activation, decreased GSH/GSSG ratio and an increase in the hypodiploid cells ($SubG_0$), leading to apoptotic SW480 cells. These anti-cancer activities may be due to the synergistic actions of bioactive compounds present in hgf-NDSCG. Additional studies are currently underway to identify more specific phytochemicals responsible for their anti-cancer activities. Also, further analysis at the molecular level may clarify the detailed mechanisms of apoptotic induction in colon cancer cells. Based on these findings, SCG is thought to be a promising antitumor dietary ingredient.

Conflict of interest

The authors declare no competing interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.01.014.

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