LUÍS FERNANDO DE SOUSA MORAES

EFFECTS OF DIETARY GREEN PEA AND *Hibiscus sabdariffa* L. ON INTESTINAL DISEASES IN MICE

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

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SUMMARY

ABBREVIATION LIST

ACF	Aberrant Crypt Foci
ANOVA	One-way Analysis of Variance
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
CAT	Catalase
CRC	Colorectal Cancer
CTLA4	Cytotoxic T Lymphocyte-associated Antigen 4
DMH	1,2-dimethylhydrazine
DSS	Dextran Sodium Sulfate
dw	Dry Weight
GAE	Gallic Acid Equivalent
GGT	Gamma Glutamyltransferase
GSK-3	Glycogen Synthase Kinase 3
HDAC	Histone Deacetylases
HFD	High Fat Diet
HPLC	High Performance Liquid Chromatography
HS	*Hibiscus sabdariffa* L.
MICA/B	MHC class I–related Chain A and B
NK	Natural Killer
NKG2D	NK group 2, member D
PD1	Programmed Cell Death Protein 1
ROS	Reactive Oxygen Species
SCFA	Short-Chain Fatty Acids
SD	Standard Deviation
SEM	Standard Error of the Mean
SOD	Superoxide dismutase

ABSTRACT

MORAES, Luís Fernando de Sousa, D.Sc., Universidade Federal de Viçosa, February, 2018. **Effects of dietary green pea and *Hibiscus sabdariffa* L. on intestinal diseases in mice**. Adviser: Maria do Carmo Gouveia Peluzio. Co-advisers: Helen Hermana Miranda Hermsdorff and Lisiane Lopes da Conceição.

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths worldwide. Present data has reported the role of anthocyanin-rich food/extract in increasing fecal short-chain fatty acids (SCFA) concentrations and NK cells infiltration in the large intestine mucosa, thus contributing to prevent preneoplastic lesions formation. We have observed that anthocyanins/anthocyanidins might prevent colorectal tumorigenesis due to stimulation of cell cycle arrest and apoptosis mechanisms, besides downregulation of pro-inflammatory and metastasis invasive signaling mechanisms. As inflammatory bowel disease, such as colitis, has been related to colorectal carcinogenesis, we have reported that green pea supplementation abrogates the severity of dextran sodium sulfate (DSS)-induced colitis in high-fat diet (HFD)-fed mice due to suppression of inflammation, mucin depletion and endoplasmic reticulum stress in the colon. We also have observed that supplementation with 5 or 10% dietary HS attenuated colonic ACF development in the distal colon. Total ACF counts per mouse was reduced by almost 29.0% in HS supplemented groups when compared to control. Fecal butyric and propionic acids concentrations, in addition to NK cell infiltration, were increased with 10% dietary HS supplementation. Hepatic catalase activity was enhanced in 10% dietary HS-treated mice when compared to control group. We might infer that dietary HS might prevent preneoplastic lesions formation due to modulation of SCFA and Natural Killer (NK) cells infiltration.

RESUMO

MORAES, Luís Fernando de Sousa, D.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Efeitos da ervilha verde e do *Hibiscus sabdariffa* L. em doenças intestinais em camundongos**. Orientadora: Maria do Carmo Gouveia Peluzio. Coorientadoras: Helen Hermana Miranda Hermsdorff e Lisiane Lopes da Conceição.

O câncer colorretal (CCR) é mundialmente a terceira maior causa de morte relacionadas aos cânceres em geral. Os dados atuais relatam o papel do alimento/extrato rico em antocianinas no aumento das concentrações fecais de ácidos graxos de cadeia curta (AGCC) e na infiltração de células NK (natural killers) na mucosa do intestino grosso, o que pode contribuir para a prevenção da formação de lesões pré-neoplásicas. Observamos que as antocianinas/ antocianidinas podem prevenir a tumorigênese colorretal devido à estimulação dos mecanismos de apoptose e bloqueio do ciclo celular, além de mitigar os mecanismos de sinalização pró-inflamatória e de metástase invasiva. Como a doença inflamatória intestinal, a exemplo da colite ulcerativa, tem sido relacionada à carcinogênese colorretal, relatamos que a suplementação de ervilha verde atenua os sintomas da colite induzida por sulfato dextrano de sódio (DSS) em camundongos alimentados com dieta rica em gordura (HFD) devido à supressão da inflamação, depleção de mucina e estresse do retículo endoplasmático no cólon. Também observamos que a suplementação com 5 ou 10% de Hibiscus sabdariffa L. (HS) na dieta atenuou o desenvolvimento de focos de criptas aberrantes (FCA) no cólon distal. A contagem total de ACF por camundongo foi reduzida em quase 29,0% nos grupos suplementados com HS quando comparados ao controle. As concentrações de ácido butírico e propiônico nas fezes, além da infiltração de células NK, foram aumentadas com a suplementação de 10% de HS na dieta. A atividade da catalase hepática foi aumentada em 10% nos camundongos tratados com HS quando comparados ao grupo controle. Podemos inferir que o HS dietético pode prevenir a formação de lesões pré-neoplásicas devido à modulação de infiltração de células SCFA e Natural Killer (NK).

1. GENERAL INTRODUCTION

Cancer is a multifactorial chronic disease mainly identified by unrestrained clonal expansion and spread of abnormal cells (1). In Brazil, cancer has been ranked as the second leading cause of death, after cardiac and cerebrovascular diseases (2). Since cancer can be initiated from an interaction between environmental, genetic, and lifestyle factors, controlling behavioral risk factors along with health services organizations has become a challenge regarding cancer control in developing countries (3).

Specifically, colorectal cancer (CRC) involves the onset of aberrant crypt foci (ACF) and micro-adenomas that will lead to an increased replication of the upper crypt cells and posterior formation of adenomatous polyps (4). Recently, in Brazil this malignancy appears as the second and third most common cancer in women and men, respectively (2).

According to a meta-analysis (5), dietary intake patterns, e.g. lower consumption of phytochemical-rich foods, have been reported as a significant risk factor for CRC development, besides inflammatory bowel disease, such as ulcerative colitis (5), a chronic inflammatory disorder that affects both mucosa and submucosa of the colon. Despite its unclear etiology, recent evidences have strongly suggested that phytochemical-rich foods reduce leukocytes infiltration, mitigate overproduction of inflammatory cytokines, and suppress mucin depletion (6-8). These events contribute to ameliorate ulcerative colitis symptoms, and therefore, might also prevent the onset of preneoplastic lesions or CRC development.

On the one hand, consumption of green pea, a notable source of fiber and polyphenolics (9, 10), has suppressed inflammation in DSS-induced colitis in mice (11). *Hibiscus sabdariffa* L. (HS) calyces, on the other hand, are a cheap and natural rich source of anthocyanin (12, 13) that can be also stimulate for human consumption, such as teas, jams, and jellies. HS extracts have been implicated as chemopreventive, anti-tumor, and hepatoprotective agents (14, 15).

In order to provide a better understanding on intestinal diseases and polyphenol-rich food, this doctoral thesis has been grounded in three main hypotheses. The first paper, entitled “Anthocyanins/anthocyanidins and colorectal cancer: what is behind the scenes?”, published in Critical Reviews in Food Science and Nutrition (2016 Impact Factor: 6.077), has elucidated the hypothesis that such phytochemicals indeed exert anti-tumor effects *in vivo* and *in vitro* by stimulating cell cycle arrest and apoptosis mechanisms, besides downregulating pro-inflammatory and metastasis invasive signaling pathways. The second paper, entitled “Dietary green pea protects against DSS-induced colitis in mice challenged with high-fat diet” and published in Nutrients (2016 Impact Factor: 3.550), has elucidated that green pea supplementation mitigates the severity of dextran sodium sulfate (DSS)-induced colitis in high-fat diet (HFD)-fed mice due to suppression of inflammation, mucin depletion and endoplasmic reticulum stress in the colon. It is worth to mention that this research was part of my doctoral training period at Washington State University – WA, United States of America, under supervision of professor Mei-Jun Zhu. In addition, it has been observed that dietary HS might prevent preneoplastic lesions formation due to modulation of fecal SCFA and Natural Killer (NK) cells infiltration. This research was supervised by professor Peluzio and the outcomes are intended to be published in The Journal of Nutritional Biochemistry (2016 Impact Factor: 4.518) with the title “Dietary *Hibiscus sabdariffa* L. mitigates preneoplastic lesions development in distal colon by increasing fecal short-chain fatty acids concentration and infiltration of Natural Killer cell”.

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2. AIMS OF THE STUDY

2.1. GENERAL AIM

To assess the mechanisms by which anthocyanins exert beneficial effects against colorectal carcinogenesis and to highlight whether dietary supplementation with green pea or HS might ameliorate the symptoms in DSS-induced colitis in HFD-fed mice or prevent preneoplastic lesions development, respectively.

2.2. SPECIFIC AIMS

- To summarize the current scientific literature linking the mechanisms how anthocyanins might prevent colorectal carcinogenesis (Article 1);

- To verify whether green pea supplementation is able to attenuate DSS-induced colitis symptoms in HFD-fed mice by reducing neutrophil infiltration (Article 2)

- To assess whether dietary HS supplementation is able to prevent preneoplastic lesions formation by (Article 3):

* Characterizing HS calyces according to its nutritional composition;

* Characterizing HS calyces according to its polyphenols and anthocyanin content;

* Conducting an *in vivo* experiment, where colorectal carcinogenesis-induced BALB/c mice will be supplemented with 5% or 10% dietary HS;

* Counting ACF in the large intestine of BALB/c mice induced to colorectal tumorigenesis;

* Quantifying fecal SCFA concentrations;

* Determining the leukocyte profile in the large intestine mucosa;

* Evaluating hepatic enzyme activity and hepatic-related serum markers.

3. RESULTS

3.1. ARTICLE 1 (REVIEW) – Anthocyanins/anthocyanidins and colorectal cancer: What is behind the scenes?

Luís Fernando de Sousa Moraes, Xiaofei Sun, Maria do Carmo Gouveia Peluzio, Mei-Jun Zhu

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Anthocyanins/anthocyanidins and colorectal cancer: What is behind the scenes?

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Anthocyanins/anthocyanidins and colorectal cancer: What is behind the scenes?

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ABSTRACT

Colorectal cancer (CRC) is one of the most common cause of cancer death. Phytochemicals, especially anthocyanins/anthocyanidins (A/A), have gathered attention of the scientific community owing to their anti-inflammatory, antioxidant, and cancer-inhibitory properties. In this review, we discussed the possible mechanisms whereby A/A exhibit intestinal anticarcinogenic characteristics. Anthocyanins/anthocyanidins inhibit the pro-inflammatory NF-κB pathway, attenuate Wnt signaling and suppress abnormal epithelial cell proliferation. In addition, A/A induce mitochondrial-mediated apoptosis and downregulate Akt/mTOR (mammalian target of rapamycin) pathway. Furthermore, activation of AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1) also contributes to the anti-carcinogenic effects of A/A. Finally, downregulation of metalloproteinases (MMPs) by A/A inhibits tumor invasion and metastasis. In conclusion, A/A exert their anti-tumor effects against colorectal carcinogenesis via multiple mechanisms, providing insights into the use of A/A as a natural chemopreventive intervention on major colorectal carcinogenesis.

KEYWORDS

Polyphenols; anthocyanins; anthocyanidins; colorectal carcinogenesis; chemopreventive intervention; cancer prevention; signaling pathway

Abbreviations: A/A: anthocyanins/anthocyanidins; ACF: aberrant crypt foci; Akt: protein kinase B; AMPK: AMP-activated protein kinase; AOM: azoxymethane; APAF1: apoptotic protease-activating factor 1; Apc: adenomatous polyposis coli; ARE: anthocyanin-rich extract; ATM: ataxia telangiectasia mutated; ATR: ataxia telangiectasia and Rad3-related; Bax: Bcl-2-associated X protein; Bak: Bcl-2-killer; Bcl-2: B-cell lymphoma 2; Bcl-xL: B-cell lymphoma-extra large; BH3: Bcl-2 homology 3; BW: body weight; CAD: caspase-activated deoxyribonuclease; caspase: cysteine aspartyl-specific protease; COX-2: cyclooxygenase-2; CRB: crumb complex; CRC: colorectal cancer; DSS: dextran sodium sulfate; ERK: extracellular signal-regulated kinases; IFN-γ: interferon gamma; IκBα: I kappa B alpha; IKKα: I kappa B kinase alpha; IL: interleukin; iNOS: inducible nitric oxide synthase; Ki-67: marker of proliferation antigen Ki-67; MAPK: mitogen activated protein kinase; MCT: medium-chain triacylglycerol; MMPs: metalloproteinases; mTOR: mammalian target of rapamycin; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; p21: cyclin-dependent kinase inhibitor; p53: tumor suppressor protein; Par: partitioning complex; PARP: poly ADP-ribose polymerase; PCNA: proliferating cell nuclear antigen; PGE_2: prostaglandin E2; pRB: retinoblastoma protein; RNS: reactive nitrogen species; ROS: reactive oxygen species; Scrib: scribble complex; SIRT1: sirtuin 1; SMAC: second mitochondria-derived activator of caspases; STAT-3: signal transducer and activator of transcription 3; TNF-α: tumor necrosis factor alpha; VEGF: vascular endothelial growth factor; Wnt: Wingless and Int; XIAP: X-linked inhibitor of apoptosis protein

1. Introduction

Colorectal cancer (CRC) appears to be the second most common cause of cancer death in the United States (Siegel, Miller, and Jemal, 2016). CRC affects more than one million patients every year worldwide (Ferlay et al., 2015). About 35% of overall cancer-related mortality is lifestyle-dependent (Doll and Peto, 1981). For instance, high dietary intake of fruits, vegetables, and whole grains has strongly sustained the inverse correlation between carcinogenesis and diet habits (Surh, 2003). Since inflammatory bowel disease (IBD) patients are predisposed to triggering the onset of colitis-associated CRC (Rhodes and Campbell, 2002) and only 15% of CRC occur due to inherited gene defect (Jackson-Thompson et al., 2006), it can be hypothesized, therefore, that inflammation management by antioxidant-rich food/extracts consumption could be a potential strategy to reduce the inflammation grade and, hence, prevent CRC onset. It is worth clarifying that although antioxidant-rich food/extract intake itself is possibly not a recommended option to either treat or cure CRC, developing good dietary habits benefits towards intestinal health against the inflammation state.

In this sense, anthocyanins/anthocyanidins (A/A) have been emerged as promising compounds capable of promoting relevant health benefits in CRC (Shashirekha, Mallikarjuna, and Rajarathnam, 2015), owing to its known antioxidant and anti-inflammatory properties (Ravipati et al., 2012). Nevertheless, the important remaining question is how A/A exert its beneficial effects on CRC. Thus, in this review, we aimed at identifying the possible mechanisms whereby A/A exhibit intestinal anticarcinogenic characteristics. In addition, the positive effects of other common polyphenols on colorectal carcinogenesis, although previously reviewed elsewhere (Juan, Alfaras, and Planas, 2012; Kotecha, Takami, and

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Espinoza, 2016; Priyadarsini and Nagini, 2012; Surh, 2003), are briefly introduced to contextualize and show the relevance of A/A as strong phytochemical compounds.

2. Polyphenols: Overview and beneficial effects on CRC

Dietary polyphenols or phenolic compounds are natural antioxidants present in plant-based foods, such as fruits, vegetables, tea, essential oils and their by-products (Zhang and Tsao, 2016), which can prevent the onset of chronic diseases, thus enhancing human health (Scalbert et al., 2007). Polyphenols can be mainly categorized into three different groups according to their chemical structures: phenolic acids, flavonoid and non-flavonoid compounds (Zhang and Tsao, 2016). Table 1 summarizes the main findings related to the beneficial effects of bioactive compounds, mostly flavonoids, on colorectal carcinogenesis in mice.

The common signaling pathway underlying lower dysplasia and tumor incidence in polyphenols-treated mice is believed to be related to cell cycle arrest and decreased expression of inflammatory markers, such as tumor necrosis factor (TNF)-α, interferon gamma (IFN-γ), interleukin (IL)-6, and cyclooxygenase -2 (COX-2) (Table 1).

In particular, A/A are synthesized via the flavonoid pathway (Holton and Cornish, 1995) and have gained attention of the scientific community owing to their anti-inflammatory, antioxidant, and cancer-inhibitory properties (Bowen-Forbes et al., 2010). Among flavonoids, A/A provide strong electron-donating ability, which are comparable to carotenoids, one of the most remarkable natural quencher of oxygen singlet (De Rosso et al., 2008). Additionally, A/A can be easily found and extracted from edible source plants (Cissé et al., 2012).

3. Anthocyanins/anthocyanidins: Overview and anticarcinogenic effects

Anthocyanins comprise over 500 water-soluble compounds, naturally found at greater quantities in most colored fruits, vegetables, leaves and flowers (Wu et al., 2006; McGhie et al., 2003). Chemically, anthocyanins are classified as glycosides of polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium (Wu et al., 2007) and, thus, consist of two benzoyl rings (A and B) in between a heterocyclic ring (C), which in turn form the flavylium cation, as shown in Figure 1.

Anthocyanins most commonly present a tri-, di- or monosaccharide unit. Hydrolyzed anthocyanins yield anthocyanidins and sugars (McGhie and Walton, 2007). Therefore, the so-called anthocyanidins or anthocyanin aglycones possess no sugar moiety attached to the molecular structure of the flavylium cation and are defined according to the substitute group – hydrogen atom, hydroxide or methoxy – that can be placed at the R1 and R2 positions (Figure 1). For instance, cyanidin is an anthocyanidin represented by the flavylium cation holding both –OH and –H substitutes at the R1 and R2 positions, respectively. Although several anthocyanidins have been properly identified, the anthocyanins mainly emerge from cyanidin, delphinidin, perlargonidin, peonidin, malvidin, and petunidin (Jing et al., 2008).

To date, studies evaluating the effects of A/A on intestinal cancer in humans are sparse. In a previous study in CRC patients, a 7-day treatment with a commercial anthocyanin-rich extract (ARE) from bilberry prior to tumor resection reduced the proliferation index, elucidated by lower Ki-67 expression, and increased the apoptotic index, observed by higher cleaved caspase-3 expression (Thomasset et al., 2009). Thus, further clinical trials should be more encouraged to provide results on A/A as a potential chemopreventive intervention.

On the other hand, the chemopreventive properties of A/A, indeed, have been successfully reported in rodent models for carcinogenesis (Hagiwara et al., 2001; Bobe et al., 2006). A 14-week supplementation with ARE from bilberry, chokeberry, and grape resulted in reduction of colonic aberrant crypt foci (ACF, preneoplastic lesions of CRC) in AOM-induced CRC rats (Lala et al., 2006). Accordingly, Shi et al. (2015) revealed reduced tumor incidence and multiplicity (number of tumors per mouse) in AOM/DSS-promoted colorectal carcinogenesis in mice after 20-week supplementation with anthocyanin-rich strawberries. Cooke et al. (2006) reported less intestinal adenomas in adenomatous polyposis coli $(Apc)^{Min}$ mice after 12-week treatment with either 0.3% of a commercial ARE from bilberry or the isolated anthocyanin type, cyanidin-3-glucoside. The number of intestinal tumors in Apc^{Min} mice was also decreased upon 7-week treatment with 0.5% of ARE from black soybean (Park et al., 2015). Likewise, Apc^{Min} mice consuming either a supplemented diet with anthocyanin-rich tart cherry, ARE from tart cherry in drinking water, or cyanidin for 10 weeks exhibited less and smaller cecal adenomas in comparison to mice under control diet or sulindac (Kang et al., 2003), a non-steroidal anti-inflammatory drug, known to inhibit tumor progression (Boolbol et al., 1996). Positively, Apc^{Min} mice fed with different dosages of ARE from tart cherry in combination with sulindac reduced total tumor area per mouse and tumor number when compared to sulindac alone (Bobe et al., 2006).

The anticarcinogenic effect of A/A has also been evaluated *in vitro*. Cyanidin and ARE from tart cherry were able to induce a dose-dependent decrement of cell proliferation in both HCT-116 and HT-29 cells with no cytotoxic effects (Kang et al., 2003). Interestingly, cyanidin was even more potent in inhibiting cell growth in comparison with ARE from tart cherry. The IC50 for cyanidin, i.e. the concentration of cyanidin inducing a 50% reduction in cell proliferation, was much lower than that for anthocyanins (Kang et al., 2003). Anthocyanidins inhibited the proliferation in stomach, colon, lung, breast and central nervous system cancer cell lines, while anthocyanins at the same concentration could not inhibit above cell growth (Zhang, Vareed, and Nair, 2005).

Similarly, ARE from Chinese blueberry suppressed the proliferation of colon carcinoma cell lines, DLD-1 and COLO-205 cells (Zu et al., 2010). The IC50 and IC90 values of Chinese blueberry were much lower in relation to ARE from bilberry. It is worth commenting that, albeit both AREs consist mainly of the aglycone delphinidin, ARE from Chinese blueberry presents higher malvidin concentration and lower cyanidin percentage than ARE from bilberry (Zu et al., 2010). Thus, not only cyanidin but also other anthocyanidin types might also strongly contribute to the antiproliferative properties and pro-apoptotic activities of A/A.

Table 1. Beneficial effects of bioactive compounds on azoxymethane and DSS-induced colorectal carcinogenesis in mice.

Bioactive compound (Group)	Carcinogen	Treatment (Trt)	Time of treatment	Main findings	Comments and limitations	Reference
Auraptene and collinin (Non-flavonoid coumarin)	1) AOM – 10 mg/kg BW on day 1; 2) DSS 1% for 7 days on day 8.	Male ICR mice (6-wk-old). Cancer-induced. Dietary treatment (Trt): 1) Basal diet; 2) Basal diet + 0.01% auraptene; 3) Basal diet + 0.05% auraptene; 4) Basal diet + 0.01% collinin; 5) Basal diet + 0.05% collinin.	1) 20 weeks. 2) Auraptene and collinin treatment started 1 week after DSS administration.	↓ Incidence of adenomas and adenocarcinomas in all Trts vs Trt 1; ↓ Total tumor multiplicity in Trt 3 and 5 vs Trt 1; ↓ Inflammation score in Trt 3 and 5 vs Trt 1; ↓ PCNA-labeling index in all Trts vs Trt 1; ↑ Apoptotic index in Trt 2, 4 and 5 vs Trt 1; ↓ Expression of COX-2 and iNOS in all Trts vs Trt 1, except Trt 4 for COX-2; ↓ Expression of nitrotyrosine in Trt 3 and 5 vs Trt 1.	1) Generally, both compounds presented better results on the highest dose. 2) No adverse effects were noticed for both dosages and compounds.	(Kohno et al. 2006)
Epigallocatechin gallate (EGCG) and Polyphenon E (Flavonoid)	1) AOM – 10 mg/kg BW on day 1; 2) DSS 2% for 7 days on day 8.	Male ICR mice (5-wk-old). Cancer-induced. Dietary treatment (Trt): 1) Control; 2) 0.01% EGCG; 3) 0.1% EGCG; 4) 0.01% polyphenon E; 5) 0.1% polyphenon E.	1) 17 weeks. 2) EGCG and polyphenon E treatment started 1 week after DSS administration.	↔ Colon length and tumor incidence between the Trts; ↓ Multiplicity of colonic adenocarcinomas in Trt 2, 4 and 5 vs Trt 1; ↓ Inflammation score in all Trts vs Trt 1; ↓ Expression levels of COX-2 in all Trts vs Trt 1; ↓ Expression of TNF-α, IFN-γ, IL-6 and IL-12 in all Trts vs Trt 1.	1) The inflammatory cytokines was significantly reduced in Trt 3 when compared to the others.	(Shirakami et al., 2008)
Resveratrol (Non-flavonoid stilbene)	1) AOM – 10 mg/kg BW on day 1; 2) DSS 1% on day 8 for 7 days + 14 days of normal water. Cycle was repeated twice.	Male and female C57 BL/6 mice (8-12-wk-old). Cancer-induced. Dietary treatment (Trt): 1) AIN-93M; 2) AIN-93M + 300 ppm resveratrol.	1) 10 weeks. 2) Resveratrol treatment started on day 8.	↓ Tumor incidence in Trt 2 vs Trt 1; ↓ Tumor multiplicity in Trt 2 vs Trt 1.	1) Trt 2 exhibited lower tumor size when compared to 1, although not significantly.	(Cui et al., 2010)
Isorhamnetin, myricetin, quercetin and rutin Flavonoid)	1) AOM – 10 mg/kg BW on day 1; 2) DSS 2% for 7 days on day 8.	Male FVB/N mice (6-wk-old). Cancer-induced. Dietary treatment (Trt): 1) AIN-93G; 2) AIN-93G + 552 ppm isorhamnetin; 3) AIN-93G +556 ppm myricetin; 4) AIN-93G + 591 ppm quercetin; 5) AIN-93G + 1099 ppm rutin.	1) 14 weeks. 2) Flavonoid treatments started 3 days after DSS administration.	↓ Morbidity in Trt 2 and 4 vs Trt 1; ↓ Tumor multiplicity, tumor burden and tumor size in Trt 2 vs Trt 1; ↓ Inflammation grade in Trt 2 vs Trt 1; ↓ Number of Ki-67-positive cells in Trt 2 vs Trt 1; ↓ β-catenin accumulation in the nucleus in Trt 2 vs Trt 1.	1) Flavonoids were added at equimolar concentrations; 2) FVB/N mice are sensitive to AOM/DSS, thus requiring only one DSS cycle; 3) No beneficial effects in Trts 3, 4 and 5.	(Saud et al., 2013)
Oroxylin A (Flavonoid)	1) AOM – 10 mg/kg BW on day 1; 2) DSS 2.5% on day 8 for 7 days + 14 days of normal water.	Male and female C57 BL/6 mice (6–8-wk-old). Cancer-induced: Dietary treatment (Trt): 1) Saline; 2) 50 mg oroxylin/kg BW; 3) 100 mg oroxylin/kg BW; 4) 200 mg oroxylin/kg BW.	1) 14 weeks. 2) Oroxylin A treatment started 1 week prior to AOM injection.	↔ Colon length between Trts; ↓ Inflammatory score in Trt 3 and 4 vs Trt 1; ↓ Tumor number and tumor burden in all Trts vs Trt 1; ↓ Tumor size in Trts 3 and 4 vs Trt 1; ↓ Number of Ki-67-positive cells in all Trts vs Trt 1; ↓ Expression of IL-6 and IL-1β in all Trts vs Trt 1.	1) Oroxylin A exhibited antiproliferative and pro-apoptotic effects.	(Yang et al., 2013)

(*Continued on next page*)

Table 1. (*Continued*)

Bioactive compound (Group)	Carcinogen	Treatment (Trt)	Time of treatment	Main findings	Comments and limitations	Reference
Carnosic acid (Non-flavonoid diterpene)	1) AOM – 10 mg/kg BW on day 1; 2) DSS 2% on day 8 for 7 days + 14 days of normal water.	Male A/J mice (4-wk-old). Cancer-induced: Dietary treatment (Trt): 1) Normal diet; 2) high-fat diet (HFD); 3) HFD + 0.01% carnosic acid; 4) HFD + 0.02% carnosic acid.	1) 11 weeks. 2) Carnosic acid treatment started on day 1.	↓ BW and epididymal fat weight in Trt 4 vs Trt 2; ↓ Total number of tumors in Trts 4 and 3 vs Trt 2; ↔ Tumor multiplicity and size among Trts; ↔ p-Akt and STAT-3 among Trts; ↓ Cyclin-D1 and Bcl-xL in Trt 4 vs Trt 2.	1) HFD accelerates tumor development (higher total number of tumors in Trt 2 vs Trt 1).	(Kim et al., 2014)
Isoliquiritigenin (ISL) (Flavonoid)	1) AOM – 10 mg/kg BW on day 1; 2) DSS 2% on day 8 for 7 days + 14 days of normal water. Cycle was repeated twice.	Male BALB/c mice (6-wk-old). Cancer-induced. Dietary treatment (Trt): 1) Saline; 2) 3 mg ISL/kg BW; 3) 15 mg ISL /kg BW; 4) 75 mg ISL/kg BW.	1) 13 weeks. 2) ISL treatment started 1 week prior to AOM injection.	↓ Colon weight-to-length ratio and histological score in Trts 3 and 4 vs 1; ↓ Tumor multiplicity and size in Trt 3 and 4 vs Trt 1; ↓ IL-6, PGE_2 levels in Trt 3 and 4 vs Trt 1.	1) Trt 4 exhibited the lowest tumor incidence. 2) IL-6 promotes inflammation through activation of STAT3 pathway.	(Zhao et al., 2014)
Tangeretin (Flavonoid)	1) AOM – 20 mg/kg BW on day 1; 2) DSS 2% for 7 days on day 8.	Male ICR mice (6-wk-old). Cancer-induced. Dietary treatment (Trt): 1) MCT suspension; 2) Blank emulsion; 3) 100 mg tangeretin in MCT suspension/kg BW; 4) 100 mg tangeretin in blank emulsion/kg BW.	1) 12 weeks. 2) Tangeretin treatment started 1 week prior to AOM injection	↔ Body weight between the Trts; ↔ Colon length and tumor incidence; ↓ Colon weight-to-length ratio and multiplicity of adenomas in Trt 4 vs Trt 2; ↓ Expression of COX-2 and VEGF in Trt 4 vs Trt 2; ↓ Expression of PCNA and β-catenin in Trt 4 vs Trt 2.	1) No signs of toxicity. 2) Trt 4 exhibited the lowest tumor incidence. 3) Goblet cells were better preserved in Trt 4.	(Ting et al., 2015)
Curcumin (Flavonoid)	1) AOM – 25 mg/kg BW on day 1; 2) DSS 2% on day 8 for 5 days + 16 days of normal water. Cycle was repeated with DSS 1%.	Female BALB/c mice (5-wk-old). Cancer-induced: Dietary treatment (Trt): 1) Normal protein diet; 2) High protein diet; 3) High protein diet + 0.02% curcumin	1) 8 weeks. 2) Curcumin treatment started on day1.	↓ Body weight and food intake in Trt 3 and 2 vs Trt 1; ↑ Number of tumors in Trt 2 vs Trt 1; ↓ Number of tumors in Trt 3 vs Trt 2; ↓ Expression of COX-2 and iNOS in Trt 3 vs Trt 2; ↔ Plasma levels of TNF-α and nitric oxide; ↓ Fecal NH_4^+/NH_3 in Trt 3 vs Trt 2.	1) DSS dosage was reduced due to severe disease activity. 2) Curcumin attenuates the effects of a high protein diet on CAC development.	(Byun et al., 2015)

↔ No difference; ↑ higher; ↓ lower. Akt, protein kinase B; AOM, azoxymethane; Bcl-xL, B-cell lymphoma-extra large; BW, body weight; COX-2, cyclooxygenase-2; DSS, dextran sodium sulfate; IFN-γ, interferon gamma; IL, interleukin; iNOS, inducible nitric oxide synthase; Ki-67, marker of proliferation antigen Ki-67; MCT, medium-chain triacylglycerol; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PCNA, proliferating cell nuclear antigen; PGE_2, Prostaglandin E2; STAT-3, signal transducer and activator of transcription 3; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

Anthocyanin-rich extract from different sources may present distinct glycosylations, leading to different anticarcinogenic activities (Koide et al., 1997). Besides, the anthocyanin structure also influences its uptake and, therefore, affects its bioavailability (Kuntz et al., 2015). In this regard, Zhao et al. (2004) have investigated whether different anthocyanin profiles with expected distinct glycosylation would trigger similar or different responses on HT-29 cell proliferation. They found that the proliferation of HT-29 cells treated with ARE from grape, containing five different anthocyanins and their acylated counterparts, was similarly inhibited (Zhao et al., 2004) when compared to treatment with ARE from bilberry, composed of non-acylated anthocyanins. However, ARE from chokeberry containing mainly monoglycosylated cyanidin derivatives showed greater inhibition compared to ARE from bilberry and grape (Zhao et al., 2004). Thus, different glycosylations attached to the anthocyanin structure will, indeed, influence its efficacy. Likewise, Jing et al. (2008) evaluated the growth inhibitory effects of ARE from different sources and found that ARE from purple corn, mainly consisting of cyanidin-3-glucoside, induced the most potent growth inhibitory activity in HT-29 cell line, followed by chokeberry and bilberry. ARE from grape, however, was able to cause moderate growth inhibition.

The growth inhibitory effects of ARE are not only dependent on the source and glycosylation pattern of anthocyanins, but also on the storage time and maturity stage (Lewis, Walker, and Lancaster, 1999; Blessington et al., 2010). At low storage temperature (4 °C), starch is converted to sugars (Isherwood, 1976). In this situation, sugars function as signaling molecules and induce the upregulation of several genes involved in the anthocyanins biosynthesis pathway (Solfanelli et al., 2006), thus increasing anthocyanin contents. Anthocyanins contribute to the main portion of polyphenols in purple-fleshed potatoes (Charepalli et al., 2015). The anthocyanin concentration is increased in purple potatoes stored up to 60 days at 3 °C (Madiwale et al., 2011). However extract from purple potatoes at 90 days of cold storage presented lower anthocyanin contents. Such decrement might be one of the reasons that the antiproliferative effects of such extracts decreased after 3 months of storage (Madiwale et al., 2011).

Black raspberries from distinct harvest locations, cultivars or maturity stages present different anthocyanin content, and, in consequence, the antiproliferative efficacy of black raspberry extracts on HT-29 cells is also influenced by their cultivars, production locations and maturation in a complex manner (Johnson et al., 2011).

4. Mechanisms responsible for anti-CRC effects of anthocyanins/anthocyanidins

Anthocyanins/anthocyanidins demonstrate strong preventive effects on intestinal tumor formation and development in preclinical animal models (Park et al., 2015; Shi et al., 2015). However, what might be the mechanisms by which A/A exert their protective properties against CRC? Accumulating studies have demonstrated the role of A/A in stimulating the expression of tumor suppressor genes and downregulating pro-oncogenic signals as well as controlling proliferation and apoptosis pathways (Forester et al., 2014; Charepalli et al., 2015).

4.1. Anthocyanins/anthocyanidins downregulate pro-inflammation and oxidation pathways

Inflammation is a crucial protective response of the host defense against pathogens, harmful stimuli or damaged tissue. However, excessive chronic inflammation, as observed in IBD, has been markedly involved in different stages of tumor growth and colitis-associated CRC (Takeuchi and Akira, 2010). During carcinogenesis, the inflammatory microenvironment represses the host anti-tumor response, and thus, cancer-promoting immune activity stimulates tumor growth, angiogenesis, and metastasis (Grivennikov et al., 2009).

Overproduction of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α in colitis-associated CRC can trigger signaling cascades that constitutively upregulate key inflammatory signaling, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 3 (STAT3) (Szlosarek, Charles, and Balkwill, 2006; Fan, Mao, and Yang, 2013). The cross-talk between inflammatory signaling and Wnt/β-catenin pathway leads to β-catenin translocation towards the nucleus (Pramanik et al., 2015), which stimulates the downstream transcription of carcinogenic growth factors, cyclin D1 and c-Myc (Mishra et al., 2013; Clevers and Batlle, 2006), and therefore results in stem cell proliferation while blocking differentiation. Both intestinal epithelial cells and crypt stem cells fail to carry out appropriate cell division. Thus, besides inflammation and dysplasia, this process leads to ACF, and even carcinoma transition depending on the severity and duration of ulcerative colitis (Terzic et al., 2010).

Inflammatory cells release high amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are the well-known triggering substances of DNA damage and mutations (Meira et al., 2008), thereby worsening the disease prognosis and inhibiting earlier remission. Based on the chemical structure, A/A have strong ability for electron donation, which explains its unique antioxidant properties (Ali et al., 2016). The intracellular ROS activity was decreased in Caco-2 cells treated with cyanidin chloride or cyanidin-3-O-β glucopyranoside (Renis et al., 2008). Coherently, ARE from red wine inhibited inducible nitric oxide synthase (iNOS) in a dose-dependent manner in HT-29 cells, accompanied with reduced levels of both nitric oxide radical and protein tyrosine nitration, a biomarker of nitrosative stress (Nunes et al., 2013).

Apoptosis-dependent tumor surveillance mechanisms are altered in HT-116 cells under treatment with delphinidin due to inhibition of the inflammatory NF-κB pathway (Yun et al., 2009). Besides inhibiting the phosphorylation and degradation of IκBα, delphinidin was able to suppress activation of I kappa B kinase α (IKKα), important to trigger IκBα activation in a dose-dependent way (Verma et al., 2012). As a result, phosphorylation of NF-κB/p65 was also inhibited by delphinidin, hence reducing the nuclear translocation of NF-κB/p65. This cascade of events subsequently led to lower transcriptional activation of inflammatory cytokines and thus, induction of apoptosis and cell cycle arrest.

4.2. Anthocyanins/anthocyanidins induce apoptosis

Anthocyanins/anthocyanidins consumption has reduced intestinal tumor incidence and/or multiplicity in animal studies

Anthocyanidins	Substitutes	
	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 1. Chemical structure of the flavylium cation (left). The main anthocyanidins are formed according to the specific substitutes at R1 and R2 positions (right). Anthocyanins, in turn, mostly present tri-, di or mono-saccharide unit incorporated into the anthocyanidin structure.

(Park et al., 2015; Silva et al., 2015). One of the mechanisms for such improvement is the fact that A/A act as antiproliferative agents *in vivo* through upregulating malignant cell apoptosis mechanisms (Seeram et al., 2006).

4.2.1. Apoptosis introduction

Apoptosis is a highly complex event of programmed cell death characterized by morphologic changes, such as chromatin condensation and subsequent nuclear and DNA fragmentation (Kroemer et al., 2009). Two major apoptosis pathways are closely regulated to induce cell destruction: the extrinsic receptor-mediated pathway, represented by the activation of death domains and death effector domains on the cell surface; the intrinsic cytotoxic mitochondrial-mediated apoptosis, in which mitochondrial membrane permeabilization will lead to cysteine aspartyl-specific protease (caspase) activation (Parrish et al., 2013). Although both pathways will trigger effector caspases, most stimuli induce apoptosis via mitochondrial outer membrane permeabilization (Lopez and Tait, 2015). Cells are stimulated to trigger cell death by apoptosis or necrosis when cells fail to repair DNA damage (Pommier, 2013). Once DNA lesions reach sufficient concentration, they activate cell cycle checkpoints and concomitant apoptosis machinery (Yoshida et al., 2008; Haince et al., 2007).

4.2.2. Cell death triggered by DNA damage

Given the potential devastating effects of gene instability, cells have developed a tight control of the main pathways of survival and death. Nevertheless, DNA damage occurs during transcription and replication. The mechanisms involved in DNA repairment are known as DNA damage response. In this context, topoisomerase I and II are key nuclear enzymes involved in the cell cycle progression and responsible to catalyze the phosphodiester backbone, thus allowing DNA unwinding for replication (Lord and Ashworth, 2012).

Anthocyanin-rich extract from bilberry and grape has also been described as topoisomerase inhibitors due to its ability to reduce topoisomerase I and II activity (Esselen et al., 2011; Habermeyer et al., 2005). However, it is noteworthy that A/A present no properties as topoisomerase poisons, since such compounds cannot stabilize the covalent DNA-topoisomerase intermediates of topoisomerase I or II, known as cleavable complex, which would also result in DNA lesions (Habermeyer et al., 2005). Interestingly, cyanidin and delphinidin, but not their isolated glycosides (cyanidin-3-glucoside and delphinidin-3-rutinoside, respectively) are effective in diminishing the catalytic activity of topoisomerases (Esselen et al., 2011; Habermeyer et al., 2005). Compounds inhibiting topoisomerase function stimulate the formation of DNA single or double-strand breaks across the genome (Lord and Ashworth, 2012). Indeed, delphinidin acts as a topoisomerase inhibitor and therefore, allows the increase in DNA strand breaks (Fritz et al., 2008). Thus, both DNA strand breaks and blocking lesions of DNA replication have been identified as downstream-apoptosis triggering lesions (Naumann et al., 2009).

The phosphatidylinositol 3-kinase-related kinases such as ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related) are crucial "sensors" of DNA lesions. Double-strand breaks and structural changes of the chromatin stimulate ATM expression and its autophosphorylation, whereas stalled DNA replication forks mainly activate ATR (Caporali et al., 2004). Besides stimulating DNA strand breaks and inhibiting the catalytic activity of topoisomerases, A/A, such as cyanidin-3-O-β glucopyranoside and its aglycone, can upregulate the expression of ATM, which in turn stabilizes tumor suppressor p53 (Renis et al., 2008). The chemotherapeutic effects are observed by cell inhibitory proliferation, inducing DNA fragmentation and hence, apoptosis.

4.2.3. Mitochondrial-mediated apoptosis

Programmed cell death can be concomitantly mediated by an intrinsic activation of a cascade involving both caspase and B-cell lymphoma 2 (Bcl-2) family of proteins (Brentnall et al., 2013). Increasing mitochondrial outer membrane permeabilization is the way by which Bcl-2 family protein determines the switch towards cell death rather than conferring survival functionality (Gavathiotis et al., 2008). Once activated over the apoptotic threshold by a diversity of cytotoxic stress stimuli, such as DNA damage or growth factor deprivation, the initiator BH3 (Bcl-2 homology 3) inhibits Bcl-2, the anti-apoptotic cell guardian. In response, the pro-apoptotic effectors Bax (Bcl-2-associated X protein) and Bak (Bcl-2-killer) are then activated and undergo translocation from the cytosol to the mitochondrial outer membrane, where they are oligomerized and, hence, form pores (Czabotar et al., 2013). The release of apoptogenic factors, such as cytochrome C and second mitochondria-derived activator of caspases (SMAC), will trigger the activation of apoptotic protease-activating factor 1 (APAF1) and the inhibition of X-linked inhibitor of apoptosis protein (XIAP), respectively. This process will activate caspase-9 and, consequently, the executioner caspase-3, -7 and -8 to carry out DNA fragmentation and degradation of cytoskeletal and nuclear proteins, thus favoring apoptosis (Li et al., 1997).

Although it remains unclear how exactly chromatin degradation takes place during apoptosis, it has been demonstrated that both caspase-activated deoxyribonuclease (CAD; also known as DNA fragmentation factor) and poly ADP-ribose polymerase (PARP)-regulated DNAS1L3, an endonuclease found in the endoplasmic reticulum, are key enzymes in this process (Errami et al., 2013). Once activated by the executioner caspases, specially caspase-3, CAD and DNAS1L3 contribute to internucleosomal DNA fragmentation. DNA fragmentation, known as "DNA ladder", is a key characteristic of apoptosis (Kello et al., 2016; Gorczyca, Gong, and Darzynkiewicz, 1993). Anthocyanin-rich extract from different blueberry cultivars,

containing mostly malvidin and peonidin glycosides, induces apoptosis in HT-29 cells as a result of increased caspase-3 activity and DNA fragmentation (Srivastava et al., 2007). In accordance, ARE from purple-shoot tea also mediates apoptosis in different colon cancer cell lines by activation of caspase-3 and its substrate PARP (Hsu et al., 2012).

Anthocyanins/anthocyanidins might also have an important role in modulating pro-apoptotic and anti-apoptotic proteins, since the expression of Bax mRNA is enhanced in HT-29 cells treated with ARE from bilberry (Wu et al., 2007). Although changes in Bcl-2 mRNA expression remain undetectable after treating cells with ARE from bilberry (Wu et al., 2007), delphinidin reduces the expression of Bcl-2 in HCT-116 cells in a dose-dependent manner with a concomitant augmentation of Bax expression, activation of caspase-9, -3 and -8, as well as the cleavage of PARP (Yun et al., 2009). In colon cancer stem cells, ARE from purple-fleshed potatoes and java plum suppresses proliferation (Charepalli et al., 2015; Charepalli et al., 2016) by activating mitochondrial-mediated apoptotic pathways through elevating Bax and cytochrome C expression in a p53-independent way (Charepalli et al., 2015). In addition, activity of caspase-3 and -7, which leads to DNA fragmentation, was also increased (Charepalli et al., 2016).

The mitogen activated protein kinase (MAPK) signaling pathways, mainly JNK/p38/ERK pathways, play a critical role in triggering apoptosis (Sui et al., 2014). Long term activation of ERK1/2 induces mitochondrial membrane disruption, leading to cytochrome C release and thus, the activation of caspase-family proteins (Zhang et al., 2004; Cagnol et al., 2006; Tentner et al., 2012). Anthocyanin-rich extract from meoru fruit inhibits cell growth and induces apoptotic cell death by activating phosphorylation of p38-MAPK and ERK1/2 with concomitant suppression of anti-apoptotic Akt and XIAP (Shin et al., 2009). Besides downregulating Akt, ARE from meoru fruit inhibits the pro-tumorigenesis mTOR pathway through AMPKα1 activation, suggesting the anticancer effects (Lee et al., 2010).

4.3. Anthocyanins/anthocyanidins suppress cancer cell proliferation by inducing cell cycle arrest

Anthocyanins/anthocyanidins can also control malignant cell proliferation through cell cycle arrest (Lazze et al., 2004; Renis et al., 2008). Cell cycle is mainly highlighted by DNA replication (S phase) and chromosome segregation, resulting in the formation of two new daughter cells (M phase). Such key events are spaced by periods of cell preparation (G1 phase) and chromatin reorganization (G2 phase) (Salazar-Roa and Malumbres, 2017). Consistently, cells commonly follow a well-controlled cell cycle, regulated by the presence and activity of different cyclin-dependent kinases and their associated cyclins (Murray, 2004) or tumor suppressor proteins (Cordon-Cardo, 2004).

4.3.1. Cell cycle blockage by cyclin-dependent kinases

Besides DNA fragmentation and activation of pro-apoptotic pathways, ARE from different berries is able to induce overexpression of $p21^{WAF1}$ and p27, two cyclin-dependent kinase inhibitors, known to restrain cell proliferation through induction of cell cycle blockage (Wu et al., 2007; Hsu et al., 2012). Anthocyanin-rich extract from chokeberry showed antiproliferative effects in HT-29 cells through dual cell cycle arrest at G0/G1 and G2/M phases, due to overexpression of $p21^{WAF1}$ and $p27^{KIP1}$ and downregulation of cyclins A and B (Malik et al., 2003). Such outcomes might be attributed to a specific anthocyanin, since almost 70% of the total anthocyanins presented in the chokeberry extract are cyanidin-3-galactoside. Consistently, pure delphinidin blocked cell cycle at G2/M phase in HT-116 cells (Yun et al., 2009). The cellular mechanism responsible to inhibit COLO 320DM cell proliferation by ARE from purple-shoot tea was mainly through cell cycle blockage (Hsu et al., 2012). Notably, cells are blocked at the G1 phase and concomitantly decreased in S phase. In addition, cyclins D1 and E expression were downregulated in a dose-dependent manner (Hsu et al., 2012). In colon cancer stem cells, which have been reported to possess an important role in forming and sustaining tumor expansion (Barker et al., 2009), ARE from purple-fleshed potato reduces cell proliferation by downregulating β-catenin levels, which in turn decreases the levels of its downstream proteins, cyclin D1 and c-Myc (Charepalli et al., 2015), both involved in cell cycle progression (Santoni-Rugiu et al., 2000).

As previously discussed, different A/A behave distinctively on cell proliferative control. Hypothetically, their effects on cell cycle progression or arrest pathways might also differ. Likewise, Caco-2 cell growth was more suppressed by cyanidin chloride when compared with cyanidin-3-O-β glucopyranoside (Renis et al., 2008). Furthermore, they were able to induce DNA fragmentation, but only cyanidin chloride treatment induced a decrease in ROS production. The ATM/p53 pathway, known to disturb cell cycle and prevent cell proliferation through the activation of p21, was only upregulated by cyanidin chloride treatment, which suggests that these anthocyanins might have different effects on cell cycle blockage.

4.3.2. Tumor suppressor proteins as cell cycle arrest inductors

It is important to highlight that tumor suppressor proteins, such as p53 and retinoblastoma protein (pRB), have critical roles in blocking abnormal cell proliferation; their mutations may lead to uncontrolled cell division (Cordon-Cardo, 2004). Most sporadic CRC development is owing to mutations in the *Apc* tumor suppressor gene (Fearon, 2011), which mediates β-catenin degradation (Kaler et al., 2009), thus contributing to adenoma-carcinoma sequence (Tarmin et al., 1995).

Anthocyanin-rich extract from illawarra plum was effective in reducing HT-29 cell proliferation associated with cell cycle blockage at the S phase and induction of p53-independent apoptosis and necrosis (Symonds, Konczak, and Fenech, 2013). Additionally, ARE treatment resulted in telomere shortening and decreased expression of telomerase reverse transcriptase, indicating ARE functions as a telomerase inhibitor. Telomerase inhibition followed by reduction in telomere length is an early event in the apoptosis pathway that will lead to restrained cell proliferation, disrupted cell cycle and subsequent apoptosis cell death (Boklan et al., 2002). Moreover, most HT-29 cells treated with ARE from illawarra plum exhibited high numbers of cytoplasmic vacuoles, suggesting cell autophagy (Symonds,

Konczak, and Fenech, 2013). Interestingly, the expression of sirtuin 1 (SIRT1), which has been demonstrated to trigger autophagy (Lee et al., 2008) and inhibit β-catenin pathway (Firestein et al., 2008), was also increased with illawarra plum extract treatment (Symonds, Konczak, and Fenech, 2013).

4.4. Anthocyanins/anthocyanidins inhibit CRC metastasis through suppressing matrix metalloproteinases

The extracellular matrix is composed of proteins and proteoglycans, which are responsible for keeping cell attachment, thus providing structural integrity to tissues (Cox and Erler, 2011). The human matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases ascribed to be involved in inflammatory tissue destruction and capable of degrading basement membrane collagen (Vandenbroucke and Libert, 2014). Accumulating evidence suggests their role in the pathogenesis of IBD (Matusiewicz et al., 2014; Nighot et al., 2015) and hence, in cancer development (Egeblad and Werb, 2002). A tumor cell can metastasize to other organs if the components of the extracellular matrix are degraded by MMPs. Therefore, MMP suppression might be one of the promising targets for cancer therapy (Gialeli et al., 2011). In this context, A/A exhibit anti-invasive activities by suppressing the expression of MMP-2 and MMP-9 in a dose-dependent manner (Shin et al., 2011; Yun et al., 2010).

Albeit MMPs are notably related to invasion and metastasis, and late events in cancer progression, studies have also emphasized its functions in immunity, such as the intertwine between MMPs and inflammation. Matrix metalloproteinases can directly or indirectly mediate the expression of several inflammation-related cytokines or pathways (Nelissen et al., 2003). For instance, the pro-inflammatory IL-1β precursor needs to be cleaved to become active (Yazdi and Ghoreschi, 2016). MMP-2, -3 and -9 can break down and activate the IL-1β precursor (Schonbeck, Mach, and Libby, 1998). Furthermore, MMPs (MMP-3, -7, -9, -12, -17) can turn latent TNF-α into bioavailable TNF-α (Haro et al., 2000; Churg et al., 2003), which results in the activation of pro-tumorigenesis NF-κB pathway (Ferrari et al., 2016). Anthocyanins/anthocyanidins can also contribute to a dual beneficial effect on tumor cell growth: reducing the expression of various pro-metastasis MMPs and additionally suppressing pro-inflammatory mechanisms via MMPs downregulation (Chen et al., 2006).

There is a positive relationship between MMPs and the Akt/mTOR signaling pathway, which has been reported

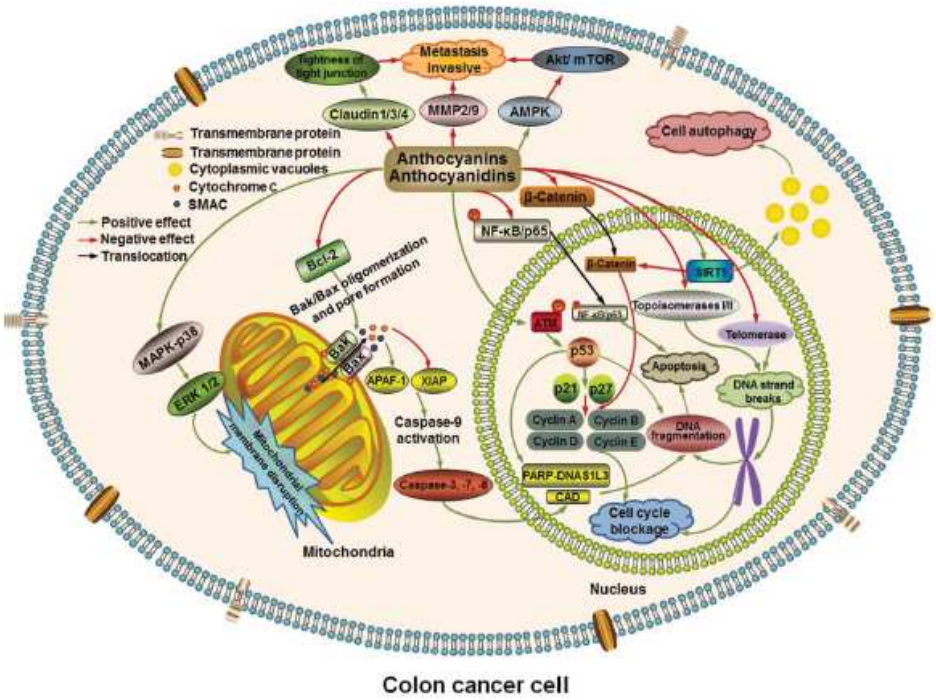

Figure 2. The possible anticarcinogenic mechanisms of anthocyanins/anthocyanidins in colon cancer cells. Anthocyanins/anthocyanidins (A/A) inhibit the pro-inflammatory NF-κB signaling pathway and β-catenin translocation to stimulate cell cycle blockage. A/A act as topoisomerase inhibitors and stimulate DNA strand break responses. Furthermore, A/A phosphorylate ATM to trigger DNA fragmentation and cell cycle blockage. A/A disrupt mitochondrial membrane to induce apoptosis. Concomitantly, A/A enhance tight junction formation, suppress metastasis invasiveness, and increase cell autophagy. Akt, protein kinase B; AMPK, AMP-activated protein kinase; APAF1, apoptotic protease-activating factor 1; ATM, ataxia telangiectasia mutated; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra large; BH3, Bcl-2 homology 3; CAD, caspase-activated deoxyribonuclease; ERK, extracellular signal-regulated kinases; MAPK, mitogen activated protein kinase; MMPs, metalloproteinases; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; SIRT1, sirtuin 1; SMAC, second mitochondria-derived activator of caspases; XIAP, X-linked inhibitor of apoptosis protein. The green arrows indicate demonstrated effects. The black dashed arrows indicate the potential effects. The red lines indicate negative effect. The black arrows indicate the translocation.

elsewhere in CRC cells (Li et al., 2016; Zhang et al., 2015). In addition, an invasive growth of CRC cells is observed when enhanced expression of MMPs takes place due to deactivation of AMPK (Banskota et al., 2015) and posterior activation of Akt/mTOR (Zhan et al., 2017). Although the role of mTOR and its downstream effectors on metastasis invasiveness activation remains speculative (Zhan et al., 2017), inhibition of MMPs, activation of AMPK and inhibition of Akt/mTOR might reduce the invasive phenotype in CRC cells. Anthocyanin-rich extract from meoru suppresses Akt/mTOR phosphorylation, in addition to triggering apoptosis, by stimulating AMPKα1 activation (Lee et al., 2010), further suggesting the potential role on metastasis prevention.

The suppressing properties of A/A on cell growth and invasiveness have also been associated with modulation of tight junction proteins, including claudin-1, -3 and -4 (Shin et al., 2011). Such claudins are crucial transmembrane proteins found to be overexpressed in CRC (Mees et al., 2009). Besides suppressing claudin-1, -3 and -4 in HCT-116 cells, ARE from meoru enhanced tight junctions (cell-cell adhesion), as indicated by increased transepithelial electrical resistance in a concentration-dependent manner (Shin et al., 2011), thus reducing cell invasion. Moreover, restoration of functional tight junction proteins has recently been reported to be related to apico-basal polarity proteins (Borovski et al., 2016). Future studies should also address the role of A/A on cell invasion by modulating tight junction-associated protein complexes, i.e. the crumb (CRB) complex, the partitioning defective (Par) complex, and the scribble (Scrib) complex.

5. Conclusions

As the second most lethal cancer in the United States, the need of new preventive approaches for CRC has become increasingly crucial. In this sense, bioactive compounds would be an easy dietary strategy to provide a therapeutic and nutritional alternative for CRC. Specially, growing evidence shows that A/A have beneficial effects on the management of CRC development. This review summarizes current literatures on anti-CRC health-promoting effects of A/A and their underlying mechanisms (Figure 2). Mainly, A/A mediate colorectal carcinogenesis via stimulation of apoptosis pathways, cell cycle arrest, inhibition of metastasis, and suppression of cell proliferation, as a result of downregulation of inflammatory and oxidative mechanisms. Most *in vitro* and *in vivo* studies, in fact, indicate the chemopreventive properties of A/A. However, due to the lack of human studies assessing the beneficial effects of anthocyanin-rich food/extracts on CRC, the results are still unclear at clinical level. In addition, more studies are needed on the interaction between A/A and the gut microbiota, in order to assess how the gut microbiota-derived anthocyanin metabolites influence the bioavailability of A/A, carcinogenesis, and growth of cancer cells, as well as the onset and development of CRC in animal models and human studies.

Disclosures statement

de Sousa Moraes, Sun, Peluzio, and Zhu have no conflicts of interests.

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3.2. ARTICLE 2 – Dietary green pea protects against DSS-induced colitis in mice challenged with high-fat diet

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Dietary Green Pea Protects against DSS-Induced Colitis in Mice Challenged with High-Fat Diet

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Abstract: Obesity is a risk factor for developing inflammatory bowel disease. Pea is unique with its high content of dietary fiber, polyphenolics, and glycoproteins, all of which are known to be health beneficial. We aimed to investigate the impact of green pea (GP) supplementation on the susceptibility of high-fat diet (HFD)-fed mice to dextran sulfate sodium (DSS)-induced colitis. Six-week-old C57BL/6J female mice were fed a 45% HFD or HFD supplemented with 10% GP. After 7-week dietary supplementation, colitis was induced by adding 2.5% DSS in drinking water for 7 days followed by a 7-day recovery period. GP supplementation ameliorated the disease activity index score in HFD-fed mice during the recovery stage, and reduced neutrophil infiltration, mRNA expression of monocyte chemoattractant protein-1 (MCP-1) and inflammatory markers interleukin (IL)-6, cyclooxygenase-2 (COX-2), IL-17, interferon-γ (IFN-γ), and inducible nitric oxide synthase (iNOS) in HFD-fed mice. Further, GP supplementation increased mucin 2 content and mRNA expression of goblet cell differentiation markers including Trefoil factor 3 (Tff3), Krüppel-like factor 4 (Klf4), and SAM pointed domain ETS factor 1 (Spdef1) in HFD-fed mice. In addition, GP ameliorated endoplasmic reticulum (ER) stress as indicated by the reduced expression of Activating transcription factor-6 (ATF-6) protein and its target genes chaperone protein glucose-regulated protein 78 (Grp78), the CCAAT-enhancer-binding protein homologous protein (CHOP), the ER degradation-enhancing α-mannosidase-like 1 protein (Edem1), and the X-box binding protein 1 (Xbp1) in HFD-fed mice. In conclusion, GP supplementation ameliorated the severity of DSS-induced colitis in HFD-fed mice, which was associated with the suppression of inflammation, mucin depletion, and ER stress in the colon.

Keywords: high-fat diet; colitis; green pea; inflammation; mucin 2; endoplasmic reticulum stress

1. Introduction

According to the latest NHANES survey (2009–2010), 31.9% of non-pregnant women 20–39 years of age are obese, and another one-third are overweight [1]. In parallel with the increased obesity prevalence, the incidence of inflammatory bowel disease (IBD), consisting of Crohn's disease (CD) and ulcerative colitis (UC), is on the rise. IBD is a chronic relapsing disorder of the gut with a complicated etiology. Increasing evidence indicates that Western dietary and life-style habits contribute to the increased prevalence of IBD by inducing intestinal inflammation [2].

The Western diet is high in fat and low in fiber, which aggravates dextran sodium sulfate (DSS)-induced colitis [3], and is further exacerbated by the intake of red meat [4]. Long-term high intake of trans-unsaturated fats is associated with an increased risk of UC in women in the USA [5]. Recently, we found that maternal HFD consumption during gestation and lactation predisposed female offspring to a higher susceptibility to DSS-induced colitis through increased inflammatory responses [6]. HFD consumption also induces oxidative and endoplasmic reticulum (ER) stress [7],

leading to mucin 2 protein misfolding in cultured colon cells [7]. Mucin 2 depletion and misfolding correlates with colitis in mice [8].

On the contrary to the HFD, high vegetable and fiber intake is associated with a decreased risk of IBD [9]. Epidemiologically, legume intake was protective against colorectal cancer in a case control study [10], and significantly reduced the risk of colorectal adenoma in a meta-analysis of three cohort studies and eleven case control studies [11]. Legumes and pulses, including peas, are rich in fiber and other phytonutrients that boost beneficial intestinal microbiota [12], producing short chain fatty acids (SCFA) and promoting epithelial barrier integrity [13]. Further, dietary soybean Bowman–Birk inhibitor concentrate [14], white and dark kidney beans [15], and cranberry bean supplements [16] suppressed colonic inflammation and reduced the severity of DSS-induced colitis in mice. Consistently, pea seed albumin extract ameliorated DSS-induced colitis in mice by reducing the expression of inflammatory markers in colonic tissues [17]. These results suggest that beans in general might have protective effects against colitis. The objective of the current study was to investigate the preventive effect of dietary green pea (GP) supplementation on DSS-induced colitis in HFD-fed female mice and further examine its underlying mechanism.

2. Materials and Methods

2.1. Green Pea (GP)

GP was purchased from Moscow Food Co-op (Moscow, ID, USA) and powdered in the cyclone mill (Model 3010-060, UDY Corp., Fort Collins, CO, USA). The powdered GP was shipped to the Research Diets, Inc. (New Brunswick, NJ, USA) for customized diet formulation.

2.2. Experimental Design and Animal Diets

Six-week-old C57BL/6J female mice (originally purchased from Jackson Laboratory, Bar Harbor, ME, USA, and inbred in our facility) were randomly divided into two groups. One group of mice ($n = 7$) was fed with the HFD (45% energy from fat, D12451, Research Diets Inc., New Brunswick, NJ, USA) (Table S1), and the other group of mice ($n = 7$) was fed HFD supplemented with GP (10% of dry feed weight) (HFDGP, D15080605, Research Diets Inc., New Brunswick, NJ, USA) (Table S1) for a total duration of 9 weeks. The dose of GP (10%) supplement was 100 g/kg of the diet. The average daily consumption by mice was 2.40 g/mouse. This equals to 240 mg GP per day for an adult mouse of 20 g (i.e., 12 g GP/day/kg body mass), which converts to 58.38 g of GP daily consumption for a 60 kg human per the published formula [18]. Colitis was induced using colitis grade DSS (Molecular Weight = 36,000–50,000) (MP Biomedicals, Santa Ana, CA, USA) after 7 weeks of dietary supplementation. Both groups were given 2.5% DSS (w/v) in drinking water for 7 days followed by a 7-day recovery period providing normal drinking water (Figure S1). We used only virgin females in the study to avoid a confounding sex effect and to minimize potential differences in female hormone cycling. Mice were monitored daily throughout the DSS treatment and recovery period for disease symptoms. All mice were housed in a temperature-controlled room with a 12 h light and 12 h dark cycle and had free access to diet and drinking water. No differences were observed in the average amount of water and feed consumption (Figure S2A) between treatment groups. All animal procedures were approved (BAF # 04316-010) by the Washington State University Animal Care and Use Committee.

2.3. Assessment of Colitis Symptoms and Disease Activity Index

Mice were monitored daily for body weight loss compared to initial weight (scored as 0–4), fecal consistency (scored as 0–4), and blood in the stool (scored as 0–4) throughout the DSS treatment and recovery period. The disease activity index (DAI) score was assessed as the combined score of the above three criteria [19].

2.4. Colonic Tissue Collection and Processing

Mice were anesthetized with CO_2 inhalation and followed by cervical dislocation. The entire colon was dissected, and a 5 mm segment of the distal colon at a constant location was fixed in freshly prepared 4% (w/v) paraformaldehyde (pH 7.0), processed, and embedded in paraffin. The remaining colonic tissue, containing both inflamed and non-inflamed areas, was rinsed in PBS, frozen in liquid nitrogen, and stored at −80 °C for later biochemical analysis.

2.5. Neutrophil Assessment

Paraffin embedded tissues were cut into 5 µm thick sections, deparaffinized, and hydrated, followed by antigen retrieval, goat serum blocking, and overnight incubation with anti-Ly-6B.2 antibody (Bio-Rad Laboratories Inc., Hercules, CA, USA). After incubation with the secondary antibody, signals were visualized using the Vectastain ABC and DAB peroxidase (HRP) substrate kits (Vector Laboratories Inc., Burlingame, CA, USA) and haematoxylin counterstaining. Images were taken using the Lecia DM2000 LED light microscope (Chicago, IL, USA). Neutrophil infiltration scores were assessed blindly by two researchers using the criteria described previously [20]. Briefly, the scores for depth of neutrophil infiltration (scored as 0–3) and staining intensity (scored as 0–4), which was the percent area positive as extent (0, none; 1, <25%; 2, 25–50%; 3, 50–75%; 4, >75%), were recorded individually. The summation of both scores resulted in a total quantified score ranging from 0 to a maximum of 7 per distal colonic section. Nine sections per animal at constant interval were used for microscopic examination and score assessment.

2.6. Immunoblotting Analysis

Immunoblotting analyses were performed as previously described [21]. Band density was quantified using the Odyssey Infrared Imaging System and Image Studio™ Lite software (Li-Cor Biosciences, Lincoln, NE, USA), and normalized to the β-actin content. Antibodies against activating transcription factor-6 (ATF-6), mucin 2, and xanthine oxidase (XO) were from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Cyclooxygenase-2 (COX-2) and interleukin (IL)-6 primary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin antibody was from Sigma (St. Louis, MO, USA). IRDye 680 goat anti-mouse and IRDye 800CW goat anti-rabbit secondary antibodies were purchased from Li-Cor Biosciences (Lincoln, NE, USA).

2.7. qRT-PCR Analysis

Total RNA was extracted from the powdered colonic tissue using Dynabeads® mRNA DIRECT™ Purification Kit (Invitrogen, Carlsbad, CA, USA) following the protocol of the manufacturer. cDNA was synthesized with the iScript™ cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). qRT-PCR was performed on a Bio-Rad CFX384 real-time thermocycler [22]. The 18S was used as the reference gene. Primer sequences are provided in Table S2.

2.8. Statistical Analysis

All data were analyzed as a complete randomized design using the General Linear Model of Statistical Analysis System (2000), expressed as mean ± standard error of mean (SEM). Student's T-test was used for calculating significance. A significant difference was considered as $p \leq 0.05$.

3. Results

3.1. GP Supplementation Counteracts Symptoms of DSS-Induced Colitis in HFD-Fed Mice

DSS-induction caused colitis symptoms in mice. The HFD-fed mice with and without GP supplementation showed similar symptomatic parameters during the DSS-treatment phase (Figure 1). However, during the recovery phase, the GP-supplemented HFD-fed group recovered faster than

mice without GP supplementation. The body weight loss and body weight loss score remained lower in the GP-supplemented HFD-fed group throughout the recovery period (Figure 1A,B). Further, a significant reduction in the fecal blood and DAI score was found in GP-supplemented HFD-fed mice (Figure 1C,D). There was no difference in body weight between the two groups before DSS-induction (Figure S2B).

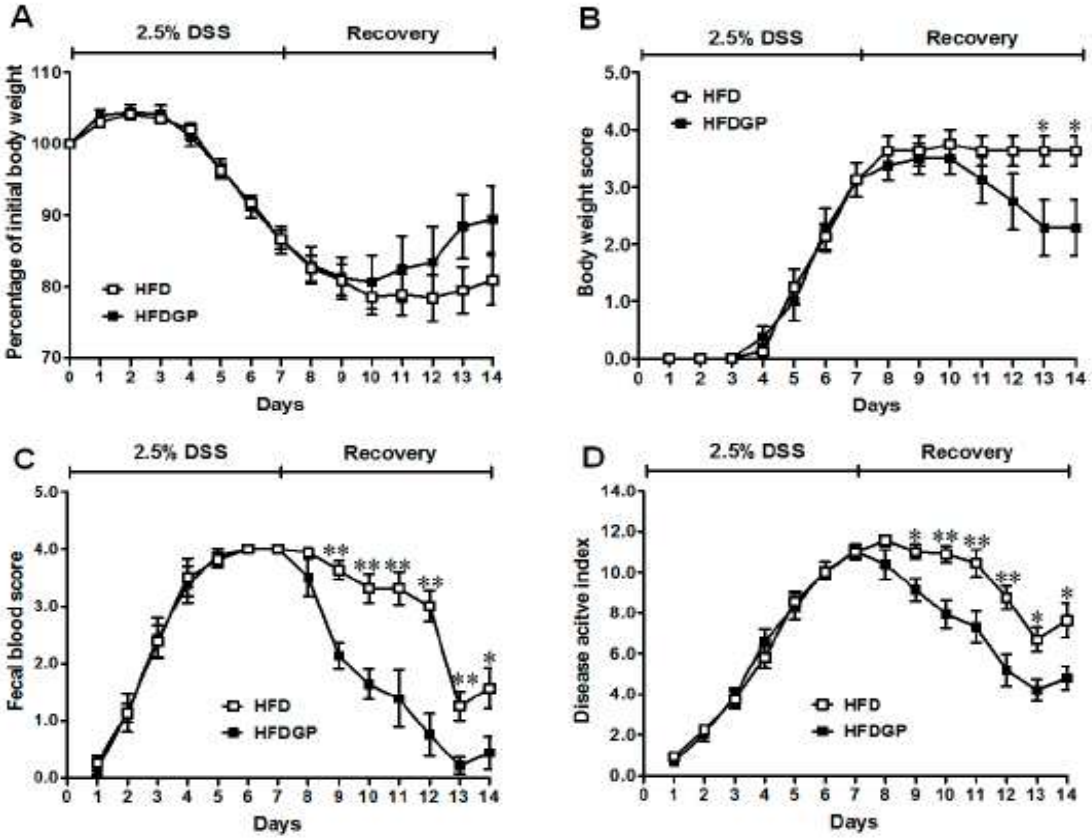

Figure 1. Symptoms of dextran sulfate sodium (DSS)-induced colitis in high-fat diet (HFD) (□) or HFD supplemented with green pea (HFDGP) (■) fed mice. (**A**) Body weight loss; (**B**) Body weight loss score; (**C**) Fecal blood score; (**D**) Disease activity index score during DSS treatment and recovery process; a higher score correlates with severer symptoms. Means ± SEM, $n = 7$, * $p \leq 0.05$, ** $p \leq 0.01$.

3.2. GP Supplementation Reduces Neutrophil Recruitment and Monocyte Chemoattractant Protein-1 (MCP-1) Expression in HFD-Fed DSS-Colitis Mice

GP supplementation reduced the neutrophil recruitment, and resultant tissue damage in the colonic tissues of HFD-fed DSS-colitis mice (Figure 2A,B). In accordance, GP supplementation reduced the gene expression of MCP-1 (Figure 2C), which enhances the recruitment of neutrophils into the mesenteric tissues [23].

3.3. GP Supplementation Reduces Inflammation and Oxidative Stress in HFD-Fed DSS-Colitis Mice

In agreement with improved epithelial damage, GP supplementation reduced the protein and mRNA expression of both interleukin (IL)-6, and cyclooxygenase-2 (COX-2) (Figure 3A,B), and reduced the mRNA levels of IL-17, interferon (IFN-γ), and inducible nitric oxide synthase (iNOS) (Figure 3C) in the HFD-fed DSS-colitis mice. Altogether, these data confirmed the beneficial effect of GP via reducing inflammation and oxidative stress in DSS-colitis.

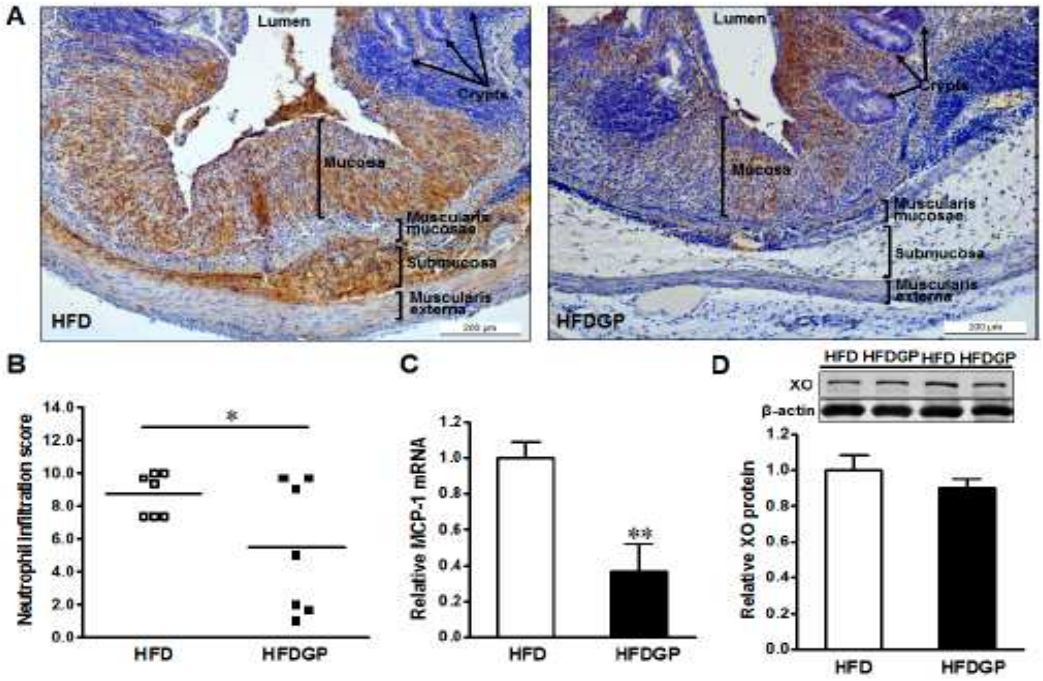

Figure 2. Immunohistochemical staining of neutrophils in distal colonic tissues of high-fat diet (HFD) (□) or high-fat diet supplemented with green pea (HFDGP) (■) fed DSS-colitis mice. (**A**) Representative images of neutrophil staining; (**B**) Neutrophil quantified score; (**C**) mRNA expression of MCP-1; (**D**) Representative immunoblotting bands and statistical data of xanthine oxidase (XO). Means ± SEM, $n = 7$, * $p \leq 0.05$, ** $p \leq 0.01$.

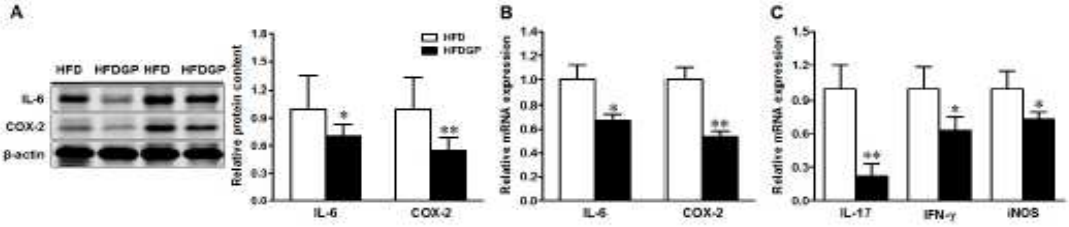

Figure 3. Inflammatory mediators in the colon of HFD or HFDGP fed DSS-colitis mice. (**A**) Representative immunoblotting bands and statistical data of IL-6 and COX-2; (**B**) mRNA expression of IL-6 and COX-2; (**C**) mRNA expression of IL-17, IFN-γ and iNOS. Means ± SEM, $n = 7$, * $p \leq 0.05$, ** $p \leq 0.01$.

3.4. GP Supplementation Enhances MUC-2 Secretion and Goblet Cell Differentiation in HFD-Fed DSS-Colitis Mice

Mucin 2 is the major mucin produced by goblet cells and provides an additional protective layer to the gut epithelium. Both the mRNA and protein levels of mucin 2 were enhanced in the GP-supplemented HFD-fed DSS-treated mice (Figure 4A,B). In agreement, the gene expression of goblet cell differentiation markers including Trefoil factor 3 (Tff3), Krüppel-like factor 4 (Klf4), and SAM pointed domain ETS factor 1 (Spdef1) were higher in the GP-supplemented HFD-fed DSS-induced mice (Figure 4C).

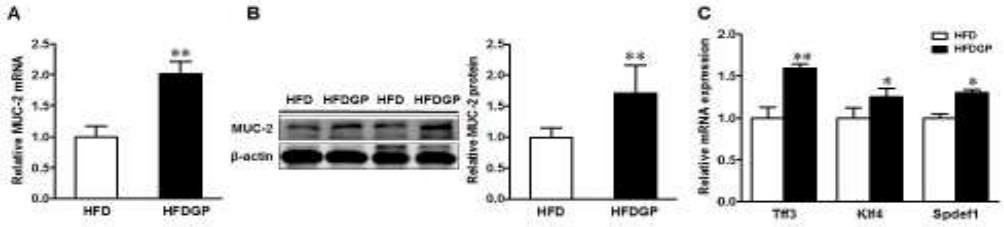

Figure 4. Mucin-2 and goblet cell differentiation markers in the colon of HFD or HFDGP fed DSS-colitis mice. (**A**) mRNA expression of MUC-2; (**B**) representative immunoblotting bands and statistical data of mucin 2; (**C**) mRNA expression of goblet cell differentiation markers, Tff3, Klf4, and Spdef1. Means ± SEM, $n = 7$, * $p \leq 0.05$, ** $p \leq 0.01$.

3.5. GP Supplementation Suppresses the Expression of Activating Transcription Factor-6 (ATF-6) and ER-Stress Markers in HFD-Fed DSS-Colitis Mice

IBD is associated with ER stress and mucin 2 misfolding [24,25]. As part of the unfolded protein response (UPR), ATF-6 triggers the transcription of genes encoding the chaperone protein glucose-regulated protein 78 (Grp78), the CCAAT-enhancer-binding protein homologous protein (CHOP), the ER degradation-enhancing α-mannosidase-like 1 protein (Edem1), and the X-box binding protein 1 (Xbp1) [26,27]. Consistently, GP supplementation reduced the protein expression of ATF-6 (Figure 5A) and mRNA expression of its downstream target genes Grp78, CHOP (Figure 5B), Edem1, and Xbp1 in the HFD-fed DSS-colitis mice (Figure 5C), showing the suppression of ER stress.

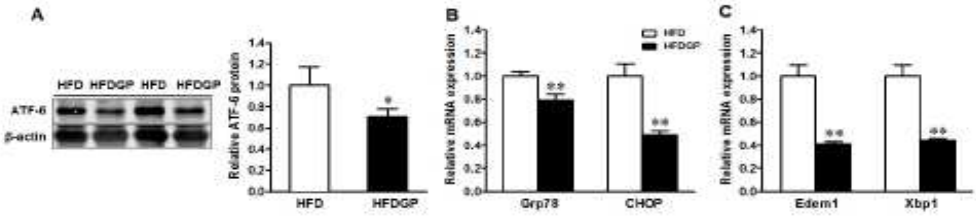

Figure 5. Endoplasmic reticulum (ER)-stress signaling in the colon of HFD or HFDGP fed DSS-colitis mice. (**A**) ATF-6 protein content; (**B**) mRNA expression of Grp78 and CHOP; (**C**) mRNA expression of Edem1 and Xbp1. Means ± SEM, $n = 7$, * $p \leq 0.05$, ** $p \leq 0.01$.

4. Discussion

Obesity is the root cause of many chronic diseases including diabetes, hypertension, and cardiovascular disease. Consumption of the HFD is associated with intestinal inflammation and increased permeability to the microbial end-products in mice [2,28]. The HFD enhances the severity of colitis in experimental colitis mice models [7,29,30], and promotes colon cancer initiation [31]. Further, inclusion of red meat in the Westernized HFD aggravated DSS-colitis in mice [4]. Peas are a valuable source of plant proteins, fiber, and polyphenolics [32], and its extract reduced inflammation in mice with DSS-induced colitis [17]. Our study shows that supplementation of GP accelerated the recovery from colitis symptoms in the HFD-fed mice as evident from decreased body weight loss and a lower fecal blood score during the recovery stage. In support of our findings, supplementation of dietary white and dark kidney beans as well as cranberry beans reduced colitis severity by reducing body weight loss, fecal blood score, and resultant DAI score in DSS-induced colitis mice [15,16]. Similarly, dietary supplementation of soybeans Bowman–Birk inhibitor concentrate reduced the severity of DSS-colitis by suppressing inflammation in the colon and improving the recovery following DSS-induction [14].

DSS causes mucosal and tissue damage in the mouse gut similar to the patterns of inflammatory responses observed in human UC [33,34]. The activation and infiltration of inflammatory cells, including neutrophils and monocytes, is one of the common features in colitis, which is a complex process driven by cytokines, chemokines, and cell adhesion molecules [35]. Cytokines mediate neutrophil infiltration into the intestinal wall and MCP-1, highly expressed in colonic mucosa in IBD [36], enhances the migration of neutrophils during chronic inflammation [23]. In DSS-induced colitis, Westernized HFD-feeding enhanced neutrophil infiltration as indicated by enhanced myeloperoxidase activity [4], and pea seed albumin extracts reduced inflammatory cell infiltration into the colon [17]. In agreement, the current study found that DSS-induction enhanced both the neutrophil infiltration and MCP-1 expression in the colon of HFD-fed mice, which were mitigated by GP supplementation. Inline, dietary white and dark kidney beans, as well as cranberry bean supplements, reduced the mRNA expression of MCP-1 in the colon of DSS-colitis mice [15,16].

Infiltrated neutrophils produce proinflammatory cytokines including IL-6, IL-17, and IFN-γ, and enhance the expression of oxidative stress enzyme, iNOS, further aggravating colitis [28,33]. IFN-γ plays an important role in the development of DSS-colitis, likely by activating and directing the leucocytes to the intestinal tissue [37]. Similarly, IL-17 stimulates epithelial cells to secrete IL-6 and helps CD34+ hematopoietic progenitors mature into neutrophils [38]. In the current study, elevated levels of IL-6, IL-17, IFN-γ, iNOS, and COX-2 caused by DSS-induction were ameliorated by GP supplementation in the colon of HFD-fed mice. Consistently, dietary white and dark kidney beans reduced the mRNA expression of IL-6 [15], and cranberry bean supplementation reduced the IL-6 protein in the colon along with reduced serum IL-6, IL-17, and IFN-γ in DSS-induced colitis [16]. The down-regulation of inflammatory cascades and oxidative stress by GP supplementation can be partially explained by the low neutrophil infiltration into the colon of HFD-fed DSS-colitis mice.

The lubricating layer of mucus that shields the epithelium from the gut luminal content predominantly consists of mucin 2 produced by goblet cells. Mucin 2 goes through heavy extensive translational modifications in the ER and Golgi complex, making it susceptible to misfolding, and thus activating the UPR signaling [39]. Disturbance in the UPR and ER stress in intestinal epithelial cells induces chronic inflammation in IBD [24,25]. Missense mutations of the MUC-2 gene in *Winnie* and *Eeyore* mice increased ER-stress-related mucin depletion, resulting in colitis [8]. Recently, Gulhane and colleagues found that the HFD induced the expression of oxidative stress marker iNOS, and ER-stress markers including UPR signaling molecules Xbp1, ER chaperone Grp78, and ERAD chaperone Edem1 in the colon of *Winnie* mice [7]. On the other hand, dietary chickpea supplementation increased colon mucus content, mRNA expression of MUC-2, and differentiation marker Klf4 with enhanced gut barrier integrity and reduced inflammation in healthy unchallenged mice [13]. In DSS-induced colitis, dietary white and dark kidney beans, as well as dietary cranberry bean supplementation, enhanced the mRNA expression of MUC-2 and Tff3, and mitigated the severity of colitis and associated inflammation [15,16]. Consistent with these observations as well as improved colitis symptoms, GP supplementation improved both protein and gene expression of MUC-2 in HFD-fed DSS-induced mice, associated with the enhanced expression of goblet cell differentiation markers in the colon. Further, ATF-6 and its downstream ER-stress markers Grp78, CHOP, Edem1, and Xbp1 [24,25] were reduced in HFD-fed mice by GP supplementation.

Legumes such as chickpeas, kidney beans, and cranberry beans contain dietary fiber, resistant starches, protein, and polyphenolics with reported beneficial effect on intestinal health [13,15,16]. The protein extract of soybeans and peas contains the active Bowman–Birk inhibitor that possesses anti-inflammatory activity and can reduce the severity of DSS-colitis in mice [14,17]. Using the whole food approach, we were not able to conclude which bioactive component in GP was responsible for protection against DSS-induced damages. Based on the previous investigations, the protective effect of GP can be attributed to the active Bowman–Birk inhibitor present in pea protein [14,17] and/or dietary fiber [12]. Dietary fiber in chickpea modulated the gut microbiota and enhanced SCFA production,

correlating with improved gut epithelial barrier function [13]. These results suggested that GP might modulate gut microbiota to exert its protective effects on DSS-induced colitis.

5. Conclusions

GP supplementation reduces the severity of DSS-induced colitis in mice challenged with the HFD by reducing inflammation, mucosal loss, and the ER-stress signaling. GP possesses anti-inflammatory properties in DSS-induced colitis in mice fed a HFD, and can be used as a potential dietary management to reduce risk of IBD development.

Supplementary Materials: The Supplementary Material are available online at www.mdpi.com/2072-6643/9/5/509/s1, Figure S1: An overview of experimental design, Figure S2: Feed intake and body weight of HFD and HFDGF fed mice before DSS-induction, Table S1: Composition of the experimental diets used in the study, Table S2: Primer sequences for quantitative reverse transcription PCR.

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Author Contributions: Shima Bibi and Meijun Zhu conceived and designed the experiments; Shima Bibi, Luís Fernando de Sousa Moraes and Noelle Lebow conducted the experiments. Shima Bibi and Meijun Zhu wrote and revised the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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3.3. Article 3 – Dietary *Hibiscus sabdariffa* L. mitigates preneoplastic lesions development in distal colon by increasing fecal short-chain fatty acids concentration and infiltration of Natural Killer cell

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Dietary *Hibiscus sabdariffa* L. mitigates preneoplastic lesions development in distal colon by increasing fecal short-chain fatty acids concentration and infiltration of Natural Killer cell

ABSTRACT

Background. Colorectal cancer (CRC) is the third leading cause of cancer-related deaths worldwide. Present data has reported the role of anthocyanin-rich food/extract in increasing fecal short-chain fatty acids (SCFA) concentrations and NK cells infiltration in the large intestine mucosa, thus contributing to prevent preneoplastic lesions formation. Here we hypothesized whether carcinogen-induced aberrant crypt foci (ACF) progression in BALB/c mice fed a diet containing dietary *Hibiscus sabdariffa* L. (HS) is suppressed by modulation of fecal SCFA and NK cells infiltration. Furthermore, we also investigate whether such supplementation might induce hepatoprotective effects. **Methods**. Nutritional composition, total phenolic and total monomeric anthocyanin content were assessed in HS calyces. Preneoplastic colorectal lesions were induced in male BALB/c mice by injecting 1,2-dimethylhydrazine (20 mg/kg body weight) intraperitoneally. Mice were fed control or supplemented diet containing either 5 or 10% dietary HS for 14 weeks. ACF counts, fecal SCFA concentrations and leukocytes infiltration were assessed. **Results**. Polyphenol and anthocyanin contents in HS calyces were found to be 57.84 mg GAE/g dw HS and 7.81 mg cyanidin-3 glucoside/ dw HS, respectively. Supplementation with 5 or 10% dietary HS attenuated colonic ACF development in the distal colon ($P < 0.01$). Total ACF counts per mouse was reduced by almost 29.0% in HS supplemented groups when compared to control ($P < 0.01$). Fecal butyric and propionic acids concentrations, in addition to NK cell infiltration, were increased with 10% dietary HS supplementation. Hepatic catalase activity was enhanced in 10% dietary HS-treated mice when compared to control group ($P < 0.01$). **Conclusions**. Colonic preneoplastic lesions in carcinogenic-induced male BALB/c mice are mitigated by HS dietary treatment probably due to modulation of SCFA and NK cell infiltration. We might also infer that 10% dietary HS is quite more effective when compared to 5%.

1. Introduction

Cancer is a multifactorial chronic disease mainly identified by unrestrained clonal expansion and spread of abnormal cells (1). Specifically, colorectal cancer (CRC), the third most common cancer in both men and women in the United States (2), involves the onset of pre-neoplastic lesions, known as aberrant crypt foci (ACF), and increased replication of the upper crypt cells (3).

A possible role of increased immune cytotoxicity has been related to ACF onset (4). Natural Killer (NK) cells are minor granular lymphocytes of the innate lymphoid cell family with potent cytolytic activity and precise cytotoxic function (5). Identified in lymphocytic infiltrates, NK cells have been increasingly speculated given its strong antitumor potential (6). NK cells functions are tightly regulated to prevent the killing of healthy cells (7). Differently from noncancerous cells, preneoplastic cells do express specific receptors on the cell surface (8). NK cells are then able to recognize abnormal cells and initiate the effector immune response by releasing granules containing perforin, a membrane-disrupting protein, tumor necrosis factors (9) and other chemokines (10). Importantly, NK cell-mediated cytolysis might be more sensitive to cancer cells in the early stage of differentiation (11).

NK cells interaction with target cancer cells seems to be involved with gut microbiota metabolites. Short-chain fatty acids (SCFA), mainly butyric acid but also propionic acid in a lesser extension, have been recognized as histone deacetylase (HDAC) inhibitors (12, 13). HDAC inhibitors can trigger hyperacetylation of histones, thus regulating the expression of silent genes related to apoptosis and cell cycle arrest (14, 15). Furthermore, the crosstalk between HDAC inhibitors and NK cells killing has been stablished (16). HDAC inhibitors activates specific pathways, which in turn improve the immunorecognition of cancer cells by NK cells (17, 18).

In this regard, because anthocyanin has contributed to increase fecal butyric acid concentrations (19) and modulate human gut microbiota *in vitro* towards a positive butyrate-producing bacteria growth (20), we here hypothesized that dietary *Hibiscus sabdariffa* L. (HS) consumption might increase fecal SCFA

concentrations and NK cells infiltration, thus contributing to abrogate 1,2-dimethylhydrazine-induced colorectal carcinogenesis in BALB/c male mice.

HS calyces are a rich source of anthocyanins (21, 22) and can be widely used for consumption, e. g. teas, jams, and jellies (23). Isolated compounds from HS are often used as chemopreventive agents (24, 25). In addition, HS or anthocyanin-rich extracts from HS have demonstrated no hepatotoxic action *in vivo* or *in vitro* (26-28). However, identifying whole foods, not only extracts, with antitumor properties is a noble research field in nutrition that provides natural and cheap alternatives to promote relevant heath benefits and, hence, CRC prevention.

2. Methods

2.1. HS calyces acquisition and Nutritional Composition

HS calyces were fully acquired from a local market in Viçosa – MG, Brazil, immediately powdered, and kept frozen (–40°C) until analysis at the Food Analysis lab. Chemical composition was evaluated following the standard methodologies proposed by the Association of Official Analytical Chemists – AOAC (29). In brief, fresh HS calyces (~ 10g) were allowed to dry until constant weight at 105°C to determine moisture (%) content (29). Crude protein (g/100g) was performed by Kjeldahl technique and calculated as nitrogen × 6.25 (29). Fat (g/100g) was determined by the Soxhlet extraction system using ethyl ether and quantified gravimetrically (30). Dietary fiber (g/100g) was assessed by the AOAC enzymatic-gravimetric method (31). Ash (g/100g) content was performed by the residue left procedure using a muffle furnace at 550°C and quantified gravimetrically (29). As proximate analysis was carried out, carbohydrate (g/100g) was obtained according to the following calculation [100 - (Moisture + Crude protein + Fat + Dietary fiber + Ash)]. All analyses were performed on a dry weight (dw) basis, except for moisture content.

2.2. Extraction of polyphenols in Hibiscus sabdariffa L. calyces

The extraction of polyphenols in HS calyces was performed at the Nutritional Biochemistry lab according to a modified version of the procedure proposed by Tseng and co-workers (32). Dried and powdered HS calyces were weighed (1 g) and mixed with ethanol: water (80:20, v/v) under continuous stirring for 30 minutes at room temperature. Extraction was performed in the dark and used to measure total phenolic compound content and total monomeric anthocyanins.

2.3. Total Phenolic Compound Assessment

The content of soluble phenols was determined at the Nutritional Biochemistry lab using the Folin-Ciocalteu method with some modifications (33). The formation of both phosphotungstate and phosphomolybdate anions turn the mixture into a bluish color and hence can be measured spectrophotometrically at 760 nm (34). Briefly, an aliquot (500 μL) of blank (distilled deionized water), standard (gallic acid, 50 g/L) or filtered HS extract was pipetted into a test tube and mixed with 2.5 mL of the Folin-Ciocalteu reagent. After standing at room temperature for 2 minutes, a solution of sodium carbonate (75 g/L, 2 mL) was added and vortexed until thoroughly mixed. The solution was left standing for 15 minutes and then immediately cooled down to room temperature. The absorbance was measured at 760 nm using a Multiskan GO microplate spectrophotometer (Thermo Scientific). Results were expressed as milligram of Gallic Acid Equivalent per gram dry weight HS (mg GAE/g dw HS). The yellowish FC reagent contains phosphotungstic and phosphomolybdic acids, which are reduced by polyphenolic antioxidants upon addition of an alkaline solution.

2.4. Quantification of Total Monomeric Anthocyanins

Total monomeric anthocyanins were determined at the Nutritional Biochemistry lab by the classical pH differential method as described by Lee and co-workers (35) with some modifications. The anthocyanin structure is reversibly modified according to the solution pH. At pH 1, the flavylium cation is predominantly formed, thus conferring a reddish color to the solution. By increasing the pH to 4.5, most anthocyanins are in a non-colored hemiketal form and, therefore, present little or

no absorbance at this pH (35). In brief, the sample appropriate dilution factor was necessary to be previously determined by mixing different sample aliquots (2.5-25.0 μL) with potassium chloride buffer (0.025 M, 200 μL, pH 1) in order to find the best absorbance within the linear range of the spectrophotometer at 520 nm. Once settled, the sample appropriate dilution factor (15 μL) was then pipetted into a 96-well plate containing potassium chloride buffer (0.025 M, 200 μL, pH 1) or sodium acetate buffer (0.4 M, 200 μL, pH 4). Distilled deionized water was used as blank. After standing for 30 minutes at room temperature in the dark, the absorbance was measured at 520 nm and 700 nm (to correct for haze) in a Multiskan GO microplate spectrophotometer (Thermo Scientific). Total monomeric anthocyanin content was calculated as milligram cyanidin-3 glucoside per gram dry weight HS (mg cyanidin-3 glucoside/g dw HS) according to the following formula:

$$TMA\ (mg/L) = [(Abs_{520nm} - Abs_{700nm})_{pH1.0} - (Abs_{520nm} - Abs_{700nm})_{pH4.5}] \times MW \times DF \times 1000 \times \varepsilon^{-1}$$,

where TMA, total monomeric anthocyanin; Abs, absorbance; MW, molecular weight of cyanidin-3 glucoside = 449.2; DF, dilution factor; ε, extinction coefficient 26,900 $L.cm^{-1}mol^{-1}$.

2.5. Animal Care and Experimental Design

Seven-week-old male BALB/c mice were obtained from the Central Bioterium (Centro de Ciências Biológicas e da Saúde) of the Universidade Federal de Viçosa, Brazil. The animals were housed at the Experimental Nutrition lab in a temperature-controlled room (22 ± 2°C) with a 12-hour light/dark cycle and *ad libitum* access to water and food. Animal protocol was approved by the Ethics Committee on Animal Experimentation from the Federal University of Viçosa, Brazil, under the process number 10/2017. Upon arrival, animals were randomly assigned to three experimental groups and fed during 14 weeks either a supplemented AIN-93M (36) containing 0 (control group, n=13), 5 (5HS group, n=14) or 10% (10HS group, n=15) dietary HS (dried powder from HS calyces), as shown in Table 1.

Table 1. AIN-93M diet for control and supplemented groups.

Ingredients (g)	*Groups*		
	Control[1]	*5HS*[2]	*10HS*[3]
Cornstarch	46.56	46.56	46.56
Casein	14.0	14.0	14.0
Dyetrose	15.5	15.5	15.5
Sucrose	10.0	10.0	10.0
Cellulose	5.0	4.54	4.08
Mineral mix	3.5	3.5	3.5
Vitamin mix	1.0	1.0	1.0
L-Cystine	0.18	0.18	0.18
Choline bitartrate	0.25	0.25	0.25
t-Butylhydroquinone	0.0008	0.0014	0.0014
Soybean oil	4.0	4.0	4.0
Dietary HS	0.0	5.0	10.0

AIN-93M, American Institute of Nutrition for maintenance. [1]Control group, AIM-93M; [2]5HS group, AIM-93M supplemented with 5% dietary HS; [3]10HS group, AIM-93M supplemented with 10% dietary HS.

According to the nutritional composition, HS calyces contain insignificant amounts of carbohydrate, crude protein and fat (Table 2). However, HS calyces presents 9.2% as total dietary fiber. Thus, all supplemented diets were properly corrected by the amount of cellulose. Food was changed on a daily basis to avoid oxidation of anthocyanins or other polyphenols.

We have supplemented the animals with 5 or 10% dietary HS (powdered calyces) taking into consideration that such dosages can be easily consumed by humans. The human equivalent amount of dietary HS consumed by male BALB/c mice was performed using the body surface area normalization method as previously describe (37). For instance, mice in 5HS group were supplemented with 5% dw dietary HS, i.e. 50 g/kg diet. In other words, dietary HS supplemented diet contains 50 g dietary HS/kg diet. In our study, the average daily food intake was 6.72 g. Therefore, 50 g/1,000 g diet x 6.72 g diet/day = 336 mg dietary HS daily. Accordingly, if the average mice body weight was 43.38 g, then 336 mg/43.38 g x 1,000 g = 7,745.5 mg/Kg diet per day. As reported by the group of Reagan-Shaw

(37), the human equivalent dose (mg/kg) = animal dose (mg/kg) x (animal K_m factor/human K_m factor). Thus, human equivalent dose (mg/kg) = 7745.5 mg/kg x (3/ 37) = 628.01 mg/kg. Considering that the average human adult weight is 60 kg, this is equal to 628.01 mg/kg x 60 kg = 37,680.6 mg, which converts to 37.7 g daily for humans approximately.

All animals at 9-week-old were received a single weekly intraperitoneal injection of 1,2-dimethylhydrazine (DMH, 20 mg/kg body weight) for 8 weeks, as shown in Figure 1.

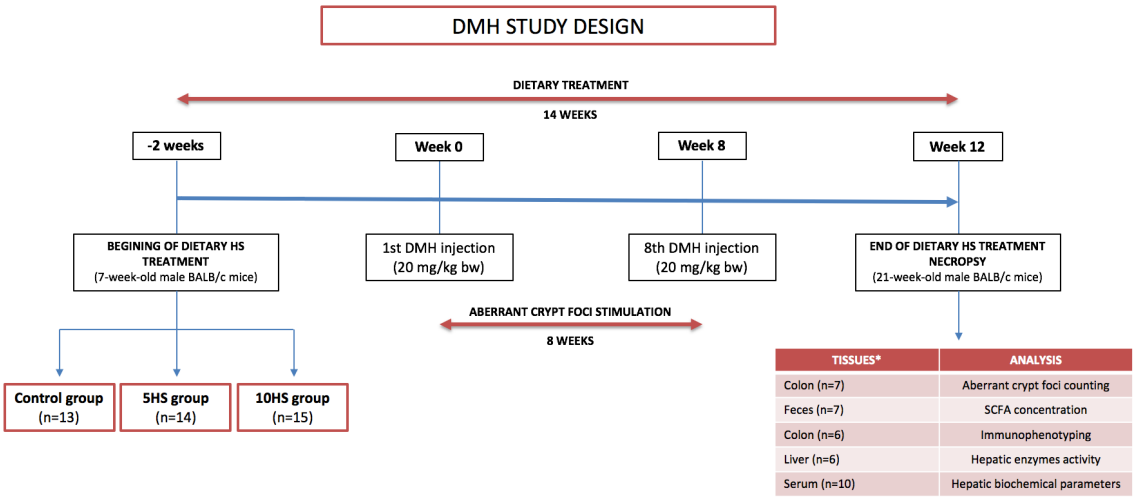


Figure 1. Experimental protocol and analysis for 1,2-dymethylhydrazine-induced colorectal carcinogenesis in male BALB/c mice supplemented with 5 or 10% dietary HS. * Number of animals randomly selected in each group.

2.6. Tissue Harvesting

Mice were anesthetized at the Experimental Nutrition lab with 3% isoflurane and blood was collected from the retro-orbital sinus. Mice were then euthanized by cervical dislocation. The entire colon was dissected, flushed with PBS buffer to remove luminal contents, cut opened along the mesenteric margin, and then fixed in karnovsky solution for 24 hours for ACF analysis. Fecal samples were collected one week before euthanasia and used for SCFA analysis. For immunophenotyping, after dissection, colon was flushed with cold PBS buffer (pH 7.2), sliced in small pieces, and incubated in DMEN medium (Sigma-aldrich™) for

90 minutes at 37°C. Meanwhile, livers were excised, weighed, immediately frozen in liquid nitrogen, and kept at −80°C until determination of hepatic enzymes activity.

2.7. Aberrant Crypt Foci Counts

Following fixation, flat colons were equally divided into three segments (proximal, medium, and distal) and stained with 0.1% methylene blue for fours minutes (Nutritional Biochemistry lab) to quantify aberrant crypt foci under a BX-60 light microscope (Olympus, Tokyo, Japan) with a magnification of 20X. Since ACF size is closely related to the risk of developing colon tumors (38), ACF were counted and categorized as containing up to three aberrant crypt focus (39).

2.8. Fecal SCFA Quantification

SCFA quantification was assessed according to Smiricky-Tjardeset (40) with some modifications. Briefly, 50 mg of frozen feces were weighted and thoroughly vortexed with deionized water (950 μL). During incubation on ice for 30 minutes, samples were homogenized every 5 minutes for 2 minutes. Samples were centrifuged (10,000 x g, 30 minutes, 4°C) three times and the supernatants were then collected. The final supernatant from each sample was filtered through a 0.45 μm membrane and transferred to vials. Acetic, propionic and butyric acids were measured at the Clinical Analysis lab by high performance liquid chromatography - HPLC (Shimadzu®) on an Aminex HPX 87H column (300 x 7,8 mm, Bio-rad®, Rio de Janeiro, Brazil) at 32°C with acidified water (0.005 M H_2SO_4) as eluent at a flow rate of 0.6 mL/minute. The products were detected and quantified by an ultraviolet detector (model SPD-20A VP) at 210 nm. Acetic, propionic, and butyric acid (SUPELCO®) standard curves were performed. Results are expressed as μmol SCFA/g feces.

2.9. Determination of Leukocytes by Immunophenotyping

Leukocytes were quantified and characterized in the large intestine mucosa as previously described (41) with some modifications. In brief, after DMEN medium

incubation for 90 minutes at 37°C, the suspension was centrifuged three times at 42 x g for 5 minutes and once again at 543 x g for 10 minutes. After each centrifugation the supernatant was pipetted and wasted. The remaining pellet was then resuspended with PBS buffer (100 μL, pH 7.2). Cell viability was assessed with Trypan blue exclusion and cells were counted in a Neubauer chamber. Leukocytes obtained were incubated with following antibodies according to the manufacturer's instructions: anti-CD4 (PeCy5), anti-CD25 FITC-conjugated, anti-CD196 or anti-CCR6 PE-conjugated, anti-CD49b or anti-PanNK APC-conjugated, anti-CD8 PECy7-conjugared (Biolegend, San Diego, CA, USA). Leucocytes ($1x10^4$ events) were acquired (FACSVerse™ and BD FACSuite software; BD Biosciences PharMingen San Jose, CA, USA) at the Microscopy and Microanalysis Center according to size (forward scatter) and granularity (side scatter). Single or two color staining was used to identify TCD4 lymphocytes ($CD4^+$), TCD8 lymphocytes ($CD8^+$), regulatory T cell ($CD4^+CD25^+$), Th17 lymphocytes (96^+) and *Natural Killer* cell ($CD49b^+$). Results are expressed as mean ± SD of the percentage of each antibody specific stained subpopulation within the gated cells.

2.10. Determination of Hepatic Enzyme Activity

Preparation of Liver Homogenate. Liver samples were weighed (150 mg) and properly homogenized in ice-cold EDTA-containing potassium phosphate buffer (1.5 mL, pH 7.4) using an Ultra-Turrax homogenizer (IKA T10 basic). The homogenate was centrifuged at 10,000 × g for 10 minutes, at 4°C. The supernatant was then pipetted into eppendorf tubes and used for further hepatic enzyme analyses. *Catalase Activity*. Hepatic catalase (CAT) activity was assessed at the Nutritional Biochemistry lab according to the method proposed by Aebi (42), where the hydrogen peroxide (H_2O_2) removal is periodically (0, 30, and 60 seconds) monitored using a Multiskan GO microplate spectrophotometer (Thermo Scientific) at 240 nm. CAT activity was normalized to protein content in the liver and expressed as units per mg protein (U CAT/mg protein). Protein content was determined as described by Lowry and co-workers (43) and bovine serum albumin

(1.6 mg/mL) was used as standard. *Superoxide Dismutase Activity*. Hepatic superoxide dismutase (SOD) activity was measured spectrophotometrically (Multiskan GO, Thermo Scientific) at 570 nm according to Dieterich and co-workers with minor modifications (44). Briefly, liver homogenate (30 μL) was pipetted into 96-well plates and mixed with EDTA-containing phosphate buffer (99 μL, pH 7.0) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (tetrazolium dye MTT, 5.5 g/L phosphate buffer, 6 μL). Pyrogallol (0.0125 g/L phosphate buffer, 15 μL) was then added and the plates were incubated for 5 minutes, at 37°C. Finally, dimethyl sulfoxide (DMSO, 150 μL) was used to stop reaction. EDTA-containing phosphate buffer (45 μL, pH 7) was used as blank. SOD activity was also normalized to the protein content in the liver and expressed as units per mg protein (U SOD/mg protein).

2.11. Hepatic Serum Markers

Serum markers of liver function, such as aspartate aminotransferase (AST) alanine aminotransferase (ALT), and gamma glutamyltransferase (GGT) were assessed at the Clinical Analysis lab by specific colorimetric assays (Bioclin®, Brazil) using a clinical chemistry analyzer BS-200 (Mindray®). Results are expressed as mean ± SD (U/L).

2.12. Statistical Analysis

The results were expressed as mean ± SEM. Data were analyzed using GraphPad Prism version 6.0. Mean values of the three groups (control, 5HS, and 10HS) were compared by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test. $P < 0.05$ was considered to be statistically significant.

3. Results and Discussion

3.1 Nutritional Composition of Hibiscus sabdariffa L. Calyces

In order to better characterize and provide valuable information on edible flowers, the general proximate nutritional composition was determined in HS calyces on a dry basis, as detailed in Table 2.

Table 2. Nutritional composition of *Hibiscus sabdariffa* L. calyces.

Nutritional Composition (g)	*Nutritional value per 100 g**
Crude protein	3.61 ± 0.26
Fat	0.66 ± 0.13
Total dietary fiber	9.22 ± 2.79
Soluble dietary fiber	1.76 ± 0.14
Insoluble dietary fiber	7.46 ± 2.64
Ash	2.69 ± 0.01
Carbohydrate	0.47 ± 0.32

* Data are expressed as mean ± SD from triplicate analyses.

3.1. Total Phenolic Compound Assessment

The amount of total phenolic compounds found in our study was 57.84 mg GAE/g dw HS. Although the content of polyphenols in plants is considerably influenced by cultivars, genetic factors, and environmental conditions (30), Borrás-Linares and co-workers (21) have observed similar total polyphenol content in HS extract (51 mg GAE/g dw HS). Interestingly, it is important to highlight that extraction-related variables, such as type of solvent, solvent ratio, temperature, and pH may contribute to differences in total phenolic content (45).

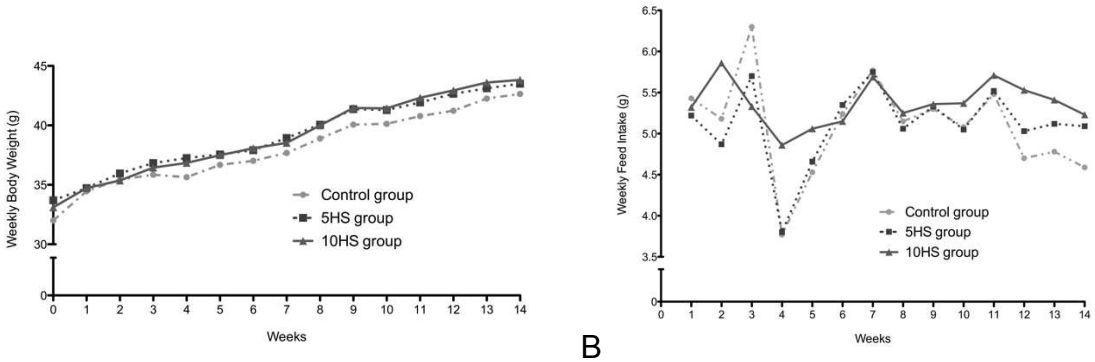
3.2. Quantification of Total Monomeric Anthocyanins

In our study, the total monomeric anthocyanin content was found to be 7.81 mg cyanidin-3 glucoside/g dw HS. The total monomeric anthocyanin content can be easily and accurately measured by the pH differential spectrophotometric method (46). It seems the amount of monomeric anthocyanins differs among different varieties (47). In a previous study (47), analysis of dried calyces from three HS varieties has revealed a range from 0.20 to 3.45 mg cyanidin-3 glucoside/g dw HS in the monomeric anthocyanin content. When compared to our results, although Gartaula and Karki (48) have reported lower amount of monomeric anthocyanin

(3.1 mg cyanidin-3 glucoside/g dw HS) detected spectrophotometrically, Abou-Arab and co-workers (49) have shown similar anthocyanin content (6.2 mg cyanidin-3 glucoside/g dw HS) by colorimetrically analysis.

3.3. Dietary HS Suppresses Aberrant Crypt Foci Development

There were no significant differences in body weight (Figure 2A) and feed intake among groups (Figure 2B).



A B

Figure 2. (A) Effects of HS dietary treatment on weekly body weight (g) in BALB/c mice. (B) Feed intake (g) during 14 weeks of HS dietary treatment.

ACF are initial recognizable premalignant lesions (39), which have been considered as a putative precursor to colorectal adenoma (50) and thus could be a useful biomarker for CRC (51). Table 3 shows the effects of supplemented diets containing either 5 or 10% dietary HS on DMH-induced ACF formation. Although ACF with higher number of aberrant crypts (ACF > 3) are more likely to progress to tumor during colorectal carcinogenesis (38), ACF > 3 were not found in our study, probably because this is a short-term study. Nevertheless, dietary treatment with 5 or 10% dietary HS for 14 weeks attenuated ACF≤ 3 development in the distal colon segment of male BALB/c mice ($P < 0.01$) by 34.5% and 47.9%, respectively. In addition, the total number of ACF per mouse was reduced by almost 29.0% in both HS supplemented groups when compared to control ($P < 0.01$). No differences were observed in the proximal and medial colon among groups. At our knowledge, this is the first study assessing the effects of dietary HS in DMH-induced colorectal tumorigenesis in male BALB/c mice. Accordingly, other

studies (52-54) have also reported protective effects of anthocyanins-rich food/extracts against drug-induced CRC tumorigenesis.

Table 3. Effects of supplemented diets containing either 5 or 10% dietary HS on DMH-induced ACF formation.

Colon segments	*Groups*		
	Control (n=7)	*5 HS (n=7)*	*10 HS (n=7)*
Proximal colon			
ACF ≤ 3	32.83 ± 13.19[a]	25.57 ± 8.94[a]	29,00 ± 8.12[a]
Medial colon			
ACF ≤ 3	30.00 ± 11.06[a]	21.29 ± 6.67[a]	23,57 ± 4.86[a]
Distal colon			
ACF ≤ 3	40.33 ± 5.31[a]	26.43 ± 8.87[b]	21,00 ± 5.85[b]
Total ACF ≤ 3*	103.2 ± 22.46[a]	73.29 ± 13.96[b]	73.57 ± 9.07[b]

Data are expressed as mean ± SD. Different letters in the same line mean statistical difference by the Tukey's multiple comparison post-hoc test ($P < 0.05$).
* Total number of ACF in colon segments (proximal, medial, and distal).

3.4. Dietary HS Increase Fecal SCFA Concentration

Figure 3 shows fecal concentrations in mice treated with 5 or 10% dietary HS. SCFA are key metabolites produced by the gut microbiota fermentation of undigested food substances, such as dietary fiber (55). The role of anthocyanin-rich food on gut microbiota modulation (56) and recently on fecal SCFA concentrations has been reported (19). Acetic acid has been implicated as anti-inflammatory and antitumor effectors via the modulation of Treg cells (57). Moreover, intracellular butyric and propionic acid, but not acetic acid, inhibit the activity of HDAC in colonocytes and immune cells, which in turn affects gene expression and cellular differentiation (58).

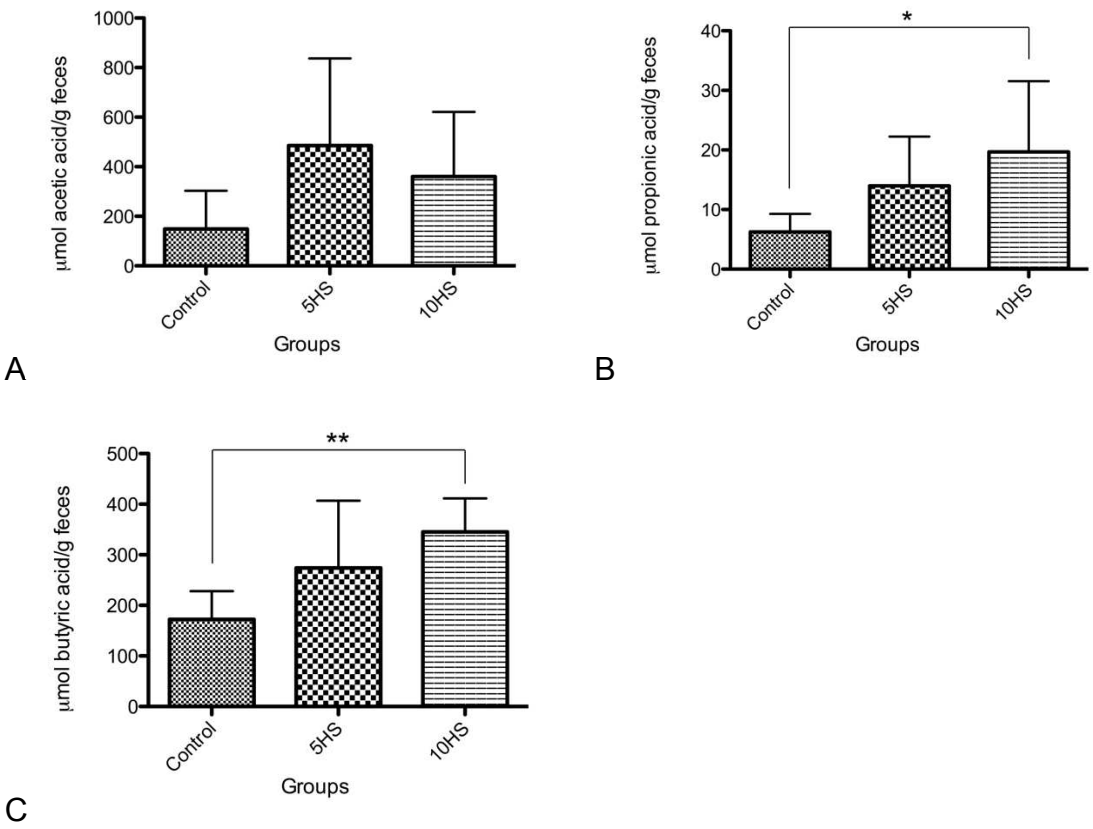


Figure 3. Fecal SCFA concentration (µmol SCFA/g feces) in DMH-induced colorectal carcinogenesis. (A) Supplemented diet with 5 or 10% dietary HS did not affect fecal acetic acid concentration. (B) 10% dietary HS supplemented diet increase fecal propionic acid concentration when compared to control diet. (C) Fecal butyric concentration is higher in BALB/c mice supplemented with 10% dietary HS when compared to controls. Data are expressed as mean ± SD (n=7). * Kruskal-Wallis followed by Dunn's multiple comparison test ($P < 0.05$). ** ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

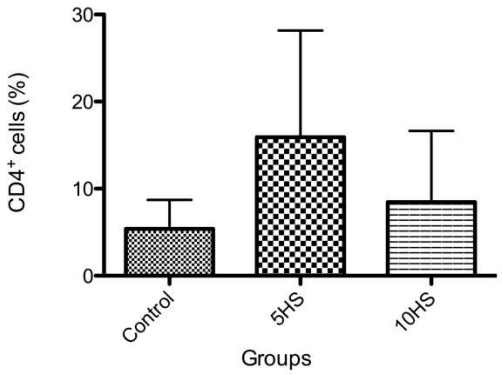
According to our results, diets supplemented with 5 or 10% HS did not affect fecal acetic acid concentration (Figure 3A). On the other hand, compared to controls, fecal propionic and butyric acid concentration were both enhanced ($P < 0.05$) with 10% dietary HS supplement diet (Figure 3B and 3C, respectively). Although the underlying molecular mechanisms are not fully understood, anthocyanins from black rice, black soybean, and purple corn have also induced an effective and positive increment in fecal butyric acid levels (19). Despite the lack of studies assessing the effects of anthocyanins or anthocyanin-rich food on fecal propionic acid concentration, anthocyanidins, i.e. anthocyanins with no sugar moiety

attached to the molecular structure (59), from strawberries were positively associated to the human fecal propionic acid content (60).

SCFA, mainly propionic and butyric acid, have an important influence on colorectal carcinogenesis *in vivo* (61). *In vitro*, such gut metabolites are also responsible to inhibit colon cancer cells growth and stimulate cell differentiation (62). Several molecular mechanisms have been proposed by the scientific community to explain the interplay between SCFA and CRC prevention (63). One of the mechanisms is related to stimulation of NK cells infiltration in the intestine mucosa.

3.5. Dietary HS Stimulates NK Cell Infiltration in the Colonic Mucosa

Figure 4 represents the percentage of leukocytes obtained from the large gut mucosa of BALB/c mice supplemented with 5% or 10% dietary HS. Our results have shown no differences in the percentage of CD4 cells (Figure 4A), CD8 cells (Figure 4B), Treg cells (Figure 4D), and Th17 cells (Figure 4E). However, 10% dietary HS supplemented diet increased NK cells infiltration in the large intestine mucosa when compared to either control or 5% dietary HS supplemented diets. Increased NK cells infiltration might partially explain why ACF counts were reduced in BALB/c mice treated with 10% dietary HS (Figure 4C). Numerous studies have found decreased NK cell function in cancer patients (64, 65). NK cells are cytotoxic lymphocytes involved in immunosurveillance against tumor formation (66). Besides releasing chemokines, NK cells can also express programmed cell death protein 1 (PD1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA4), which have been already implicated in cancer therapy (67). Furthermore, as we have previously demonstrated, BALB/c mice have shown higher fecal butyric acid concentrations when also supplemented with 10% dietary HS.



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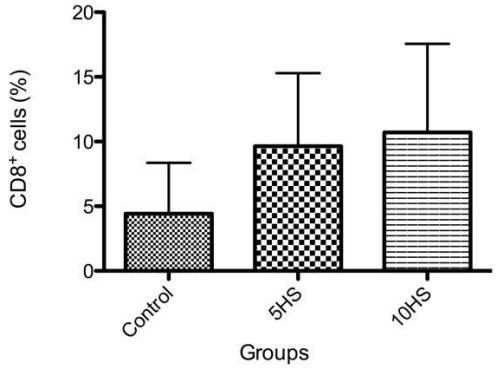

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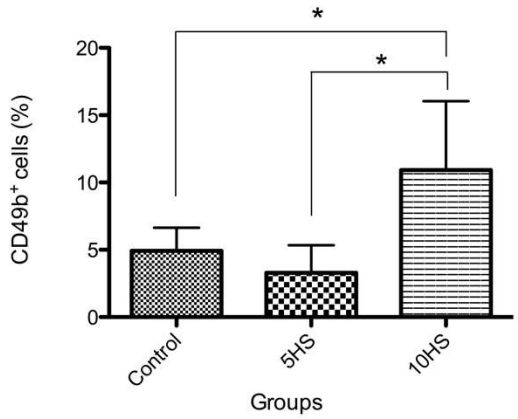

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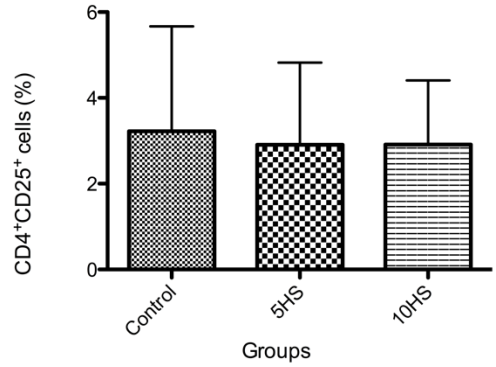

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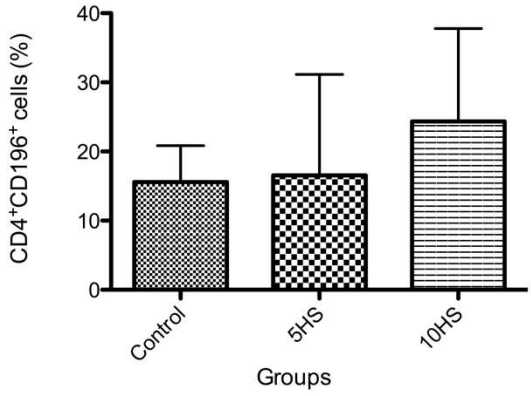

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Figure 4. Leukocytes obtained from the large gut mucosa of BALB/c mice supplemented with 5% or 10% dietary HS after 14 days of experiment. (A) Percentage of CD4; (B) Percentage of CD8; (C) Percentage of NK; (D) Percentage of Treg; (E) Percentage of Th17. Data are expressed as mean ± SD (n=6). * ANOVA followed by Tukey's multiple comparison test ($P < 0.01$).

The mechanisms how butyric acid may prevent ACF formation is possibly related to the activation of MHC class I–related chain A and B (MICA/B) receptors on the cancer cell surface by HDAC inhibitors, which in turn activates receptors expressed by NK cells, known as NK group 2, member D (NKG2D). Such receptors stimulate MICA/B expression apparently due to enhanced glycogen synthase kinase-3 (GSK-3) activation. The upcoming response is an enhanced NK cell responsiveness to kill colon tumor cells (8).

3.6. Dietary HS Provides No Hepatotoxic Effects

Since we have noticed that supplementation with 10% dietary HS induces effective changes in BALB/c mice metabolism towards cancer prevention, it is also of great scientific interest to demonstrate whether such dietary HS intake amount would cause harmful side effects on liver function. Anthocyanin-rich HS extracts have been hepatoprotective and helpful against diseases where oxidative stress is related to its etiopathogenesis, probably owing to its antioxidant and free radical scavenging effects (68). Antioxidant free radical scavenging enzymes, such as (CAT) and (SOD), play a key role on the first line of cellular antioxidant defense system against reactive oxygen species (ROS) (39). Overproduction of ROS, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH•), has been widely documented in the development and progression of overall non-transmissible diseases (69). SOD transforms the superoxide anion into hydrogen peroxide, which is then converted to water by CAT (70). Figure 5 shows the effects of dietary HS supplementation on hepatic CAT and SOD activities. According to our results, increased hepatic CAT activity (Figure 5A) was noticed in 10% dietary HS-treated mice when compared to control group ($P < 0.05$). No difference was noticed on hepatic SOD activity among groups (Figure 5B). Other studies have also corroborated the hepatoprotective and chemopreventive potential of increased CAT activity upon treatment with dietary HS (68, 71). The hepatoprotective effect may be also predicted by measuring the levels of liver serum marker enzymes, such as ASL, ALT, and GGT (72). Figure 6 shows the effects of dietary HS on serum marker enzymes.

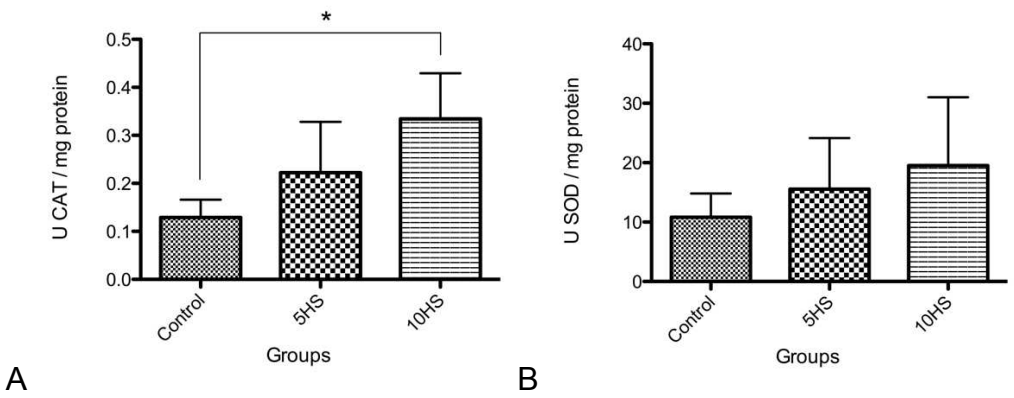

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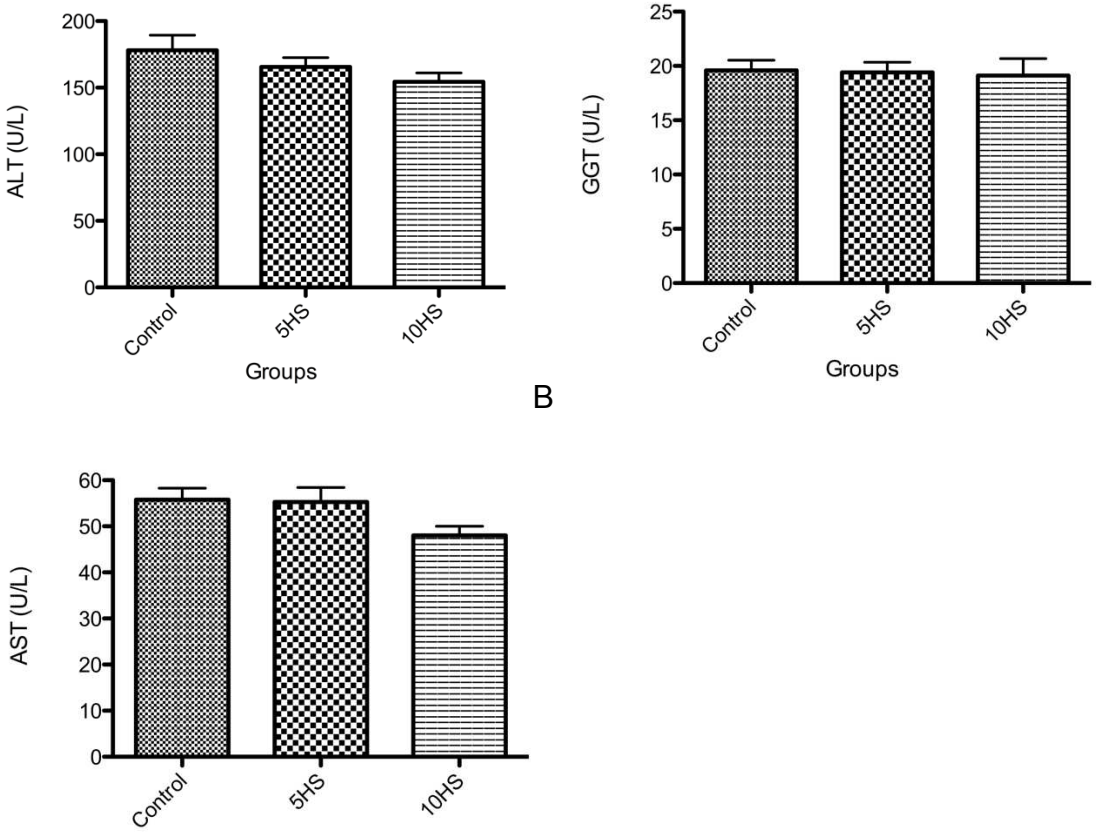
Figure 5. Effects of dietary HS supplementation on hepatic CAT and SOD activities. Data are expressed as mean ± SD (n=6). (A) Units of CAT per mg protein. (B) Units of SOD per mg protein. * $P < 0.05$.



A B

C

Figure 6. Effects of supplementation with dietary HS on serum marker enzymes. (A) Alanine aminotransferase – ALT (U/L); (B) Gamma glutamyltransferase (U/L); (C) Aspartate aminotransferase – AST (U/L). Data are expressed as mean ± SD (n=10). No significant differences by the Tukey's multiple comparison test.

In our study, BALB/C mice supplemented with dietary HS did not ameliorate ALT (Figure 6A) or GGT (Figure 6B) when compared to controls. Albeit we have observed no statistically differences (P = 0.06) on AST serum levels among groups (Figure 6C), we might consider that BALB/c mice supplemented with 10% dietary HS (48 ± 6.46 U/L) do exhibit a physiological and important reduction on such parameter when compared to controls (55.8 ± 7.80 U/L), which might also help liver protection. Lower levels of hepatic enzymes upon treatment with anthocyanin-rich extracts have been reported *in vivo*, thus conferring hepatoprotective effects (73). No studies assessing the effects of dietary HS on serum liver parameters were found.

4. Conclusions

Diet supplemented with 10% dietary HS prevent colorectal ACF formation possibly owing to its anthocyanin content, which might positively modulate HDAC inhibitors, such as butyric and propionic acids, whose response leads to NK cell infiltration in the large intestine. Dietary HS has provided hepatoprotective effects by stimulating the hepatic antioxidant enzyme system, such as catalase activity, along with a tendency to reduce the serum levels of aspartate aminotransferase.

It is worth to mention that the beneficial and protective effects above reported in male BALB/c mice consuming a diet supplemented with 10% dietary HS might also be observed in humans if 75 g of dietary HS is daily intake (human equivalent dose). Thus, our group is now intrigued to further reveal whether the potential of dietary HS to prevent preneoplastic lesions development is indeed caused by the upregulation of related proteins, such as HDAC, MICA/B, NKG2D and GSK-3.

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4. GENERAL CONCLUSIONS

Anthocyanins/anthocyanidins has effectively contributed to mitigate colorectal carcinogenesis in both *in vivo* and *in vitro* experiments through several molecular mechanisms. The intake of green pea, as a source of dietary fiber and polyphenols, might confer protective effects against inflammatory bowel disease, such as ulcerative colitis, by suppressing inflammation, mucin depletion and endoplasmic reticulum stress in the colon. In addition, we may infer that dietary HS intake reduces preneoplastic lesions development as a result of increased fecal butyric and propionic acids concentrations and NK cell infiltration, thus preventing colorectal carcinogenesis. More studies are still needed to determine the effects of dietary green pea or HS supplementation on intestinal diseases in humans.