Hibiscus (*Hibiscus sabdariffa* L.) supplementation increases butyrate synthesis and reduces inflammatory cells, attenuating the formation of aberrant crypt foci in BALB/c mice induced to pre-neoplastic lesions

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This peer-reviewed article has been accepted for publication but not yet copyedited or typeset, and so may be subject to change during the production process. The article is considered published and may be cited using its DOI

10.1017/S0007114522001222

The British Journal of Nutrition is published by Cambridge University Press on behalf of The Nutrition Society
Abstract

The development of colorectal cancer involves some morphological changes, and in the initial stage, pre-neoplastic lesions called aberrant crypt foci (ACF) appear. Thus, an intervention with sources of bioactive compounds such as Hibiscus sabdariffa L., rich in phenolic compounds and anthocyanins, could attenuate the risk of developing these lesions due to its antioxidant, anti-inflammatory and anti-proliferative properties. Therefore, the aim of this study was to evaluate the effects of 5% and 10% supplementation of dehydrated H. sabdariffa calyces (DHSC) during the development of 1,2-dimethylhydrazine-induced preneoplastic lesions in male BALB/c mice. The characterization of DHSC was carried out. The in vivo experiment lasted 12 weeks, and the animals were randomly divided into 3 experimental groups: the control group (CON) and the supplemented groups with 5% DHSC and 10% DHSC. The activities of liver enzymes catalase and superoxide dismutase were determined. In addition, ACF, short chain fatty acids (SCFA), presence of inflammatory infiltrates, goblet cells and leukocytes in the colonic mucosa were quantified. There was a significant reduction in ACF and the presence of inflammatory infiltrates in the colon of animals in groups 5DHSC and 10DHSC. In addition, the 10DHSC group showed an increase in the activity of the catalase enzyme, in the production of butyrate and in the presence of NK cells in the colon, in addition to more hypertrophied goblet cells. Based on these findings, it is suggested that DHSC supplementation may be recommended to attenuate cellular responses in the early stage of preneoplastic lesions.

Keywords: phenolic compounds, anthocyanins, antioxidants, pre-neoplastic lesions.
Accepted manuscript

Introduction

Colorectal cancer (CRC) is the third most common type of cancer and the second with the highest mortality rate among men and women worldwide, and in Brazil it is the second with the highest incidence accompanied by high mortality rates\(^{(1,2)}\). CRC is a neoplasm that includes morphological and pathological changes that affect the entire segment of the large intestine, and this process involves the appearance of pre-neoplastic lesions called aberrant crypt foci (ACF)\(^{(3-5)}\). ACF can be identified microscopically on the surface of the entire colonic mucosa, being considered a biomarker for evaluating the progression of CRC\(^{(6)}\).

Colorectal carcinogenesis can be influenced by genetic factors and modifiable factors such as food intake, in which a greater consumption of processed and ultra-processed foods and a reduction in fresh foods is observed\(^{(1,3)}\). In this context, sources of bioactive compounds with antioxidant, anti-inflammatory, anti-carcinogenic properties, among others, are of great scientific interest, as they are related to reducing the risk of developing chronic diseases such as cancer. Among the sources of bioactive compounds, the Hibiscus sabdariffa L. (H. sabdariffa) of the Malvaceae family stands out, commonly known as hibiscus, roselia, sorrel or sour okra, and widely cultivated in Africa, Southeast Asia, and some countries in America. It is consumed in the form of tea, jellies, fermented products, among others, due to its considerable concentration of phenolic compounds and anthocyanins\(^{(7-9)}\).

Plant derived phenolic compounds include simple phenols, phenolic acids, tannins, flavonoids, among others\(^{(10)}\). Anthocyanins belong to the group of flavonoids and are the pigments responsible for the coloration of H. sabdariffa. These compounds play anti-inflammatory, anti-proliferative and hepatoprotective functions, in addition to a powerful antioxidant capacity, acting in the neutralization of free radicals, reducing the risk of damage to the DNA molecule, and the development of diseases or injuries associated with oxidative stress\(^{(11-14)}\).

Given the information above, phenolic compounds and total anthocyanins from DHSC can be used to reduce oxidative stress, the inflammatory state, and consequently to suppress the carcinogenesis process\(^{(15-18)}\). Therefore, the aim of the study was to evaluate the effects of supplementation of 5% and 10% of DHSC during the development of pre-neoplastic lesions induced by 1,2-dimethylhydrazine (DMH) in BALB/c mice.
Methodology

Sample acquisition and preparation

Ten kg of *H. sabdariffa* calyces were originally obtained from a producer in Coimbra - Minas Gerais (latitude: -20,830,127, Longitude: -42,801,351) and purchased from a local market in Viçosa - Minas Gerais. *H. sabdariffa* calyces were then pulverized in a knife mill and stored at -20 °C.

DHSC characterization

Centesimal composition and dietary fiber

The determination of the proximate composition of the DHSC followed the methodologies proposed by the Association of Official Analytical Chemists – AOAC (19). Proteins were determined by the Kjeldahl method. Lipids were quantified by extraction with ethyl ether in Soxhlet. Ashes were determined (fixed mineral residue) by a gravimetric process. The total fiber content was quantified by the gravimetric-enzymatic method. Finally, carbohydrates were calculated by difference [100 – (moisture + protein + lipids + fiber + ash)].

Total phenolic compounds, total anthocyanins and antioxidant activity

The extraction of total phenolic compounds was performed according to Borrás-Linares et al. (8), in which 20 grams of DHSC was macerated in 100 mL of extraction solution (70% ethanol acidified with 0.1% HCl) at a ratio of 1:10 (p/v). The extraction took place for 24 hours, at refrigeration temperature (8 ± 2 °C). Finally, the extract was filtered and concentrated in a rotary evaporator at 40 °C.

The determination of total phenolic compounds was performed according to the Folin-Ciocateu colorimetric method, and the results expressed in mg of Gallic Acid Equivalents / 100 grams of sample (20). Total anthocyanins were quantified by the differential pH method, and the results expressed in mg of cyanidin-3-glucoside/100 grams of sample (21).

The scavenging capacity of the DPPH radical was determined using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) as previously described (8,22). The antioxidant activity of DHSC was defined using a trolox standard curve, and the result expressed as μmol equivalent of trolox per gram of DHSC.
Experimental design

Forty-five male BALB/c mice, seven weeks old, initial weight between 20 - 40 grams, purchased from the Animal Facility of the Biological Sciences Center of the Universidade Federal de Viçosa were used. Animal protocol was approved by the Ethics Committee on Animal Experimentation from the Universidade Federal de Viçosa, Brazil, under the process number 10/2017. BALB/c mice were used because they develop neoplasms more easily, in addition, male mice were defined due to the hormonal variability of females. The sample calculation was performed according to Mera, Thompson and Prasad (23) who consider the effect of treatment between the experimental groups on the outcome variables of interest. The animals were housed in collective cages, with controlled temperature (22 ± 2 °C), 12-hour photoperiod and ad libitum access to water and feed. After two weeks of acclimatization, the animals were randomized according to body weight and distributed in ascending order in the cages in three experimental groups: control group - CON (diet AIN-93M, American Institute of Nutrition for Maintenance, n = 15), group 5DHSC (diet AIN - 93M + 5% DHSC, n = 15) and the 10DHSC group (diet AIN-93M + 10% DHSC, n = 15) (Table 1). The 5% and 10% DHSC supplementation was defined in order to provide about 100 - 200 mg of anthocyanins. Such amounts can be incorporated into the human diet. The difference in the average weights between the animals was not greater than 5% and the cages were properly identified. The experimental period was 12 weeks, and during the first eight weeks all animals received an intraperitoneal injection in a single dose per week of the drug DMH (20 mg / kg of body weight) for induction of pre-neoplastic lesions.

At the end of the experimental period, the mice were fasted for 12 hours, then anesthetized in the Experimental Nutrition Laboratory with 3% isoflurane (Cristália®, Brazil), followed by total exsanguination through the cervical retro-orbital sinus. The entire colon was dissected, washed with PBS buffer to remove luminal content, cut along the mesenteric margin and then fixed in Karnovsky's solution for 24 hours for ACF analysis. Fecal samples were collected one week before euthanasia and used for SCFA analysis. For immunophenotyping, after dissection, the colon was washed with cold PBS buffer (pH 7.2), cut into small pieces and incubated in DMEN medium (Sigma-aldrich™) for 90 minutes at 37 °C. Another part of the colon was fixed in Carson's formalin solution for 24 hours for histological analyses. Livers were excised, weighed, immediately frozen in liquid nitrogen and stored at -80 °C until liver enzyme activity was determined.
Antioxidant Liver Enzymes and Serum Markers

The activity of liver antioxidant enzymes was determined in liver homogenate. Catalase (CAT) concentration was determined according to Aebi (24), and superoxide dismutase (SOD) concentration was determined based on the ability of this enzyme to reduce pyrogallol auto-oxidation (25). All readings were performed in a spectrophotometer (Thermo Scientific®, model Multiskan GO, Vantaa, Finland), and data were expressed in units (U) / mg protein. The determination of the concentration of proteins resident in the liver tissue used in the CAT and SOD analyzes was carried out according to Lowry et al. (26). Readings were performed in a spectrophotometer (Thermo Scientific®, model Multiskan GO, Vantaa, Finland) at a wavelength of 700 nm.

Colonic ACF score

The colon was measured and divided into three equidistant segments, identified as proximal, medial, and distal in relation to the cecum. To count the ACF, the colon segments were stained with 0.1% methylene blue solution for two minutes. Counting was performed with the aid of an optical microscope (Olympus America InC., Model CBA, Pennsylvania, USA) at 100x magnification by two trained double-blind observers. The categorization of ACF was performed based on the number of aberrant crypts per focus, defined as foci with more than three (ACF > 3) or less than three aberrant crypts (ACF ≤ 3) (27).

Quantification of Fecal Short Chain Fatty Acids (SCFA)

SCFA extraction and quantification followed the Smiricky-Tjardeset et al. (28) and Marcon et al. (29). Acetic and butyric acids were detected and quantified by an ultraviolet detector (model SPD-20A VP) at 210 nm. Results were expressed as µmol SCFA / g of feces.

Histological analyzes in the colon

The colon fragments were fixed in Carson's formalin and subsequently dehydrated in increasing concentrations of ethanol and cleared with xylene and embedded in paraffin (Sigma Aldrich®, St. Louis, USA). 5 µm-thick cross sections were obtained on a rotating microtome (Olympus America InC., Model CUT 4055, Pennsylvania, USA). The slides were stained with hematoxylin and eosin (HE) to verify the presence of inflammatory infiltrates. For analysis of goblet cells, slides were stained with Alcian Blue (AB, pH 2.5) and periodic
acid Schiff (PAS). All slides underwent a pre- and post-staining step and were then analyzed under an optical microscope (40x) (Leica Microsystems®, Inc.).

The counting of inflammatory infiltrates and the determination of goblet cells were performed with the aid of image analysis software (Image Pro Plus 4.5, Media Cybernetcs Inc, Rockville, USA).

**Determination of leukocytes in the colon mucosa by immunophenotyping**

Leukocytes were quantified and characterized in the colon mucosa as previously described Belkaid, Jouin e Milon (30) and Marcon et al. (29). The colon was removed and washed in ice-cold PBS, cut into small fragments, and incubated in cell culture medium, DMEM, pH 7.2 (Sigma-aldrich™) for 90 minutes at 37°C. Thereafter, the suspension was centrifuged three times at 42 g for 5 minutes and the supernatant removed. Then it was centrifuged again at 543 g for 10 minutes. Afterwards, the remaining sediment was resuspended with PBS buffer (100 µL, pH 7.2). Cell viability was assessed using Trypan blue dye and, after staining, the cells were counted in a Neubauer chamber. The leukocytes obtained were incubated with the following antibodies, according to the manufacturer's instructions: anti-CD4 (PeCy5), anti-CD25 FITC-conjugate, anti-CD196 (anti-CRC6) PE-conjugate, anti-CD49b (anti- PanNK) APC-conjugated, PECy7-conjugated anti-CD8 (Biolegend, San Diego, CA, USA). Leukocytes (1x10⁴ events) were acquired (FACSVers™ and BD FACSuite software; BD Biosciences PharMingen San Jose, CA, USA) according to size (direct scatter) and granularity (side scatter). One or two stains were used to identify CD4 T lymphocytes (CD4+), CD8 T lymphocytes (CD8+), regulatory T cells (CD4+ CD25+), Th17 lymphocytes (CD4+ CD196+) and Natural Killer cells (CD49b +). Results are expressed as mean ± SD of the percentage of each subpopulation stained with the specific antibody within the blocked cells.

**Statistical analysis**

Results are presented as mean ± standard deviation. The means of the three groups (CON, 5DHSC and 10DHSC) were compared to each other by means of analysis of variance (ANOVA) complemented with Tukey's test, to verify the difference between the control group and the groups supplemented with *H. sabdariffa*. A significance level of 5% (p < 0.05) was considered. Data were analyzed using Graph Pad Prism 5 software, version 5.01.
Results and discussion

Centesimal characterization, dietary fiber, and phenolic compounds of DHSC

The chemical composition of the *H. sabdariffa* calyces in the present work showed a predominance of total dietary fiber, with the highest concentration of insoluble fiber, followed by proteins, ash, total phenolic compounds, lipids, carbohydrates, and anthocyanins (Table 2). Differences in the chemical composition of *H. sabdariffa* calyces between different studies are common and attributed to factors such as different cultivars, planting conditions, storage, climate type, soil type, harvest time, among other factors \(^{(8,9,31)}\).

Differently of our results, Jabuer et al. \(^{(32)}\), after to analyze the proximate composition of DHSC was observed higher concentrations of carbohydrates, followed by ash, proteins, and lipids. Regarding dietary fiber, in the present study, the concentration of soluble and insoluble fibers in the DHSC was higher than that reported by Kalla et al. \(^{(33)}\), who found a total dietary fiber concentration of 6.15 g / 100 g, with 5.86 g / 100 g of insoluble fiber and 0.29 g / 100 g of soluble fiber. However, higher concentrations of dietary fiber were reported by Sáyago-Ayerdi et al. \(^{(31)}\), with a total dietary fiber concentration ranging from 36 to 39 g / 100g, in which soluble fiber ranged from 7.06 to 8.32 g / 100 g and insoluble fiber from 13.0 to 17.08 g / 100 g among different cultivars of *H. sabdariffa* calyces whole flower stems analyzed in the study.

As for bioactive compounds, the concentration of total phenolic compounds in the present work was lower than that found by Maciel et al. \(^{(9)}\) with a total concentration of phenolic compounds of 1.71 g in 100 grams, but the value of anthocyanins was like our study, with a concentration of 0.24 g in 100 grams. The antioxidant activity of DHSC in the present study was similar to that reported by Borrás-Linares et al. \(^{(8)}\), with a concentration ranging from 27.4 to 112 µmol of trolox / gram of dehydrated calyx. The therapeutic potential of anthocyanidins already was demonstrated by Hemmati et al. \(^{(34)}\) and Casao et al. \(^{(35)}\), which relate to the action of this compound in inflammatory diseases due to its antioxidant and anti-inflammatory properties. It indicates that its therapeutic action is probably related to the high level of phenolic compounds associated with Anthocyanins.

Body weight and food intake of mice

Body weight did not differ between the groups (Figure 2-A). However, considering the total food consumption per week (Figure 2-B), all groups showed a reduction in diet
intake during the second, third, and fourth experimental weeks. The control and the 5DHSC groups presented lower food intake compared to the 10DHSC group between the second and fourth week. Interestingly, such decrement in food consumption matches the beginning of DMH protocol. After the fifth week, no difference in food consumption was observed.

According to the average DHSC consumption of the animals, the 5DHSC group consumed 250 mg of DHSC/day which provided 23.05 mg of total dietary fiber, with 4.4 mg of soluble fiber and 18.65 of insoluble fiber, in addition to 2.73 mg of total phenolic compounds and 0.55 mg of total anthocyanins. In contrast, the 10DHSC group was supplemented with twice the amount of DHSC in the diet, and this group consumed 530 mg of DHSC/day containing 48.87 mg of total dietary fiber, with 9.33 mg of soluble fiber and 39.54 mg of insoluble fiber, in addition to 5.77 mg of total phenolic compounds and 1.17 mg of total anthocyanins. After, it was possible to calculate the quantity of DHSC possibly ingested by humans. This amount was determined through the method of normalization of the body surface area considering the mice’s diet consumption. According to the calculations of Reagan-Shaw, Nihal and Ahmad (36), considering the average weight of an adult to be 70 kg, the equivalent ingestion of DHSC for a human would be approximately 36 g/day (5DHSC) and 75 g/day (10DHSC) incorporated in preparations such as cakes, jams, ice creams, and yogurts, for example (8,9).

**Liver enzyme activity and serum markers in serum**

No significant differences in SOD enzyme activity were observed between the experimental groups (Table 3). However, there was an increase in CAT enzyme activity in the group supplemented with 10% DHSC. The drug DMH is a powerful carcinogen that induces oxidative stress, in addition to hepatotoxicity through its hepatic metabolism (37,38). Therefore, phenolic compounds and anthocyanins, due to their antioxidant properties, can help reduce the risk of oxidative damage, in addition to contributing to hepatoprotective effects by increasing the activity of the antioxidant enzymes CAT and SOD as shown in the literature (39-42).

These enzymes are extremely important in the fight against the generation of free radicals within the cell. When the tissue is DMH exposure, occur the generation of reactive oxygen species (ROS) usually increases, which changes the lipids, protein, and DNA of the cells, causing tissue stress and decreased cellular function (34,43). Therefore, the formation of these radical species is controlled by the antioxidant enzymes, and it is desirable that occur
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the increased of these enzymes to protect the tissue from the activity of these radicals. We believed that the increase in the antioxidant system was stimulated by 10% DHSC was important to reduce oxidative damage and, consequently, to reduce the risk of liver disease. The mechanism of action of antioxidant enzymes begins with the action of the SOD enzyme, which converts the superoxide anion into hydrogen peroxide, then, by the action of the CAT enzyme, this peroxide is transformed into water, reducing oxidative damage \(^{(37,44)}\).

**Effect of DHSC on the development of ACF in the colon**

In the present study, it was observed that, in the proximal and medial segments of the colon, there was no difference in the number of ACF between the groups (Table 4). However, in the distal portion of the colon, the groups supplemented with 5% or 10% of DHSC demonstrated a reduction of 34% and 48%, respectively, in ACF compared to the control group. Still, considering the entire portion of the tissue (proximal, medial, and distal), there is a reduction in ACF in the 5% and 10% DHSC groups compared to the control group. Similarly, studies in the literature have shown that phenolic compounds are able to attenuate the formation of ACF in animals induced to CRC, reducing the risk of progression of the carcinogenesis process \(^{(39,45-47)}\). ACFs can be observed in Figure S1 in the supplementary material.

Have been reported that DMH exposure causes deep modifications in tissue associated with inflammation and oxidative stress and consequently promotes intestinal crypt proliferation, and increase of ammonia concentration, which can damage healthy cells. The lesions induced by this drug, present epithelial origin, and histologic, morphologic, and anatomic characteristics considered highly reproducible for CRC studies. Furthermore, they are highly specific, leading to the initiation and promotion of carcinogenesis in a dose-dependent manner \(^{(6,48)}\).

In our study, we believe that the reduction in ACF after DHSC exposure occurred because the DHSC interferes in the inflammatory and oxidative process, as well as promotes a regression in the morphologic alteration imposed by the carcinogenic process. Corroborating with our study, Chewonarin et al. \(^{(49)}\), showed that chemopreventive effects are more effective in the early stages, in which the *H. sabdariffa* calyces extract reduced the formation of ACF in the early stage of carcinogenesis, but in the post-initiation stage the group treated with *H. sabdariffa* showed a formation of ACF similar to the untreated group, especially the ACF that had four or more crypts / focus. Phenolic compounds in general
contribute to attenuate the progression of colorectal carcinogenesis acting as natural chemopreventives, promoting inhibitory effects on the colorectal carcinogenesis process (50-52).

It is noteworthy that ACF with more than 3 aberrant crypts per focus (ACF > 3) are more likely to progress to tumor during colorectal carcinogenesis (52). In the present study, no ACF > 3 crypts / focus were found. It is suggested that this result is associated with the antioxidant properties of phenolic compounds and anthocyanins present in DHSC, since the increase in antioxidant defenses helps to reduce the risk of developing pre-neoplastic lesions.

Effects of DHSC supplementation on butyrate production

SCFA (acetic, butyric, and propionic acids) are the main metabolites produced by the fermentation of dietary fibers through the bacteria that make up the intestinal microbiota (53). It was observed that the concentration of acetic and butyric acid varied between the experimental groups (Figure 3-A,B). The diet supplemented with 5% or 10% DHSC did not significantly influence the concentration of fecal acetic acid (Figure 3-A), however, butyric acid was higher in the 10 DHSC group compared to the control group (Figure 3-B). Among the experimental weeks, a reduction in butyric acid is noted in the group supplemented with 10% DHSC from the 1st to the 12th week. However, it should be noted that all fatty acids showed a reduction in their concentrations from the beginning to the end of the experimental period, but this reduction, even if significant, was smaller in the 10DHSC group compared to the control and 5DHSC groups.

According to Liu et al. (54), animals treated with myrityl extract rich in anthocyanins showed a high representation of Lachnospiraceae and Ruminococcaceae in the microbiota, in which the presence of members of these families is considered an important producer of butyric acid. This result was consistent with the increase in butyric acid concentration in the supplemented group. These changes in the structure of the intestinal microbiome of animals treated with anthocyanins contribute to increased SCFA production, especially butyric acid.

The ingestion of dietary fiber and its fermentation by the intestinal microbiota increases SCFA production. Fiber intake significantly alters the microbiota population, increasing SCFA-producing bacteria, their metabolite levels, and may play a protective role in colon cancer (55). Therefore, it is suggested in the present study that such increase in butyric acid concentration in the 10DHSC group can be attributed to the higher consumption of DHSC and, consequently, fiber, phenolic compounds and anthocyanins. Acetic acid is
absorbed in the colonic epithelium, transported and metabolized in the liver where it can be used by some ways such as lipogenesis, ketogenesis, production of cholesterol, glutamine and glutamate. Another part of the acetic acid reaches the circulation, is captured and oxidized by the muscle for energy production (56). Butyric acid, however, is absorbed in the colonic epithelium, so its bioavailability is mainly restricted to the colon. It is later metabolized to produce ATP, providing 70% of energy to colonocytes, in addition to playing other roles in maintaining colon homeostasis and epithelial integrity (56-59).

Butyric acid can exert immunomodulatory effects, such as the inhibition of histone deacetylases (HDAC), by inhibiting the activity of HDCA, butyric acid induces the expression of the p21^{Waf1/Cip1} gene, which can interrupt cell proliferation (57,59). The anti-inflammatory mechanisms of butyric acid involve the suppression of activation of the nuclear factor kappa-β (NF-Kβ). The inhibition of NF-Kβ by butyric acid can result in the reduction of myoperoxidacies, COX-2, adhesion molecules and pro-inflammatory cytokines. In addition, butyric acid can inhibit interferon-γ production and regulate peroxisome proliferator activated receptor γ (PPARγ) signaling. Activation of this receptor on colonic epithelial cells can inhibit the production of inflammatory cytokines, promoting anti-inflammatory effects (56).

Intake of fiber and/or polyphenols has been associated with reduced inflammation and inflammatory diseases due to the production of metabolites from polyphenols (valerolactones, aromatics, among others) and fiber (SCFAs) (60,61). Therefore, the increased production of butyric acid in the 10DHSC group in the present study may be associated with a reduction of ACF and the presence of inflammatory infiltrates in this group.

**Histological analyzes of the distal colon**

The groups treated with 5% and 10% DHSC in the diet showed a significant reduction in the percentage of inflammatory infiltrates compared to the animals in the control group (Figure 4).

Possibly, the phenolic compounds present in the DHSC showed antioxidant and anti-inflammatory properties during the development of pre-neoplastic lesions. In our study, the inflammation observed in Control group probably is related to DMH exposure, since already been known that this drugs causes oxidative stress through the methylation of epithelial cell biomolecules resulting in an inflammatory state that contributes to the progression of these lesions (62). To better understand the inflammatory process that occurred, it is known that, after the appearance of a tissue injury, the recruitment of inflammatory cells to the injury site
begins. The infiltration and enhanced activation of inflammatory cells in the injured tissue can initiate and promote the progression of the carcinogenesis process, since these inflammatory cells can produce inflammatory cytokines, several of which play crucial roles during this process (63-65). Furthermore, it is noteworthy that with the progression of the inflammatory process, the intestinal mucosa is exposed to oxidative processes, which contributes to a greater risk of transformation of normal cells into malignant ones (66). We believed that the reduction in inflammatory infiltrates after DHSC exposure occurred due to the presence of phenolic compounds and anthocyanins may involve some mechanisms such as, the neutralization of free radicals, regulation of pro-inflammatory compounds and inflammation-associated cells (67). Thus, the antioxidants present in the DHSC may be able to alleviate the damage to the colon caused by free radicals, with less infiltration of inflammatory cells in the tissue of the treated animals.

In relation to the goblet cells, based on area and diameter values (Figure 5-A,B), the group supplemented with 10% DHSC had more hypertrophied goblet cells compared to the CON group. In addition, the CON group demonstrated a reduction in the volume density of goblet cells per tissue area, that is, a depletion of these cells in relation to the 10DHSC group (Figure 5-C). The mechanisms that could explain this hypertrophy is that after the development of tissue inflammation, the immune system induces goblet cells to extensive mucus release to remove intruders, making them larger due to increased mucus production (68,69).

Bioactive compounds can help maintain the integrity of goblet cells in the colon as reported by Almagrami et al. (70), in which the authors observed that animals in the control group induced to CCR with Azoxymethane showed a significant reduction in the size of goblet cells and a depletion of mucin. On the other hand, the group of animals treated with Acanthus ilicifolius Linn in the diet (250 mg / kg of body weight) showed a slight reduction in these cells. Acanthus ilicifolius Linn is a prickly herb that is a source of flavonoids, terpenes, and alkaloids. Furthermore, Rehman et al. (71) demonstrated that prophylactic treatment for 14 days with the flavonone miracetin (25 and 50 mg / Kg of body weight) during cisplatin-induced colon toxicity was able to maintain the integrity of the goblet cells in addition to preventing the mucin depletion. Goblet cells perform necessary functions to combat oxidative damage, inflammatory disorders and intestinal infections, in addition to being part of the innate immune system of the mucosa to defend the host against possible pathogens (72).
Determination of the presence of leukocytes in the colonic mucosa

No significant differences were observed in the percentage of CD4, CD8, Th17 and Treg cells between groups (Figure 6-A,C,D,E). However, the group that was supplemented with 10% DHSC showed an increase in NK cell infiltration into the colonic mucosa (Figure 6-B).

NK cells are cytotoxic lymphocytes that play a role in immune defense. These cells through class I major histocompatibility complex (MHC) can distinguish between normal and altered cells, and can lyse malignant cells, acting against the formation and development of tumors (73,74).

Unlike normal cells, pre-neoplastic cells express specific receptors on the cell surface. These receptors are recognized by NK cells and then the immune response is initiated, through the release of granules containing perforin, a protein that breaks the cell membrane and induces cell apoptosis (75). Cell death mediated by NK cells may be more effective in the early stage of differentiation of carcinogenic cells (76). Thus, the greater infiltration of NK cells in the colonic mucosa of animals in the 10DHSC group may be associated with the reduction in ACF that was observed in this same group of animals.

Conclusion

Supplementation with 5% and 10% of DHSC promoted a significant reduction in ACF, and this result is consistent with the reduction in the presence of inflammatory infiltrates in these groups. Supplementation with 10% DHSC was more effective, as in addition to these changes in ACF and inflammatory infiltrates, there was an increase in hepatic CAT enzyme activity, butyrate production and a greater infiltration of NK cells in the colon. Therefore, it is suggested that the consumption of DHSC attenuates cellular responses in the early stage of development of pre-neoplastic lesions. Intervention in the initial phase is relevant as it can help reduce the risk of progression of the carcinogenesis process. Furthermore, oxidative stress can be attenuated due to the potential antioxidant capacity of phenolic compounds and anthocyanins present in the DHSC included in the diet. The reduction of the oxidative process is another important factor to reduce the risk of developing pre-neoplastic lesions.
Interest conflicts:

The authors declare that there are no conflicts of interest.

Acknowledgments:

This study was supported by the Coordination for the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) Brazil, the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ), the Fundação de Amparo à Pesquisa of the State of Minas Gerais (FAPEMIG) and the Universidade Federal de Viçosa (UFV). All authors contributed significantly to the manuscript.

Supplementary Materials:

Complete checklist according to ARRIVE guidelines and image of aberrant crypt foci. Study data are available upon request to the author.
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Figure 1. Flow diagram of the study steps.
Figure 2. Effect of DHSC supplementation on body weight (A) and food intake (B) of mice induced to preneoplastic lesions with DMH.
Figure 3. Fecal concentration of acetic and butyric acid (µmols SCFA/g feces) in mice induced to pre-neoplastic lesions with DMH and supplemented with DHSC. SCFA were quantified on weeks 1, 5 and 12 of the experiment. Data are expressed as mean ± standard deviation (n=5). * Means statistical difference according to ANOVA complemented with Tukey’s Test (p < 0.05).
**Figure 4.** Percentage (%) of inflammatory infiltrates (A) in the control group (B), 5DHSC (C) and 10DHSC (D) in the distal colon portion of BALB/c mice induced to preneoplastic lesions with DMH and supplemented with DHSC. Arrows indicate inflammatory infiltrates. Data are expressed as mean ± standard deviation (n=7). Different letters mean statistical difference according to ANOVA complemented with Tukey's test (p < 0.05).
Figure 5. Area (µm), diameter (µm) and percentage (%) volumetric density of goblet cells present in the colonic mucosa of BALB/C mice induced to pre-neoplastic lesions with DMH and supplemented with DHSC. Staining was performed with Alcian Blue pH 2.5 (Control - D, 5DHSC - E, 10DHSC - F) and periodic acid Schiff (Control - G, 5DHSC - H, 10DHSC - I). Arrows indicate goblet cells. Data are expressed as mean ± standard deviation (n=10). * Means statistical difference according to ANOVA complemented with Tukey's Test (p < 0.05).
Figure 6. Leukocytes quantified in the colon mucosa of BALB/c mice induced to pre-neoplastic lesions with DMH and supplemented with DHSC. Data are expressed as mean ± standard deviation (n = 6). Different letters between bars mean statistical difference according to ANOVA complemented with Tukey's Test (p < 0.05).
Table 1. Composition of experimental diets AIN-93M* (g 100g-1).

<table>
<thead>
<tr>
<th>Ingredients (g)</th>
<th>CON(^1)</th>
<th>5HS(^2)</th>
<th>10HS(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch</td>
<td>39,75</td>
<td>39,75</td>
<td>39,75</td>
</tr>
<tr>
<td>Casein</td>
<td>20,00</td>
<td>20,00</td>
<td>20,00</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>13,20</td>
<td>13,20</td>
<td>13,20</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10,00</td>
<td>10,00</td>
<td>10,00</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>7,00</td>
<td>7,00</td>
<td>7,00</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5,00</td>
<td>5,00</td>
<td>5,00</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>3,50</td>
<td>3,50</td>
<td>3,50</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>1,00</td>
<td>1,00</td>
<td>1,00</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>0,30</td>
<td>0,30</td>
<td>0,30</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0,25</td>
<td>0,25</td>
<td>0,25</td>
</tr>
<tr>
<td>t-butylhydroquinone</td>
<td>0,0014</td>
<td>0,0014</td>
<td>0,0014</td>
</tr>
<tr>
<td>Dietary DHSC</td>
<td>-</td>
<td>5g</td>
<td>10g</td>
</tr>
</tbody>
</table>

\(^1\) Control group (AIM-93M); \(^2\) 5DHSC group (AIM-93M supplemented with 5% dietary DHSC); \(^3\) 10DHSC group (AIM-93M supplemented with 10% dietary DHSC). *AIN-93M (American Institute of Nutrition for Maintenance).
Table 2. Characterization of the centesimal composition, dietary fiber, and phenolic compounds of DHSC.

<table>
<thead>
<tr>
<th>Nutritional composition (g)</th>
<th>Nutritional value per 100 grams</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>3.61 ± 0.26</td>
</tr>
<tr>
<td>Lipids</td>
<td>0.66 ± 0.13</td>
</tr>
<tr>
<td>Ashes</td>
<td>2.69 ± 0.01</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>0.47 ± 0.32</td>
</tr>
<tr>
<td>Total Dietary Fiber</td>
<td>9.22 ± 2.79</td>
</tr>
<tr>
<td>Soluble Dietary Fiber</td>
<td>1.76 ± 0.14</td>
</tr>
<tr>
<td>Insoluble Dietary Fiber</td>
<td>7.46 ± 2.64</td>
</tr>
<tr>
<td>Total Phenolic Compounds</td>
<td>1.09 ± 0.02</td>
</tr>
<tr>
<td>Total Anthocyanins</td>
<td>0.22 ± 0.04</td>
</tr>
<tr>
<td>DPPH (μmol / g)</td>
<td>67.45 ± 0.11</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation of the analyzes in triplicate.
**Table 3.** Effects of DHSC supplementation on liver enzyme activity and liver serum markers in BALB/c mice.

<table>
<thead>
<tr>
<th>Markers / Liver Enzymes</th>
<th>Groups</th>
<th>CON</th>
<th>5DHSC</th>
<th>10DHSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td>12.89 ± 3.70a</td>
<td>22.24 ± 10.58a</td>
<td>33.46 ± 9.47b</td>
<td></td>
</tr>
<tr>
<td>SOD</td>
<td>10.80 ± 4.01a</td>
<td>14.57 ± 8.26a</td>
<td>17.85 ± 11.38a</td>
<td></td>
</tr>
</tbody>
</table>

Catalase (CAT), superoxide dismutase (SOD) (n=10); control (CON); 5% DHSC (5DHSC), 10% DHSC (10DHSC). Data are expressed as mean ± standard deviation. Different letters mean statistical difference according to ANOVA complemented with Tukey's test (p < 0.05).
Table 4. Effect of DHSC supplementation (5% and 10%) on ACF formation in mice with DMH-induced preneoplastic lesions.

<table>
<thead>
<tr>
<th>Colon segments</th>
<th>Groups</th>
<th>CON</th>
<th>5DHSC</th>
<th>10DHSC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proximal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACF ≤ 3</td>
<td></td>
<td>32.83 ± 13.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.57 ± 8.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.00 ± 8.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Medial</td>
<td></td>
<td>30.00 ± 11.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.29 ± 6.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.57 ± 4.86&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Distal</td>
<td></td>
<td>40.33 ± 5.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.43 ± 8.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.00 ± 5.85&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total ACF ≤ 3</td>
<td>*</td>
<td>103.2 ± 22.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.29 ± 13.96&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73.57 ± 9.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation (n=7). Different letters in each line mean statistical difference according to ANOVA complemented with Tukey’s Test. * Total number of ACF in all colon segments (proximal, medial and distal).