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The effect of the hydroalcoholic extract of *Helianthus annuus* roots in pre-neoplasic lesion and colorectal structural organization in rats exposed to the carcinogen 1.2 dimethylhydrazine

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ABSTRACT

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world. Different organs of many plants, including *Helianthusannuus (Asteraceae)*, have secondary metabolites that exhibit high cytoprotective activity and many of which have a cytotoxic effect on colorectal cells. The objective of this study was to investigate the *in vivo* anti neoplastic action of the hydroalcoholic root extract of *H. annuus* in carcinogenesis induced by 1.2-dimethylhydrazine (DMH). The experimental groups were evaluated for 10 weeks with 0.9% NaCl (negative control), DMH+0.9% NaCl (positive control) and three different concentrations of the *H. annuus* extract (10, 100, 200 mg/kg) combined with DMH. The results of the qualitative phytochemical survey of the extract indicated the presence of phenolic compounds, flavonoids and tannins. There was a significant increase in the number of aberrant crypt foci (ACF) and organizer regions of the nucleolus of enterocytes in treatments with DMH and the lowest concentration of sun flower extract, contrasting with the animal groups that received higher doses (100 and 200 mg/kg), groups which also showed a significant reduction in the serum level of nitrite/nitrate. The level of antioxidant minerals such as copper, magnesium, selenium and zinc was significantly reduced in the epithelium of ACF in animals receiving DMH alone or a smaller dose of the extract, compared to the control. The hydroalcoholic extract of *H. annuus* roots, especially at the highest dose used (200mg/kg), minimized colorectal morphological remodeling, reducing the development of precancerous lesions in animals exposed to the carcinogen.

Keywords: *colorectal cancer, Helianthus annuus (sunflower), 1.2-Dimethylhydrazine*.

1. INTRODUCTION

Overall, colorectal cancer (CRC) is the third most commonly diagnosed cancer; it is the third leading cause of cancer death among men and the second among women [1, 2], 25% of patients are diagnosed in metastatic stages [3] and the median overall survival rate at 5 years is around 55% in developed countries and 40% in developing countries, [1, 2, 4] whereas the diagnosis in asymptomatic stages increase the chances of a cure and the patient's quality of life [2, 5, 6]. In Brazil, about 32600 new cases were estimated for the year 2014, being prevalent among women [4] with a rising projection in the number of cases in the population by 2025 [7].

With etiologically multifactorial cancer, there is a high chance of recurrence, metastasis and chemo-resistance, associated with family history, body weight, diabetes, hypertension, age, smoking and alcoholism [4, 8, 9, 10]. Colorectal carcinogenesis is characterized by molecular heterogeneity with genetic [3, 11, 12] and epigenetic changes such as in DNA methylation, for example [13, 14, 15], which results in differences in survival of patients [9, 16]. Deregulation of epigenetic machinery components is a key event in carcinogenesis, affecting normal gene regulation, cell cycle, DNA repair, cell growth and differentiation and apoptosis, but which can be modulated by many plant compounds, including flavonoids [17, 18, 19].

Preventive measures, including changes in lifestyle and diet are strong strategies to reduce the incidence of this cancer. Different organs of many plants, including *Helianthus annuus* (sunflower) have chemical constituents that exhibit high antioxidant activity and phenolic compounds such as flavonoids, phenolic acids, tannins and coumarins which are commonly the most responsible [20, 21], many of which have *in vivo* and *in vitro* cytotoxic effects [17] on CRC cells [8, 22, 23]. A plant-based diet, fruits and whole grains, for example, is inversely associated with the risk of CRC by the presence of fibers and many secondary metabolites with anticarcinogenic properties [4, 8, 24, 25].

Colorectal tumorigenesis may morphologically present itself as discrete microscopic alterations, called aberrant crypt foci (ACF), considered as colonic mucosal precancerous lesions, used as markers for the risk of developing colorectal cancer [17] and may be induced chemically by the administration of the carcinogen 1.2-dimethylhydrazine (DMH) or azoxymethane (AOM) in animal models, whereas DMH exhibits greater carcinogenic efficiency with properties in promoting DNA hypermethylation of colorectal epithelial cells [17, 26, 27].

Plants and phytochemicals with *in vitro* and *in vivo* anti-tumor properties on different tumors are already known [28, 29], including CRC [17]. *Heliantus annuus* L. (*Asteraceae),* one of the 67 species of the genus, has potential therapeutic action by chemical constituents that have phytosterols, vitamins and minerals [30, 31] with *in vivo* non-genotoxic effects of dose-, time- and gender-independent, besides antigenotoxicity action when associated to chemotherapy [32], which gives a promising scientific target. Lutein, a carotenoid found especially in *H. annuus,* exhibits, anti-inflammatory, antioxidant, anticarcinogenic properties, among others, and suppresses the number of ACF *in vivo* [33].

Nitric oxide is a reactive oxygen species that, along with other radicals, is involved in oxidative stress and cell damage contributing to various diseases such as inflammation of the gastrointestinal tract, ulcerations, and gastric and colorectal tumors [30]. In addition to the endogenous antioxidant machinery, vitamins and minerals in one's diet have cytoprotective properties, such as vitamin A, C and E, and minerals such as iron, zinc, copper, manganese and selenium, constituents of enzymes and intracellular antioxidant protein pathways; polyphenols such as flavonoids, phenols, phenolic acids, lignins and tannins are also important dietary antioxidants [30, 34].

2. EXPERIMENTAL SECTION

2.1. Preparation of the hydroalcoholic extract of *Helianthus annuus*.

The extract was obtained from the roots of the plant. The collected material was washed in running water and dried in an oven at 40°C with controlled relative humidity. A 10 g sample of dried and ground material in a knife mill (Marconi® - Model 340) was added to a 100 mL water-alcohol solution (30%) for ultrasonication (Unique® - MaxiClean 1400), for 60 minutes (25°C). The extract was filtered to the vacuum for subsequent chemical analysis. The plant herbarium specimens are deposited under the VIC number 38971 of the Herbarium of the Federal University of Viçosa.

2.2. Phytochemical Screening.

The hydroalcoholic extract of the *H. annuus* root was submitted, in triplicate, to a series of reactions to characterize phenolic compounds, naphthoquinones, characterization of flavonoids, tannins, coumarins, triterpenes, steroids, cardiotonic glycosides and saponins [35].

2.3. Animals and experimental model.

The Central Animal Facility of the Federal University of Viçosa provided 35 female Wistar rats (*Rattus norvegicus*), at 8 weeks of age with an initial weight of 130.8 ± 27.1 g. The animals were allocated individually in boxes with an automated ventilation system (Ventilife Alesco®, São Paulo, Brazil), kept in an environment with regulated temperature (20° ± 2°C), humidity (60% - 70%) and light (12/12 h light/dark), receiving food and water *ad libitum*. The animals were divided into 5 experimental groups with 7 animals in each: Group 1 (negative control): 0.9% NaCl; Group 2 (control of induction): DMH + 0.9% NaCl; Group 3: DMH + hydroalcoholic extract of *H. annuus* (10 mg/kg); Group 4: DMH + hydroalcoholic extract (100 mg/kg); Group 5: DMH + hydroalcoholic extract (200 mg/kg). Colorectal carcinogenesis was induced by subcutaneous injection of DMH (Aldrich Chemical Co., Milwaukee, USA) at a dose of 20 mg/kg weekly for 10 weeks. From the 11th week forward, the hydroalcoholic extract was administered by gavage 3 times, for 14 weeks, with animals in groups 1 and 2 receiving only 0.9% NaCl (700 µL). The animals' weight was recorded weekly. At the end of the 25 weeks, the animals were euthanized by cervical dislocation under anesthesia (ketamine 10 mg/kg; and xylazine 2 mg/kg i.p.) after fasting for 12 hours. This study was conducted in accordance to the internationally accepted standards for work with experimental animals and approved by the Ethics Committee for Animal Research of the School of Biological and Health Sciences - UNIVIÇOSA (protocol 00002/2012-I).

2.4. Screening of aberrant crypt foci.

After euthanasia, the large intestine was removed *in totum*, washed in 0.9% NaCl, opened longitudinally, and fixed for 24 hours in paraffin plates containing 10% buffered formalin, pH 7.2 [36]. The intestines were measured and divided into three equal pieces (proximal, middle and distal colon) in relation to the cecum. Afterwards, they were stained with 0.1% methylene blue for one minute and rinsed in a phosphate buffer, pH = 7.2, for microscopic analysis. For ACF identification and quantification, the mucosal surface of the large intestine was observed from the intact pieces of the colon using a light microscope (Olympus BX-60®, Tokyo, Japan) with a 10 objective lens [36]. The ACF categorization was performed by determining the observed frequency of aberrant crypts for each focus (1 to 3, 4 to 10, above 10 aberrant crypts, and tumor).

2.5 Histopathological and histomorphometric analysis.

The intestinal fragments were destained with successive washes in 50% ethanol, dehydrated in ethanol, diaphanized in xylene and embedded in paraffin. Sections at 4 µm-thick were obtained in a rotary microtome (Leica Multicut 2045®, Reichert-Jung Products, Germany), stained with hematoxylin and eosin (HE), Alcian blue (AB) and Periodic Acid-Schiff (PAS) for marking of mucous secreting cells [37, 38].

To avoid the histological analysis of the same histological area 1 in every 20 tissue sections were used. Sections were viewed and images were captured using a light microscope (Olympus BX-60®, Tokyo, Japan) connected to a digital camera (Olympus QColor-3®, Tokyo, Japan). For each staining and group investigated, there were sixty microscopic fields with a 40x magnification randomly selected, so that a total area of 8.27×10^5 μm^2 was analyzed for each intestinal segment.

The histopathological parameters analyzed were the presence of tissue dysplasia, crypt dilatation and morphology of enterocytes [38], morphometric parameters and volume of the crypts. Cross sectioned crypts were used to determine the area of each crypt, its lumen and the number of mucus-secreting cells per histologic unit area. In these same sections, the volume density

(Vv) occupied by acidic (AB+) and neutral (PAS+) mucins in the histological area was estimated by point counting using the following formula: Vv[AB+, PAS+] = P_P [AB+, PAS+] / P_T; where P_P is the number of points that relate to each type of mucin and P_T is the total number of test points from a point system with 72 points applied on a standard histological area of 20.0×10^3 μm^2 [39].

The marking of nucleolar organizer regions (AgNORs) was performed from 4 µm sections of the three intestinal segments incubated in colloidal silver solution in the dark for 60 minutes at room temperature [40]. AgNORs were visualized under a light microscope with an increase of 1000 times and counted in 90 randomly sampled enterocyte nuclei in 30 intestinal crypts of each intestinal segment for each animal. The results were expressed throughout the whole intestine as the number of AgNORs per nucleus. The entire histomorphometric analysis was performed using the software Image Pro-Plus 4.5® (Media Cybernetcs, Silver Spring, MD, USA).

2.6. Statistical analysis.

Data were analyzed by analysis of variance (Kruskal-Wallis One Way Analysis of Variance on Ranks) followed by Dunnett's test and the results expressed as mean and standard deviation (mean ± SD). Values of $p < 0.05$ were considered significant.

3. RESULTS SECTION

3.1. Phytochemical screening.

The results of the qualitative phytochemical screening of the the hydroalcoholic extract of *Helianthus annuus* roots indicated the presence of phenolic compounds, flavonoids and tannins (Table 1).

Table 1. Phytochemical screening of the hydroalcoholic extract of the analyses with *H. annuus* roots.

Chemical Constituents	Hydroalcoholic Extract 30%
Phenolic compounts	+
Naphthoquinones	-
Flavonoids	++
Tanins	+
Cumarins	-
Triterpens e steroids	-
Cardiotonic Glycosides	-
Saponins	-

(+) positive result and (-) negative result

3.2. Biometric analysis and aberrant crypt foci.

There was no significant difference in body weight and intestinal length (data not shown) between the experimental groups after 25 weeks of evaluation.

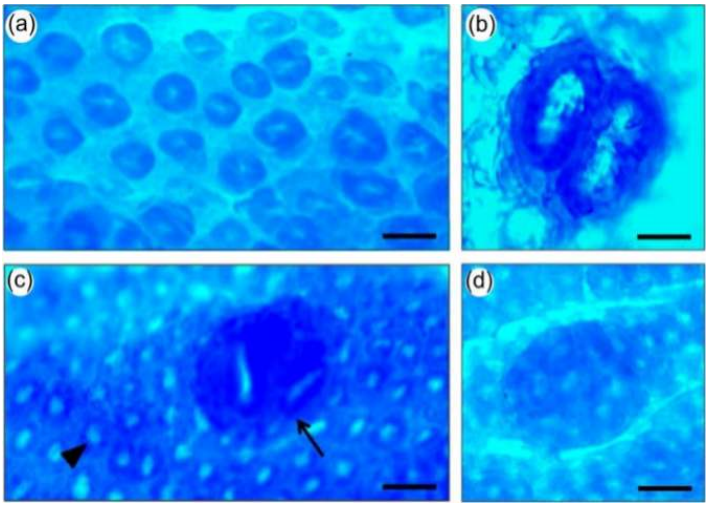

Figure 1. Representative photomicrographs of the distal colon crypts of the control rats (a) and exposed to 1.2 dimethylhydrazine (DMH) (b, c and d) obtained by complete bowel assembly. In (a) homogeneously distributed normal crypts. Animals exposed to DMH are identified targets with 2 (b and c) and about 10 (d) aberrant crypts, characterized by swelling of the intestinal lining epithelium and protrusion toward the intestinal lumen. In (c) normal crypts (arrowhead) around the aberrant crypt focus (arrow) (Methylene Blue Staining, bar = 40 µm).

Table 2. Analysis of aberrant crypt foci in the large intestine of rats exposed to 1.2 dimethylhydrazine (DMH) and treated with hydroalcoholic extract of *Helianthus annuus* roots. Values expressed in relation to the mean and standard deviation $p < 0.05$ *vs. G1 (negative control), 0.9% NaCl; †vs. G2 (induction control), DMH 20 mg/kg + 0.9% NaCl; G3, DMH-treated hydroalcoholic extract of 10 mg/kg of *Helianthus annuus*; G4, DMH-treated hydroalcoholic extract of 100 mg/kg of *H. annuus*; G5, DMH-treated hydroalcoholic extract of 200 mg/kg of *H. annuus*.

Intestinal Segment/ Number of aberrant crypt foci	Experimental Groups				
	G1	**G2**	**G3**	**G4**	**G5**
Proximal					
1-3	0.0 ± 0.0	18.1 ± 2.1*	26.8 ± 3.8*†	20.0 ± 3.9*†	17.8 ± 3.6*†
4-10	0.0 ± 0.0	3.0 ± 1.0*	3.0 ± 1.3*	1.8 ± 0.7*	1.3 ± 0.5*†
> 10	0.0 ± 0.0	1.3 ± 0.4*	0.2 ± 0.1*†	0.0 ± 0.0	0.0 ± 0.0
Tumor	0.0 ± 0.0	0.5 ± 0.1*	0.4 ± 0.1*	0.0 ± 0.0†	0.0 ± 0.0†
Middle					
1-3	2.5 ± 0.1	72.7 ± 4.6*	53.4 ± 6.8*	48.2± 4.4*	34.8 ± 2.5*†
4 to 10	0.0 ± 0.0	9.7 ± 3.4*	6.4 ± 2.0*	6.5 ± 1.9*	4.2 ± 1.2*†
> 10	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1*†	0.2 ± 0.1*†	0.5 ± 0.1*†
Tumor	0.0 ± 0.0	0.7 ± 0.2	0.0 ± 0.0†	0.0 ± 0.0†	0.5 ± 0.1*†
Distal					
1-3	0.7 ± 0.3	42.6 ± 8.9*	29.0 ± 2.8*	29.2 ± 3.6*	29.8 ± 3.0*
4-10	0.0 ± 0.0	4.7 ± 2.4*	3.8 ± 1.7*	1.0 ± 0.4*†	0.5 ± 0.1*†
> 10	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Tumor	0.0 ± 0.0	0.5 ± 0.1*	0.4 ± 0.1*	0.2 ± 0.1*†	0.2 ± 0.1*†

The intestinal analysis showed the presence of ACF with different levels of development throughout the mucosal surface in all intestinal segments in groups exposed to DMH. There was a predominance of foci containing between 1 and 3 aberrant crypts surrounded by normal crypts spread over the entire length of the large intestine of the animals (Figure 1).

The quantification of mucosal lesions showed increased numbers of the three ACF intestinal segments in all groups exposed to DMH, especially in animals that received the root extract (G2). In general, higher dosages of the extract (100 and 200 mg/kg) caused significant reduction in the total number of aberrant crypts per focus (Table 2).

3.3. Histopathological, immunohistochemical and histomorphometric analysis.

The histopathological analysis showed marked morphological reorganization of the intestinal crypts (Figure 2).

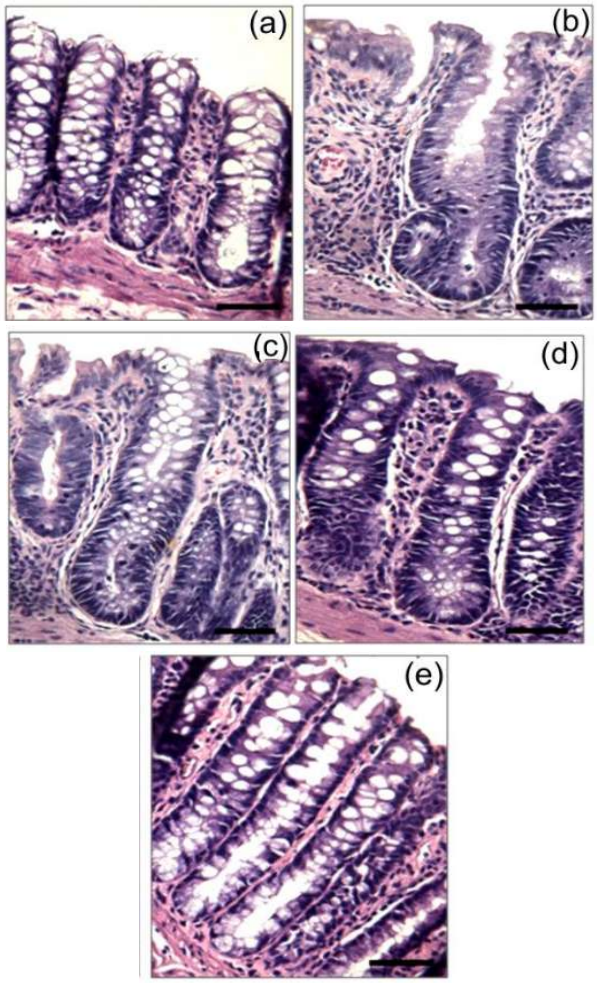

Figure 2. Photomicrographs of longitudinal sections of crypts of the distal colon of rats exposed to 1.2 dimethylhydrazine (DMH) and treated with the hydroalcoholic extract of *Helianthus annuus.* G1 (negative control, Figure 2a), 0.9% NaCl; †*vs*. G2 (induction control, Figure 2b), DMH 20 mg/kg + 0.9% NaCl; G3 (Figure 2c), DMH-treated hydroalcoholic extract of 10 mg/kg of *H. annuus*; G4 (Figure 2d), DMH-treated hydroalcoholic extract of 100 mg/kg of *H. annuus;* G5, DMH-treated hydroalcoholic extract of 200 mg/kg of *H. annuus* (H & E Staining, bar = 50 µm).

There was a dose-dependent inhibitory effect of the hydroalcoholic extract of *H. annuus* on ACF in all intestinal segments studied, especially in the experimental group (G5) with the higher dosage of the extract (200 mg/kg). The average intestinal segment was that which showed the largest number of ACF in the three treated groups.

DMH (G2) induced hypertrophic intestinal crypts with severe dysplasia; an increase in volume and density of enterocytes with an elongated cytoplasm and nuclei, reduction in the density of goblet cells to the fullest extent of the crypts, dilation of luminal space and pronounced infiltration of inflammatory cells. In G3 and G4 there was evident reduction in crypt dysplasia, with the presence of elongated enterocytes and considerably enlarged luminal space. The higher dose of the extract (200 mg/kg) resulted in morphological remodeling with crypts similar to healthy animals.

Table 3. Morphometric analysis of crypts of the large intestine of rats exposed to 1.2 dimethylhydrazine (DMH) and treated with the hydroalcoholic extract of *Helianthus annuus* roots. Values expressed in relation to the mean and standard deviation $p < 0.05$ **vs.* G1 (negative control), 0.9% NaCl; †*vs.* G2 (control of induction), 1.2-dimethylhydrazine-DMH 20 mg/kg + 0.9% NaCl; G3 (DMH-treated hydroalcoholic extract of 10 mg/kg of *H. annuus*); G4 (DMH-treated hydroalcoholic extract of 100 mg/kg of *H. annuus*); G5 (DMH-treated hydroalcoholic extract of 200 mg/kg of *H. annuus*).

Intestinal segment	Width (µm)	Length (µm)	Cript area (µm^2)	Lumen area (µm^2)	Cript volume (µm^3 x 10^3)	Luminal Volume (µm^3 x 10^3)
Proximal						
G1	222.6 ± 30.1	34.9 ± 7.1	1801.3 ± 551.7	146.2 ± 47.8	403.9 ± 75.2	39.5 ± 7.1
G2	337.5 ± 40.3*	71.9 ± 18.5*	3717.5 ± 659.1*	463.5 ± 60.2*	698.7 ± 100.1*	110.7 ± 17.9*
G3	243.4 ± 29.3†	44.7 ± 6.4†	2016.8 ± 421.5†	155.8 ± 47.3†	482.6 ± 80.9†	57.5 ± 9.1†
G4	215.1 ± 53.2†	33.0 ± 6.3†	1134.4 ± 495.4†	140.7 ± 40.8†	444.9 ± 81.9†	42.1 ± 10.3
G5	212.5 ± 46.5†	38.8 ± 9.0†	1692.6 ± 621.9†	146.3 ± 53.3†	417.7 ± 78.3†	38.9 ± 10.9
Middle						
G1	242.6 ± 28.4	37.0 ± 8.1	1709.5 ± 398.3	177.3 ± 65.2	382.1 ± 49.5	39.9 ± 9.2
G2	379.6 ± 51.8*	70.3 ± 11.0*	3106.1 ± 605.4*	531.8 ± 119.0*	937.4 ± 121.6*	120.5 ± 24.1*
G3	300.9 ± 40.3	51.3 ± 10.8	2891.1 ± 597.8*	329.2 ± 51.8*	615.8 ± 81.4*†	71.5 ± 14.2*†
G4	268.0 ± 35.7†	45.2 ± 7.3†	1818.5 ± 504.1†	232.2 ± 51.8†	518.9 ± 68.0*†	45.9 ± 11.4†
G5	245.3 ± 50.3†	46.0 ± 8.1†	1725.4 ± 437.7†	202.5 ± 47.7†	500.2 ± 97.4†	53.7 ± 15.9†
Distal						
G1	261.7 ± 19.4	44.3 ± 6.8	1425.6 ± 418.3	121.7 ± 46.0	399.6 ± 73.1	42.3 ± 6.5
G2	400.8 ± 53.7*	68.7 ± 9.5*	2900.4 ± 411.7*	340.4 ± 75.1*	849.8 ± 117.3*	80.2 ± 11.9*
G3	350.6 ± 29.6*	56.9 ± 6.0*	2716.2 ± 445.1*	285.6 ± 50.2*	690.0 ± 90.8*	60.4 ± 11.6*
G4	219.7 ± 33.6†	47.6 ± 6.1†	1885.4 ± 451.8†	129.1 ± 62.1†	504.3 ± 95.1†	37.3 ± 7.5†
G5	254.9 ± 32.1†	47.1 ± 7.2†	1605.9 ± 498.6†	130.5 ± 56.7†	483.8 ± 84.2†	38.5 ± 10.1†

The histomorphometric analysis of the crypts showed a significant increase in the three segments of the colon in all parameters evaluated in the experimental group treated only with DMH (G2). The effect of this plant extract resulted in a reduction of the parameters evaluated in the fullest extent of the large intestine, but less efficiently in its lowest dose (10 mg/kg), particularly in middle and distal segments and better results in a high concentration (200 mg/kg) (Table 3).

Based on the histochemical analysis, a reduced expression of cells producing acidic and neutral mucins in the crypts of the animals exposed to DMH compared to the control was observed. In healthy animals, wide and homogeneous density distribution of goblet cells were found whereas DMH caused a reduction of density in crypts and goblet cells, epithelial hypertrophy and a greater area occupied by the connective tissue surrounding the crypts (Figure 3).

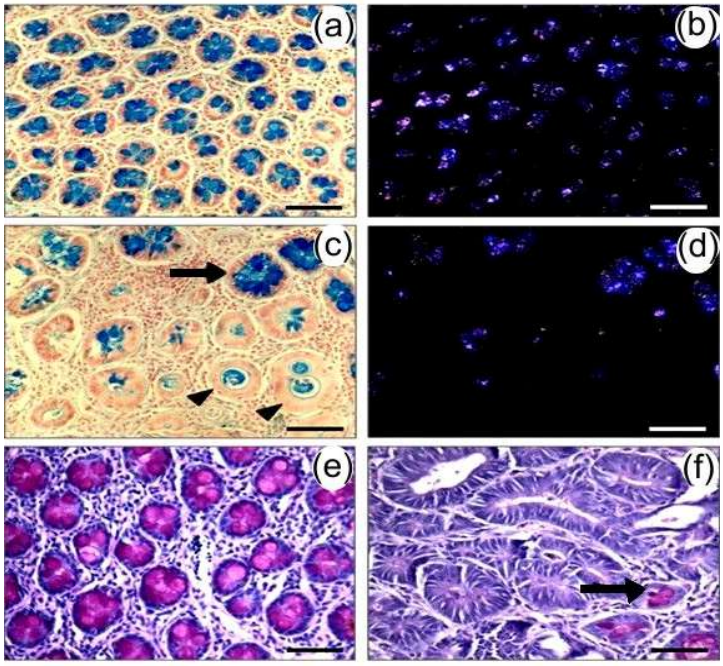

Figure 3. Acidic mucin-producing cells (a-d) and neutral cells (e-f) in the crypts of the distal colon of healthy rats (G1) and exposed to 1.2 dimethylhydrazine (DMH) (G2). The panel (a and e) shows a wide density and homogeneous distribution of goblet cells in the healthy animal crypts (a, Alcian blue staining, bar = 110 µm; and, Periodic Acid-Schiff Staining and Hematoxylin, bar = 70 µm). In the panel (b) the same image (a) is observed under polarized light. Note the uniform distribution of polarization and mucus throughout the tissue area (bar = 110 µm). In the panel (c) dilated dysplastic crypts are observed (arrow) from an animal exposed to DMH, observe the foci with 1 and 2 confluent aberrant crypts (arrowhead). Note the reduced density of crypts and goblet cells, and the largest area occupied by the connective tissue surrounding the crypts (Alcian blue staining, bar = 110 µm). In (d) reduction of the area occupied by mucus is clearly seen in the image under polarized light (bar = 110 µm). In (f) dysplastic crypts with hypertrophic epithelium show drastic reduction of goblet cells (arrow) (Periodic Acid-Schiff Staining and Hematoxylin, bar = 70 µm).

3.4. Endocrine cells and AgNORs.

In general, in all groups exposed to DMH, there was a significant reduction in the number of endocrine cells compared to the control (G1). In G5 (200 mg/kg) the number of endocrine cells was significantly higher compared to G2, but not compared to G1 (Figure 4 a–b). For all intestinal segments of the animals receiving DMH there was a significant increase in the number of nucleolar organizer regions per nucleus of enterocytes (AgNORs/nucleus), except for animals (G5) who received the highest dose of the plant extract (200 mg/kg) wherein the AgNORs/core number was significantly lower, comparable to healthy animals from G1 (Figure 4 c–g). In all investigated groups, crypt epithelial cells were observed in different stages of cell division, mainly in the dysplastic epithelium and tumor tissue in G2.

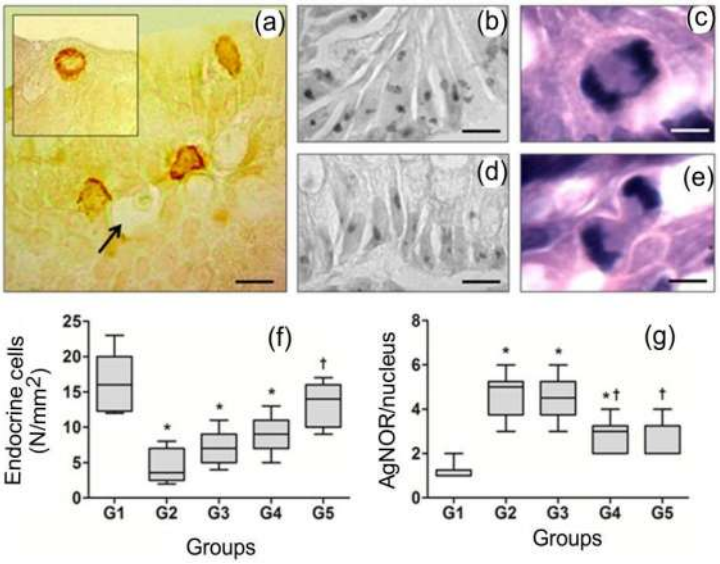

Figure 4. Endocrine cells of the large intestine, nucleolus organizer regions by the nucleus of enterocytes (AgNORs/core) and crypt epithelial cells in cell division of the rats exposed to 1.2 dimethylhydrazine (DMH) and treated with the hydroalcoholic extract of *Helianthus annuus*. (a) Representative photomicrograph of endocrine cells at the base of the intestinal epithelium (Grimélius method, bar = 25 µm). (b) Distribution of endocrine cells by histological area in the groups investigated. Values are expressed as median and interquartile ranges for the whole intestine. Representative photomicrograph from groups 2 and 1 displaying cell nuclei with up to 5 (c) or 2 (d) AgNORs, respectively (bar = 15 µm). (g) Distribution of AgNORs/nucleus in the investigated groups. Crypt epithelial cells in division in anaphase (e) and telophase (f). G1 (negative control), 0.9% NaCl; G2, (induction control) DMH + 0.9% NaCl; G3, DMH + hydroalcoholic extract of *H. annuus* (10 mg/kg); G4, DMH + hydroalcoholic extract of *H. annuus* (100 mg/kg); G5, DMH + hydroalcoholic extract of *H. annuus* (200 mg/kg). Statistical difference $p < 0.05$; **vs*. G1 †vs. G2.

4. CONCLUSIONS

The treatment with this plant extract partially contributed to the morphological reorganization of the intestinal epithelium, attenuating the formation of ACF, the total number of aberrant crypts per focus, the intensity of the epithelium dysplasia of the crypts, the number of AgNORs/nuclei of enterocytes, the nitrosative stress and increasing mucus expression, the density of its producer cells and the distribution of endocrine cells.

The induced animals exhibited a reduction in the number of mucin-producing goblet cells, with severe epithelial dysplasia of intestinal crypts. Although these changes were attenuated by treatment with the hydroalcoholic extract of *H. annuus*, the role of goblet cells in intestinal cancer still remains unexplored.

The significant reduction in the number of AgNORs/nuclei displayed in the group of animals treated at the highest concentrations of *H. annuus extract* (100 and 200 mg/kg) indicates

a decreased tumor cell proliferation in the colonic epithelium, a clear inhibitory effect of tumorigenic progression on the intestinal tissues.

Tissue remodeling of intestinal crypts, and the inhibitory effect on the progression of the lesions observed mainly at the highest dose of the extract may be related to their anti-inflammatory and anticarcinogenic chemical compounds, such as flavonoids [17], and the carotenoid lutein [33], many of which have properties similar to known chemotherapeutic agents acting in key points of colorectal oncogenic biochemical pathways [41, 42]. Deregulated oncogenic pathways in colorectal cancer, such as Wnt/β-catenina signaling, Ras and p53 [12] can be modulated by flavonoid action.

Associated with advances in metabolic bioengineering with prospects of increasing concentrations of desirable bioactive plant compounds or introduction to new biosynthetic pathways [43], *H. annuus* may have potential therapeutic action with chemopreventive properties at least in part by their ability to modulate aberrant epigenetic mechanisms. A potential source of antioxidants [44], non-genotoxic action and *in vivo* antigenotoxicity mechanism exhibited by *H. annuus* when associated with chemotherapy are even more promising studies with this plant [32].

Lastly, the results presented in this study demonstrate that *H. annuus* has the potential inhibitory effect on initial intestinal carcinogenic proliferation by limiting the formation of ACF and preventing deleterious action of DMH by increased antioxidative cytoprotective mechanisms. The chemical composition of this extract and its synergy among its compounds may contribute together with the effects presented here and further evaluation in pre-clinical and clinical studies will produce safer scientific results.

5. REFERENCES

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