RESEARCH ARTICLE

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Antineoplastic Activity Evaluation of Brazilian Brown Propolis and Artepillin C in Colorectal Area of Wistar Rats

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Abstract

Objective: The study's aim was to evaluate Brazilian Brown Propolis (BBP) and Artepillin C (ARC) chemopreventive action in Wistar rats' colons. **Methods:** Fifty male Wistar rats were divided into ten experimental groups, including control groups, groups with and without 1,2-dimethylhydrazine (DMH) induction, and BBP, ARC, and ARC enriched fraction (EFR) treatments, for sixteen weeks. Aberrant crypt foci (ACF) were classified as hyperplastic or dysplastic, and proliferating cell nuclear antigen (PCNA) expression was quantified. **Result:** ACF amounts in experimental groups (induced or not) decreased in both colon portions, while the isolated Aberrant Crypt (AC) number increased. Experimental groups of animals showed higher hyperplasia and dysplasia amounts compared with control groups. The ACF dysplastic amount present in groups induced and treated, in both colon portions, had similar values to IDMH (DMH induction group without treatment). In addition, DMH was effective in ACF inducing and there was positive staining for PCNA in basal and upper dysplastic foci portions in all experimental groups, in the mitotic index (MI) evaluation. To conclude, considering all the experimental groups, the one treated with EFR (fraction enriched with ARC) had the lowest rates of cell proliferation. **Conclusion:** BBP and its derivatives prevented crypt cell clonal expansion.

Keywords: Chemoprevention-Aberrant Crypt Foci (ACF), 1,2-Dimethylhydrazine (DMH)- Brazilian Brown Propolis

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Introduction

Colorectal cancer (CRC) is one of the biggest western world health problems [1, 2]. Several risk factors (genetic and environmental) contribute to humans neoplasms increased incidence, including colorectal. Environmental factors influence this disease as they interfere in cells' oxidative state. In addition, rich red and processed meats diets, as well as alcoholism, smoking, obesity, advanced age, and physical inactivity are important risk factors when it comes to disease development [3, 4].

1,2-Dimethylhydrazine (DMH) is considered a potent carcinogen commonly used as a CRC inductor due to its colon cells' high specificity and similar pathogenesis between rodents and humans [5]. This compound can generate oxidative stress capable of causing intestinal mucosa damage, leading to Aberrant Crypts (AC) formation, which is characterized by its elliptical shape and the elevated area around the crypt compared to normal ones. A set of AC is known as Aberrant Crypts Foci (ACF). On the rats' colonic mucosa surface stained with methylene blue and treated with carcinogens, it is possible to easily see AC and ACF presence [6, 7].

Important biological activities have been discovered in Brazilian Brown Propolis (BBP) [8]. Antitumor action is one of the most important properties studied in this compound, which makes it a source for drug formulation based on propolis extract [9]. A large amount of Artepillin C (ARC) (a simple phenolic acid), can be found in Brazilian Brown Propolis [10, 11], and its potent antitumor activities in gastrointestinal cancer cell lines have been reported for its inhibitory effects on cell proliferation [12]. However, the ARC mechanism of action has not been clarified yet [13].

Therefore, this study aimed to evaluate BBP, ARC and EFR chemopreventive activity regarding colorectal carcinogenesis through Wistar rats' colon pre-neoplastic lesions evaluation.

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Materials and Methods

Alcoholic extract of BBP and ARC: acquisition and chemical characterization

The alcoholic extract of BBP and ARC sample was provided by the pharmacology department of the Federal University of Mato Grosso do Sul (UFMS), in partnership with Apiário Vovô Pedro Ltda., Campo Grande city, state of Mato Grosso do Sul. It was produced using a propolis original sample collected from December 2015 to March 2016 in Mato Grosso do Sul, as described by Dembogurski [14]. The extractor was cleaned with hexane, and fractions were obtained with Dichloromethane, Ethyl Acetate and Ethanol, which demonstrated the presence of Artepillin only in the Dichloromethane portion, characterized as propolis Enriched Fraction (ERF). This fraction was used for isolation by HPLC (High-Performance Liquid Chromatography), generating pure Artepillin, and its structural confirmation was performed by NMR (Nuclear Magnetic Resonance) (300 MHz, Bruker DPX-300).

Medicines

DMH (1,2-dimethylhydrazine 98%) (SIGMA-ALDRICH - batch BCBL2593V) was used at a concentration of 40 mg/Kg as a neoplasm-inducing drug, diluted in a phosphate buffer solution with EDTA 1.5%, pH 6, 5. The animals received four doses of DMH twice a week intraperitoneally (IP), for two weeks, on the right side of their lower abdominal quadrant.

BBP, FRE (enriched fraction), and ARC were administered by oral gavage, using a 5% suspension of Carboxy Methyl Cellulose (CMC) in a dose corresponding to 80 mg/kg of propolis extract body weight and 10 mg /kg Artepillin C body weight, mixed by a manual stirring process, as determined by Shimizu [15]. Gavage was performed with a rigid stainless-steel cannula, carefully introduced into animals' mouth, passing through esophagus, and reaching the stomach, where the substances suspension was dispensed, not exceeding the body weight maximum volume: 10 mL/ kg.

Experimental design

Fifty male Wistar rats, supplied by the State University of Maringa (UEM) Central Animal Facility, and weighing between 150 - 200 grams, were used. The animals were transferred to the Department of Pharmacology and Therapeutics (LIFIN) sectoral vivarium and were placed in environments with controlled temperature (20ºC+2ºC), controlled humidity (60-70%), and light and dark cycles (12/12h), inside collective cages containing at most three animals, with special food for rats (Nuvital CR1) and sterilized water ad libitum.

The rodents were divided into ten experimental groups and the experiment lasted sixteen weeks. They were weighed on the experiment first day to determine drug doses administered and they would be weighed weekly for dose correction until the end of the experiment.

1. PBS - DMH diluent negative control, PBS: animals without induction and treatment, inoculated via intraperitoneal (IP) with DMH dilution solution - EDTA 1.5% in phosphate buffer pH 6.5, three times a week for fifteen weeks.

2. CMC - Gavage negative control: animals without induction and treatment, treated only with gavage vehicle, three times a week for fifteen weeks.

3. IDMH – Positive control induction: DMH induction twice a week in the first and second week of the experiment (total: 4 doses), and euthanasia in the sixteenth week.

4. IBBP – Experimental BBP treatment group: DMH induction twice a week in the first and second week of the experiment (total: 4 doses), and treatment with BBP three times a week for fifteen weeks.

5. IEFR – Experimental EFR treatment group: DMH induction twice a week in the first and second week of the experiment (total: 4 doses), and treatment with EFR three times a week for eight weeks.

6. PBBP – Prophylactic experimental treatment group with BBP: animals were treated with BBP during fifteen weeks three times a week, and induced with DMH only in the 6th and 7th weeks, twice a week.

7. IARC – Experimental ARC treatment group: DMH induction twice a week in the first and second week of the experiment (total: 4 doses), and treatment with ARC three times a week for eight weeks.

8. CBBP – Control experimental treatment BBP: animals treated only with BBP three times a week for fifteen weeks, without DMH induction.

9. CEFR – Control experimental treatment EFR: animals treated only with EFR three times a week for eight weeks, without DMH induction.

10. CARC – Control experimental treatment ARC: animals treated only with ARC three times a week for eight weeks, without DMH induction.

Twelve hours before euthanasia, the animals were deprived of food. In order to obtain biological material, they were euthanized by injecting an anesthetic at a dose three times higher than that used in general anesthesia. Thus, the doses adjusted for euthanasia were Xylazine Hydrochloride (30mg/kg) and Ketamine Hydrochloride (240mg/kg), administered intraperitoneally, in accordance with topic 9.1.2.2 of Normative Act nº 37 of the CONCEA Euthanasia Practice Guideline. All procedures were performed after the project had been approved by the UEM Ethics Committee on the Use of Animals (CEUA nº 3301180520), following recommendations for laboratory animal use based on Brazilian regulations. Then, the entire length of the large intestines was removed and the organs were washed in saline solution, sectioned along mesenteric line, and distended on Styrofoam trays for fixation with paraformaldehyde (4%) for six hours. Subsequently, tissue was transferred from a flask to be stored in 70% ethanol.

Histopathological analysis and quantification of AC and ACF

Colonic mucosas were stained with 1.0% methylene blue [16]. Subsequently, aberrant crypts were identified and quantified, according to criteria established by Bird [6], and tabulated as isolated AC and ACF with 02 to 03, 04 to 09, or more than 10 AC/focus. After that, tissue samples from both the proximal and distal areas were processed in baths of increasing alcohol solutions, diaphanized in xylene, and embedded in paraffin. Paraffinized blocks

were sectioned using a semi-automatic microtome into 4-5 μm-thick fragments and adhered to histological slides. Four to five samples from each colon region were placed on each slide, subjected to successive baths of xylene and alcohol, and stained with Hematoxylin Eosin (HE). An average of 20 fields/slice were analyzed under 200x magnification, and the ACF were histologically classified as hyperplastic and dysplastic according to Yoshimi [17] classification.

Immunohistochemistry Analysis of AC and ACF

Previously silanized slides received tissue samples from each experimental group and were submitted to the Immunohistochemistry (IHC) reaction protocol, comprising the following steps: a) blocking of endogenous peroxidase with a solution of hydrogen peroxide (3.5%) in methanol; b) antigen retrieval with 10 mM citrate buffer pH 6.0; c) blocking of nonspecific binding with BSA (Bovine Albumin) and Donkey Serum d) incubation with proliferating cell nuclear antigen (PCNA) primary Ac (monoclonal PCNA PC-10 – Invitrogen) (overnight); e) washing with PBS and incubation with the SuperPicTure™ Polymer Detection Kit –(Invitrogen); f) incubation with DAB chromogen (diaminobenzidine) (Invitrogen) and g) against staining with Hematoxylin.

Quantitative Analysis of PCNA Expression

The PCNA quantitative determination expression was performed (at a 1:200 concentration) using a computerized system consisting of a light microscope (Opticam microscopy technology), and the images collected along the entire histological section were captured by a camera attached to it (Opticam Lopt 14003 brand). Positive and negative cells for PCNA expression were counted in at least 20 normal-appearing perpendicular crypts, well oriented at 400x magnification in the most intensely stained areas. After digitizing the images, 100 mucosal epithelium cells were counted in random fields [18] with the Image Processing and Analysis software – Image-Pro plus. The index of colonic crypts cells expressing the Proliferating cell nuclear antigen (PCNA) was found to be positive in cells with clear dark brown nuclear staining. The protein expression index was calculated using the following formula: [(number of cells labeled with PCNA) / (total number of cells)] x100, thus obtaining each group's mitotic index (MI) [19].

Statistical analysis

Experiments were described as mean ± standard deviation of aberrant crypt and aberrant crypt foci numbers per animal/group, and the data were analyzed by statistical tests. The normality of AC, ACF, PCNA, and HE records per group was tested by using the Shapiro-Wilk test. The homogeneity of variance, in turn, was tested by the Levene test, while boxplots were used to test the presence of outliers. Based on these assumptions, Oneway ANOVA and Tukey HSD or Kruskal-Wallis and Nemeniy tests were used, as parametric and non-parametric tests, respectively. The significance level adopted was 5%.

Results

BBP ethanolic extract was used as a starting material for ARC isolation through HPLC-DAD on a semi-preparative scale. The isolated substance was structurally characterized by NMR and HPLC-DAD-MS techniques, and ARC showed 89% chromatographic purity (Figure 1 A-F).

Histological analyses performed by staining with methylene blue are shown in Table 1, as the mean ± standard deviation. Our results show that in control groups few AC and ACF were visualized, but without statistical significance. In IDMH, high values in proximal and distal portions, both of AC and ACF, were found.

Regarding isolated AC (Figure 2a), in both portions, there was an increase in AC without significant difference in all groups with experimental treatment induced with DMH compared to IDMH, except for the IEFR group, in which there was a reduction in AC in the proximal part (Table 1).

As for ACF (Figures 2b, 2c, and 2d), the results show a significant difference between IDMH and IEFR for ACF with 2-3 crypts/focus in the proximal region, and in ACF with 4-9 and more than 10 crypts/focus in the distal region. Furthermore, between IBBP/IEFR and IEFR/PBBP for ACF with 2-3 crypts/focus in the proximal and distal regions, respectively, significant differences were also found (Table 1).

In addition to these parameters, histological sections of intestines, stained with HE, were evaluated and quantified regarding the presence of ACF with hyperplasia and dysplasia in the proximal and distal portions. Along the colon's entire length, the presence of crypts with hyperplasias (Figure 3b) and dysplasias (Figure 3c) was observed in most animals.

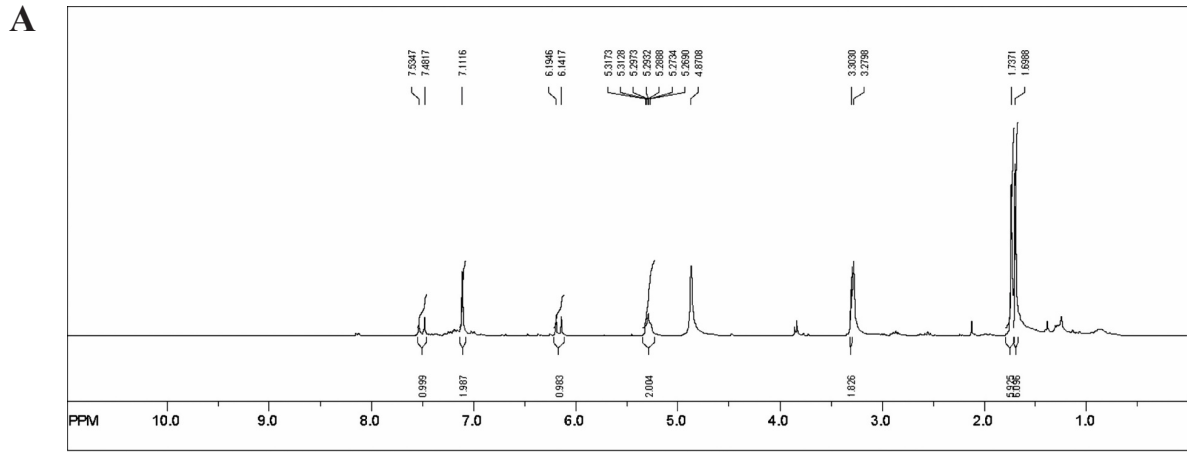
In groups with no DMH induction, only a few hyperplastic and dysplastic foci were identified, yet, in lower amounts compared to other groups (Figure 4). Animals in experimental groups presented higher amounts of hyperplasia and dysplasia compared with the control groups (PBS, CMC, CBBP, CEFR, and CARC) and may present up to three times greater amounts of dysplasia than those of the control groups. The IBBP, IEFR, PBBP, and IARC groups, in both colon portions, showed no statistical difference compared to IDMH. In addition, only ACF with dysplasia was observed in IDMH. Hyperplastic ACF was not visualized. However, this group showed the highest amount of dysplasia in the distal colon portion compared to the others, proving that DMH was an effective ACF inductor. In our experiment, there were no occurrences of tumors in any of the experimental groups.

Through analyses with the immunohistochemical technique (IHC), our results showed cells positively labeled for PCNA in all experimental groups (Figure 5). We found marked nuclei both in the proliferative (basal region) and apical crypts regions.

Through the mitotic index (MI) values obtained (Figure 6), we found results with a significant difference in both portions (proximal and distal) between IDMH (36% and 54%, respectively) and IEFR (18% and 32% respectively). In addition, IBBP/IEFR, IEFR/PBBP, and

Table 1. Means of Aberrant Crypts (AC) and Aberrant Crypts Foci (ACF), in the Colon Proximal and Distal Regions, with DMH (1,2 dimethylhydrazine) Experimental Induction, and Treatments with Brazilian Brown Propolis and Artepillin C.

Groups	Proximal colon				Distal colon			
	AC	ACF			AC	ACF		
	1 crypt	2-3 crypts	4-9 crypts	≥ 10 crypts	1 crypt	2-3 crypts	4-9 crypts	≥ 10 crypts
PBS	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0,16 ± 0.4	0 ± 0.0	0 ± 0.0
CMC	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0,16 ± 0.4	0 ± 0.0	0 ± 0.0
IDMH	25,3 ± 11.2	29,6 ± 12.9 a	3,4 ± 3.3	0 ± 0.0	26,1 ± 7.5	101,6 ± 26	96,3 ± 26.5 a	4,0 ± 2.9 a
IBBP	33,5 ± 8.6	28,6 ± 10.5 b	2,8 ± 2.6	0 ± 0.0	35,6 ± 20.2	87,1 ± 24.5	70,8 ± 16.9	1,3 ± 1.2
IEFR	17,6 ± 5.2	6,6 ± 4.1 a,b	0,16 ± 0.4	0 ± 0.0	37 ± 21.2	66,3 ± 13.3 b	30,5 ± 19.9 a	0,16 ± 0.4 a
PBBP	34,6 ± 25.6	29,5 ± 25.1	2,3 ± 3.3	0 ± 0.0	61,5 ± 32.5	114,3 ± 31.3b	59 ± 47.5	0,6 ± 1.2
IARC	29 ± 0.0	10 ± 0.0	0 ± 0.0	0 ± 0.0	44 ± 19.7	84,5 ± 24.7	51 ± 21.2	0 ± 0.0
CBBP	0 ± 0.0	0,16 ± 0.4	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
CEFR	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0,3 ± 0.5	0 ± 0.0	0 ± 0.0
CARC	0 ± 0.0	0,5 ± 0.7	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0



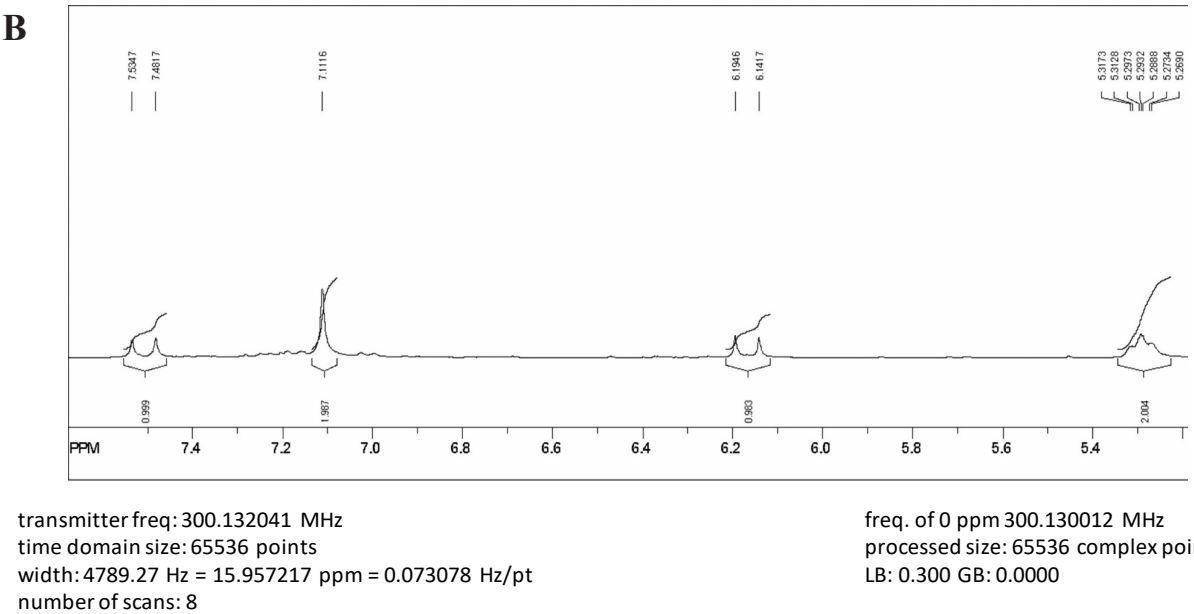
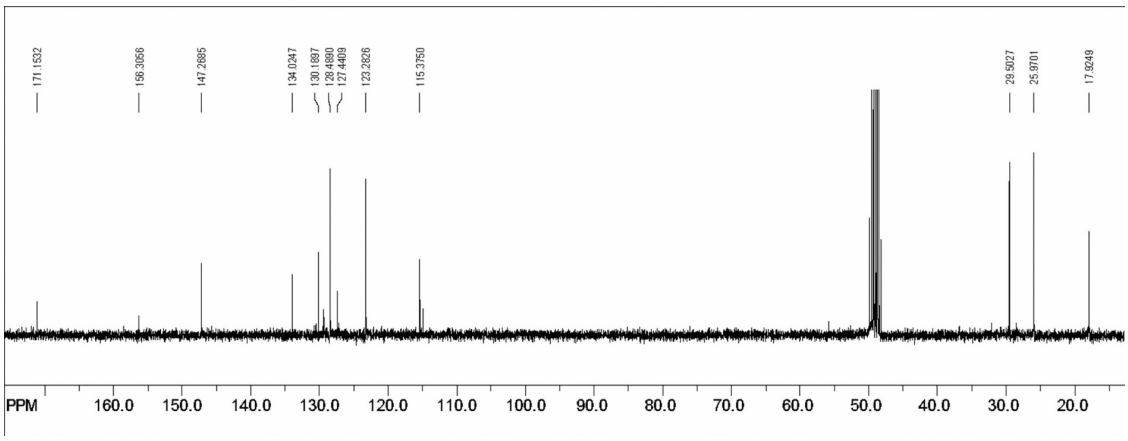


Figure 1. Chemical Characterization of BBP and ARC Alcoholic Extract. A, ^{1}H NMR spectrum (300 MHz, CD3OD) of Artepillin C; B, Magnification of Artepillin C ^{1}H NMR spectrum (300 MHz, CD3OD).

C



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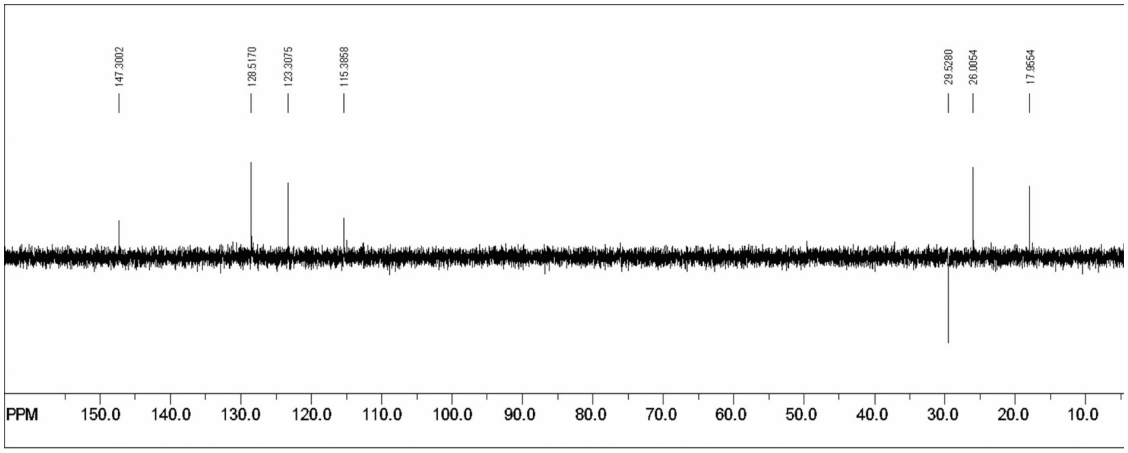

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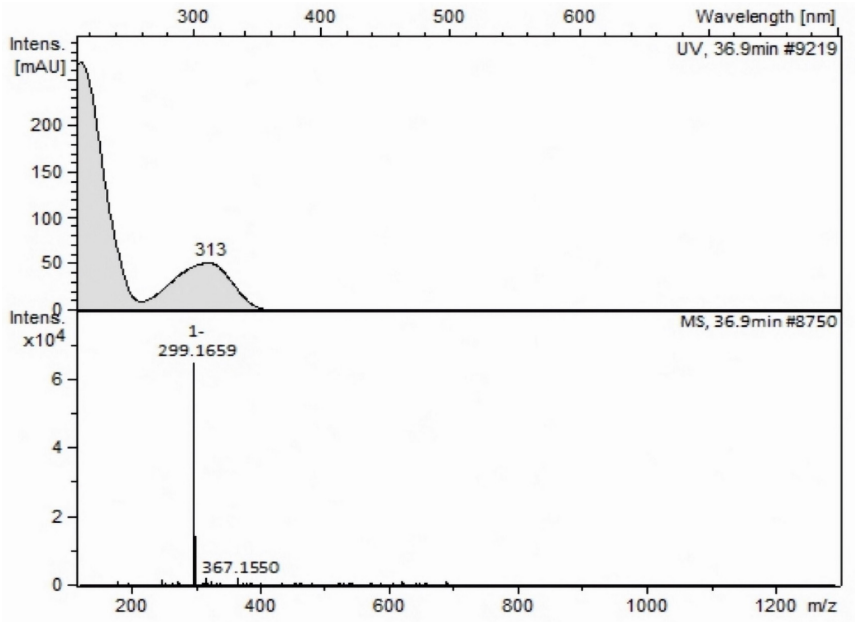


Figure 1. Chemical Characterization of BBP and ARC Alcoholic Extract.C, . ^{13}C NMR spectrum (75 MHz, CD3OD) of Artepillin C; D, DEPT 135º spectrum (75 MHz, CD3OD) of Artepillin C; E, UV and mass spectra (HPLC-DAD-MS) of Artepillin C

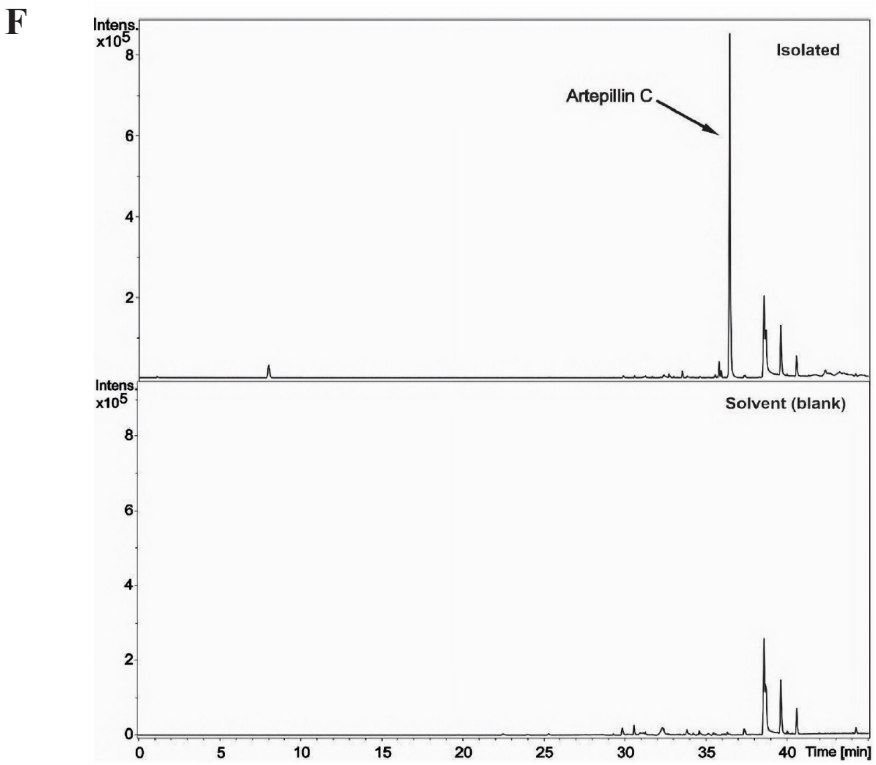


Figure 1. Chemical Characterization of BBP and ARC Alcoholic Extract. F, HPLC-DAD-MS chromatogram of Artepillin C and blank (solvent) at wavelength 210 to 400 nm.

PBBP/IARC also achieved results with a significant difference, coinciding with the ACF analysis using methylene blue staining, which is shown in Table 1. However, results obtained from IM to CARC showed a large increase of labeled nuclei compared to the other control groups.

In relation to the proximal portion, the distal portion mitotic index (MI) values were higher in all groups, except for group 10. These data are confirmed by the results presented in Figure 4, which show a considerable increase in hyperplastic and dysplastic ACF in the distal portion compared to the proximal one. In addition, we

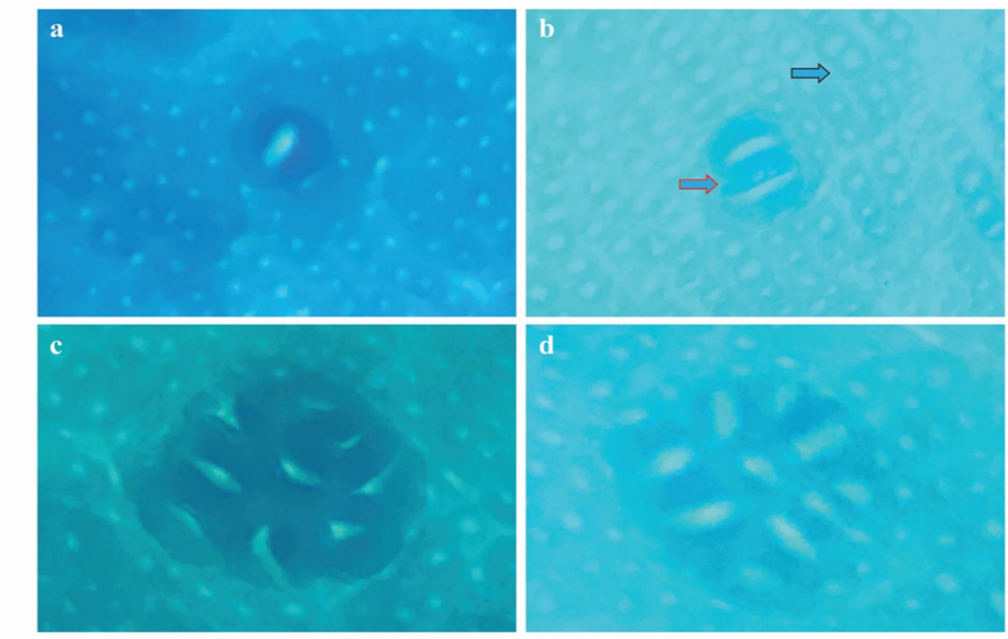


Figure 2. Representative Crypts and Aberrant Crypt Foci Photomicrographs of Rats' Distal Colon Exposed to 1,2-dimethylhydrazine (DMH). (A) isolated aberrant crypt (B,C,D) aberrant crypt foci with 2 (B), 9 (C), and more than 10 (D), characterized by intestinal lining epithelium dilated and protrusion towards the intestinal lumen. On panel, normal crypts (black arrow) are observed around aberrant crypt focus (red arrow) (Methylene Blue Stain). 20X objective.

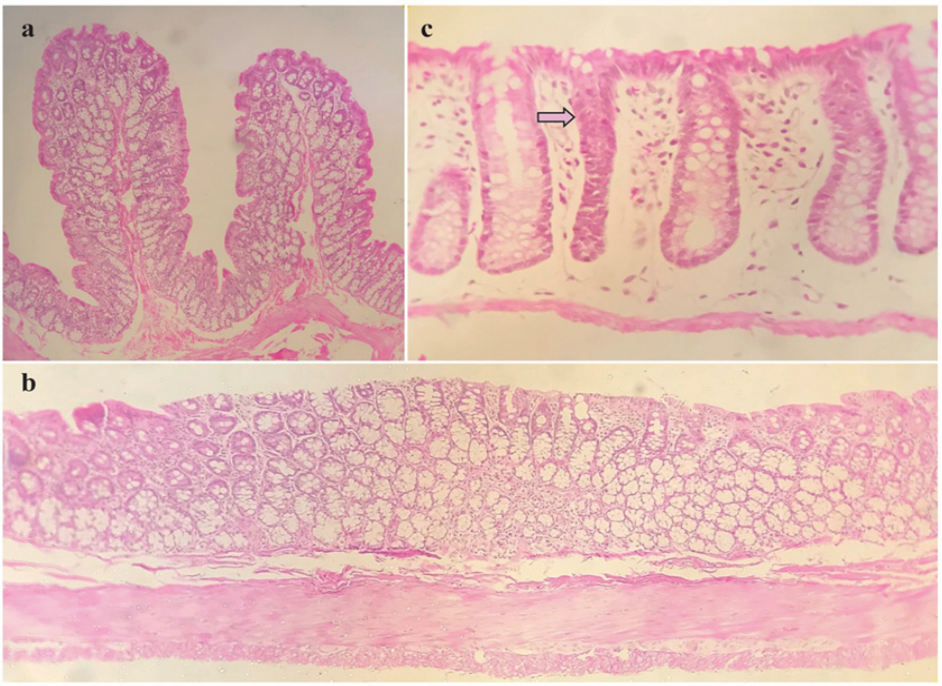


Figure 3. Illustrative Image of the Colon of Wistar Rats sStained with HE. (A) normal colon proximal region (B) proximal colon with gland hyperplasia and goblet cells (C) colon proximal region with dysplastic crypt (arrow). 20X objective.

again observed a significant reduction in MI values in IEFR in comparison with the other induced groups. All in all, considering all the experimental groups, the one treated with EFR had the lowest rates of cell proliferation.

In the macroscopic and microscopic histological analyses of the renal and hepatic parenchyma, there were no morphological alterations, which means that BBP and ARC were not harmful to the analyzed tissues.

Discussion

This study was carried out to investigate whether BBP and its main component ARC, when administered orally after carcinogen induction, could act or not as probable chemoprotective agents against induced carcinogenesis in rodents' colon.

Analysis of ACF quantification in experimental groups showed that both colon portions had a reduction in treated groups compared to the induction control group, while isolated AC numbers increased. These results may suggest that BBP and its derivatives at first cannot control regular crypt transformation into aberrant crypts, although it can probably inhibit cell clonal expansion that composes ACF and, consequently, ACF formation. This reinforces the hypothesis that the experimental treatment with BBP and its derivatives, despite not controlling completely AC and ACF appearance, had a chemoprotective action by controlling crypts/focus multiplicity. This is

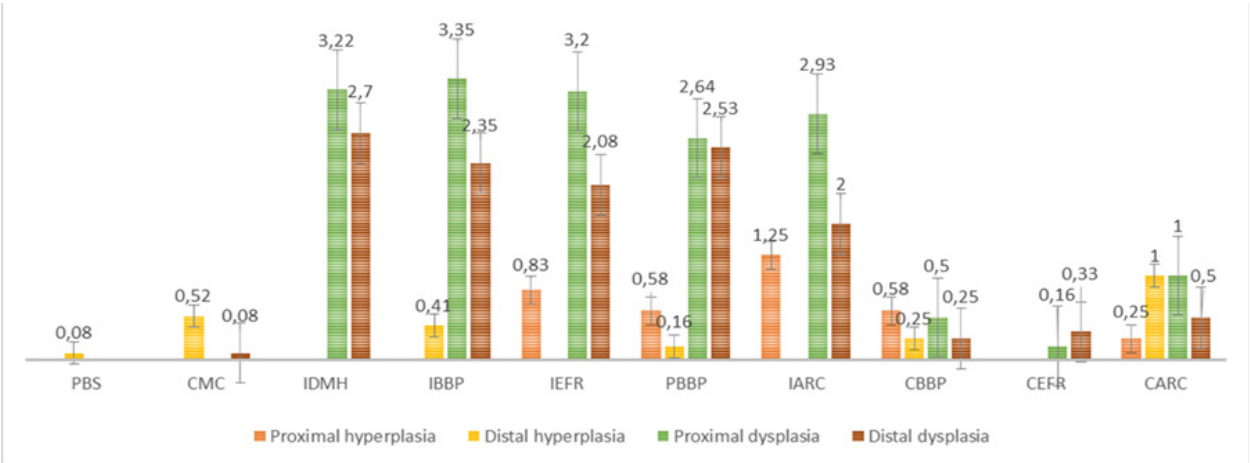


Figure 4. Number of Hyperplasia and Dysplasia in the Proximal and Distal Portions of the Groups after the HE Technique. Mean and standard deviation were calculations based on each group's number of animals. Results were expressed as means observed in animals of the same group ± standard deviation.

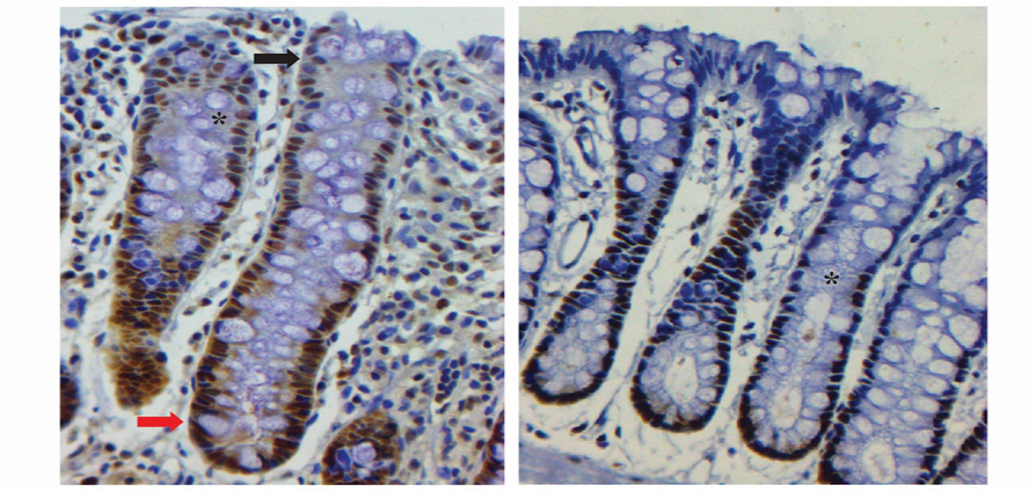
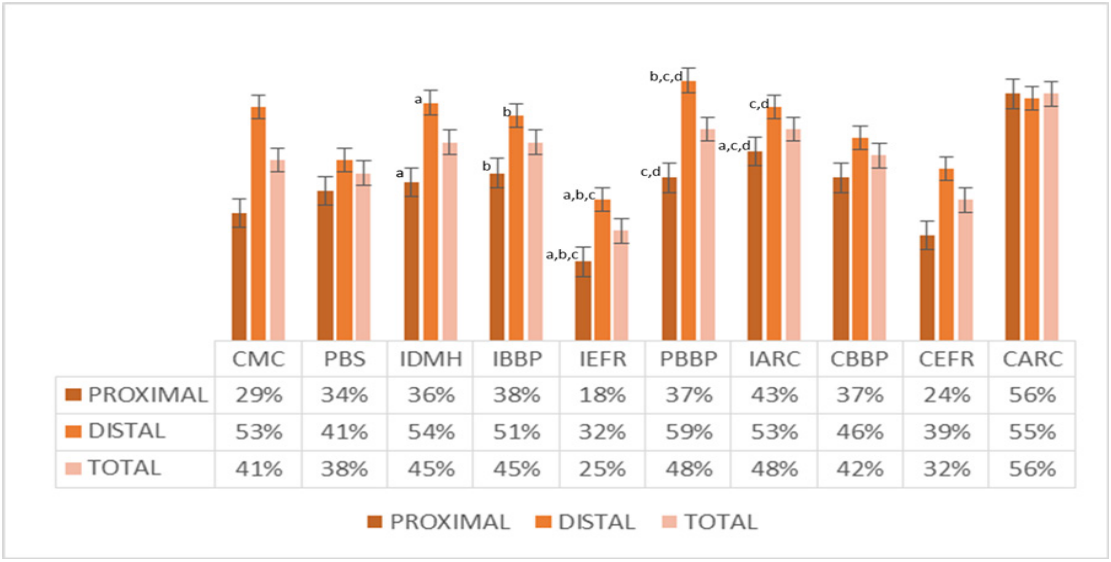


Figure 5. Photomicrographs of a Colon Tagged with PCNA and Stained with DAB. Crypt cells in the crypt basal region (red arrow) and crypt apex (black arrow) with marked expression of PCNA; the presence of nuclear stratification, and mucin production reduced (*). 20X objective.



	CMC	PBS	IDMH	IBBP	IEFR	PBBP	IARC	CBBP	CEFR	CARC
PROXIMAL	29%	34%	36%	38%	18%	37%	43%	37%	24%	56%
DISTAL	53%	41%	54%	51%	32%	59%	53%	46%	39%	55%
TOTAL	41%	38%	45%	45%	25%	48%	48%	42%	32%	56%

Figure 6. Expression of PCNA-tagged Proteins. Results are expressed as percentages by calculating the mitotic index [(number of PCNA-labeled cells) / (total number of cells)] x100. *p < 0,05 by the oneway ANOVA test; **p < 0,05 by Kruskal-Wallis test. For Proximal, the difference between groups IDMH and IEFR, IDMH and IARC, IBBP and IEFR, IEFR and PBBP, IEFR and IARC, and PBBP and IARC (Tukey's test); For Distal, the difference between groups IDMH and IEFR, IBBP and IEFR, IBBP and PBBP, IEFR and PBBP, IEFR and IARC and PBBP and IARC (Nemeniy's test). a = IDMH; b = IBBP; c = IEFR; d = PBBP.

possibly related to bee products' antioxidant capacity, which is proven capable of scavenging free radicals and neutralizing the effects of oxidative stress underlying pathogenesis [20], thus, preventing disease progression.

Animals treated with EFR showed more efficient results compared to other compounds tested, which suggests that the portion isolated in dichloromethane (DCM) is the richest one in phenolic compounds because it is the main responsible for the antioxidant capacity of propolis [21, 22]. However, these results also imply that the antioxidant activity of propolis may vary according to the compounds that act together with it, since EFR, which had only some components of propolis, had lower results of AC and ACF compared to the group treated with BBP, which, in turn, contains all the propolis components. This suggests that some of its constituents together can act against antioxidant action. Therefore, caffeic acid phenethyl ester (CAPE) and quercetin were the flavonoids found in the propolis samples that showed the best antioxidant activity according to a study that compared the significant differences in vitro antioxidant activity and in the phenolic profile between some types of propolis [23]. Such findings may help in the investigation of the identification of compounds present in the DCM portion, which, with ARC, significantly reduced the amount of pre-neoplastic lesions in the colon of rodents.

The results obtained in our research are of great interest, as previous studies have found that epithelial cells undergo pathogenesis for CRC from aberrant crypts foci, a location that favors malignant tumor formation. In addition, other studies report that ACFs with increasing crypt multiplicity are more resistant to apoptotic cell

death [24, 25], and according to Chiu [26], propolis can act on the apoptosis mechanism, inducing chromatin condensation in CRC cells, consequently causing their programmed death.

The analysis with HE staining showed that groups of animals without DMH induction had only a few hyperplastic and dysplastic foci in much lower amounts than the other groups (Figure 4). By observing that ACF amounts in induced and treated groups (IBBP, IEFR, PBBP, and IARC), in both portions of the colon, had similar values to IDMH, with a reduction in PBBP and IARC, we can hypothesize that BBP and its derivatives were effective in controlling clonal expansion cells that were multiplying considerably, because based on other studies [27, 28] BBP and its derivatives are capable of inhibiting proliferation through several mechanisms.

Considering the results of the IDMH group, only ACF with dysplasia were found, and no hyperplastic ACF was observed. However, this group showed the highest dysplasia amount in the distal portion among all the groups, which proves that DMH was an ACF effective inductor.

MI evaluation showed an increase in the proliferation of cells that compose the ACF. Besides, these dysplastic lesions had positive markings for PCNA in the basal and apical crypt portions (Figure 5) in all experimental groups. That indicates that the cells marked in the upper portions of the ACF were probably in the G1 phase of the cell cycle, during which cells undergo cell division more quickly, especially when subjected to stimulators of cell multiplication [19], as was the case in this study.

In this sense, even though BBP and its derivatives did not cause a reduction in MI values in the experimental groups (except in IEFR), we can conclude that the compounds we tested, especially EFR, were able to control cell proliferation. We can also say that their presumable antioxidant activity has an important neutralizing action of free radicals produced at the moment of harmful stimulus to the cell, which induces mucosa cell renewal/proliferation, resulting in a potential risk of cancer reduction [29].

Chiu [26] reported that compounds with antioxidant activity in molecular events at all process stages could affect the carcinogenesis process, resulting in a potential decrease in the risk of cancer. Such compounds could inactivate reactive oxygen and nitrogen species that play an important role in carcinogenesis, preventing processes commonly catalyzed by cytochrome P-450 enzymes. The main constituents of propolis produced in temperate zones are phenolic compounds [30], known for playing an antioxidant role, that is, scavenging and promoting the decomposition of radicals [31]. Likewise, ARC chemoprotective activity in gastrointestinal cancer cell lines has been reported to have inhibitory effects on cell proliferation [12] and a cytotoxic action that inhibits human malignant tumor cells' growth in vitro and in vivo [13].

The microscopic analyses demonstrating tissue integrity suggest the viability of hepatic and renal functions, indicating that the compounds used in this study were not harmful to the animals' organs, which makes it safe to be used.

Therefore, we can suggest that BBP and its derivatives have the ability to inactivate reactive oxygen and nitrogen species due to its antioxidant capacity, which modified rats' colon carcinogenesis in this AC and ACF quantitative histopathological evaluation bioassay. This is probably because other studies can prove the antioxidant capacity of propolis [31, 22, 20, 23, 21] although in our experiment this specific type of property was not tested.

In addition, since cell proliferation is necessary for genetic alterations fixation during carcinogenesis, our findings suggest that ACF are parts of the intestine with greater probabilities of developing neoplasms due to their high proliferative activity. Moreover, they are excellent biomarkers in bioassays for the chemopreventive evaluation of compounds with probable antineoplastic and/or antitumor activity. Furthermore, the predictive value of a neoplasm precursor lesions evaluation is increasingly necessary for deciding on cancer treatments or preventive measures [32].

Author Contribution Statement

All authors contributed to the study conception and design. Material preparation were performed by Djaceli Sampaio de Oliveira Dembogurski, Denise Brentan da Silva, Brenda Barroso Pelegrini and Tânia Cristina Alexandrino Becker. Data collection and analysis were performed by Brenda Barroso Pelegrini, Amanda Alexandrino Becker, César Agostinho Ferreira, Gregório Rossetto Machado, Murilo Gauer, Sabrina Roledo Mazarin and Tânia Cristina Alexandrino Becker. Alice Maria de Souza Kaneshima revised it critically for important intellectual content. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Ethical Declaration

All procedures were performed after the project had been approved by the UEM Ethics Committee on the Use of Animals (CEUA nº 3301180520), following recommendations for laboratory animal use based on Brazilian regulations.

This study is part of an approved student thesis, Brenda Barroso Pelegrini, from State University of Maringa, for the title of master.

Conflict of Interest

The authors have no financial or proprietary interests in any material discussed in this article.

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