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Butia capitata (sour coconut) prevents the appearance of Aberrant Crypt Foci in the colon of rats

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ABSTRACT: Colorectal cancer is a life-threatening disease. This study evaluates whether sour coconut (*Butia capitata*) prevents the appearance of aberrant crypt foci (ACF) in the colon of rats, induced with 1,2-dimethylhydrazine (DMH). The test subjects were divided into four treatment groups: I - Control; II - aqueous extract of sour coconut pulp; III - DMH and IV - Aqueous extract of sour coconut pulp + DMH. The colons of the subjects were submitted to ACF count, analysis of gene expression and histopathological. The count of ACF differed in the proximal portion of the colon between animals treated III and IV, with a lower count in Treatment IV ($p < 0.05$). The interleukin-1 (*IL-1*) gene ($p < 0.05$) was less expressed in Treatment IV. In the histopalogic analysis of the colons in Treatment IV, less dilation was observed at the base of the crypts and the lumen was moderately dilated. The sour coconut acted in preventing the appearance of ACF, probably due to its high composition in bioactive compounds, such as phenolic compounds, polyunsaturated fatty acids and vitamin E. The anti-inflammatory capacity reduces the expression of the *IL-1* gene and preserves the histo-architecture of the colon.

1. INTRODUCTION

Colorectal cancer (CRC) results from a series of genetic alterations that lead to a progressive and irreversible loss of normal control of cell growth and differentiation. There are several molecular pathways from normal mucosa to colorectal carcinoma, which explains the existence of intestinal tumors of different biological nature and may afford specific targets for both prevention and cure (De Leon & Percesepe, 2000). This biology provides an excellent way to study genetic abnormalities involved in tumor formation. Results of such research led to the formulation of a general model for colorectal tumorigenesis, in which the accumulation of genetic alterations is responsible for successive waves of clonal expansion that occur during tumor promotion and progression (Fearon & Vogelstein, 1990).

Aberrant crypt foci (ACF) are the first preneoplastic lesions observed in rodent colorectal carcinogenesis models, which suggests that the ACF are precursor lesions of adenomas and cancers (Bird, 1987; Hurlstone et al., 2005; Kim et al., 2008; Seike et al., 2006; Takayama et al., 1998). ACF consists of large, thickened intestinal crypts with varying degrees of atypia or nuclear dysplasia, which are easily visible in the colon of test subjects treated with a carcinogen and stained with methylene blue (Gregorio et al., 1997; Roncucci et al., 1991).

Neoplasms result from genetic alterations that affect the stages of cell development, including the mechanisms of proliferation and death by apoptosis (Curioni et al., 2001; Tajra et al., 2007; Wenjing et al., 2020). When cell damage becomes irreversible, tumor-suppressor proteins activate the intrinsic pathway of apoptosis and trigger cell death (Akhter et al., 2014; Beckerman & Prives, 2010; Einsele-Scholz et al., 2016; Grivicich et al., 2007; Villunger et al., 2003).

Many cancers lead to mechanisms that allow affected cells to escape death or lose the ability to interrupt the cell cycle (Akhter et al., 2014; Opferman, 2016). The increased expression of

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some genes belonging to the family of anti-apoptosis proteins and inflammatory cytokines activate transcriptional regulators at the tumor site, which keep cells alive and increase tumor mass (Grivennikov et al., 2010; Voronov & Apte, 2015).

Like other diseases of multifactorial causes, cancer is apparently associated with oxidative stress in the cell. Preventive measures against damage can be adopted, which include a diet rich in antioxidant compounds. The main compounds with this property are of vegetable origin, such as vitamin C, vitamin E, carotenoids, and phenolic compounds (Degáspari & Waszczynskyj, 2004; Silva et al., 2010).

Brazil has a wide variety of fruits distributed throughout different regions and many are still largely unexplored, such as the "coquinho azedo" or sour coconut (*Butia captata*), which is a palm tree of the Arecaceae family native to the Cerrado region in the states of Bahia, Goiás, and Minas Gerais. The fruits are spherical and become yellow when ripe. They have a fibrous edible pulp, which is both sweet and sour. The nut is also edible. Being palatable and nutritional, the fruits are broadly consumed not only unprocessed but also as juices, popsicles, jellies, liqueurs, cakes, and ice cream (Lima et al., 2010).

Our analysis of the chemical composition of 100g of sour coconut pulp demonstrated its high nutritional value and antioxidant activity: total energy (83.34 Kcal.100g^{-1}); carbohydrate (12.51%); lipid (3.42%); protein (0.74%); vitamin C (53.57 mg.100g^{-1}); β-carotene (8.56 mg.100 g^{-1}); vitamin E (121.07 μg.100g^{-1}); α-tocopherol (39.84 μg.100g^{-1}); α-tocotrienol (28.02 μg.100g^{-1}); γ-tocoferol (22.99 μg.100g^{-1}); γ-tocotrienol (11.11 μg.100g^{-1}); β-tocoferol (19.11 μg.100g^{-1}); cupper (1.8 mg.100g^{-1}); phenolic compounds total (493.60 mg.Eq^{-1} of galic acid) and antioxidant activity (4.74 μM of Trolox.g^{-1}), reference tab (Barbosa et al., 2021). Other data not evaluated by our group were tabulated from the reference (Faria et al., 2008): tannic acid (116 mg.100g^{-1}) and 10.1% of fiber.

The sour coconut may become a source of bioactive compounds important for growth, development and protection against diseases, thereby complementing its acceptance as a nutritional source. Mindful of this possibility, our group evaluated whether the aqueous extract of sour coconut pulp acts in the prevention of ACF or pre-neoplastic lesions, chemically induced in the colon of rats with 1,2-dimethylhydrazine (DMH).

2. MATERIALS AND METHOD

2.1. Test subjects and diets

The forty male Wistar rats used in our experiments were twelve-weeks old and weighed approximately 120-150 grams. The animals were kept in individual cages at the Experimental Nutrition Laboratory of the Department of Nutrition and Health of the Federal University of Viçosa, Viçosa, Minas Gerais, Brazil, at a temperature of 22 °C ± 2 °C, in a 12-hour photoperiod. The test subjects were divided into four treatment groups: I - Control; II - Aqueous extract of the sour coconut pulp (pulp:water; 50:50); III – DMH; and IV - Aqueous extract of the pulp of sour coconut + DMH. Animals from all treatments received water ad libitum, Nuvilab CR1 rodent chow (QUIMTIA®, Paraná, Brazil) and aqueous extract of *Butia capitata* pulp by gavage, 1.0 mL/day for 9 weeks (treatments II to IV). DMH (Sigma® Chemical Co., St. Louis, Missouri, USA), was dissolved in 0.9% saline solution containing 1 mM EDTA (Sigma® Chemical Co., St. Louis, Missouri, USA) and 10 mM sodium citrate (Sigma® Chemical Co., St. Louis, Missouri, USA), pH 8, animals in groups I and received only the vehicle without DMH. The Ethics Committee on the Use of Animals - CEUA / UFV, approved the experiment, process N$^{o.}$ 17/2014.

2.2. Induction of the appearance of ACF with the administration of 1,2-Dimethylhydrazine (DMH) and collection of material

Test subjects which were submitted to the appearance of ACF received an intraperitoneal injection of DMH (Sigma® Chemical Co., St. Louis, Missouri, USA) twice a week and over a period of two weeks, with a dose of 40mg/kg of body weight (Newell & Heddle, 2004). Seven weeks after the final application of DMH (Sigma® Chemical Co., St. Louis, Missouri, USA), the animals of all treatment groups were euthanized with anesthesia with 5% isoflurane (Cristália®, São Paulo, Brazil), using an inhalation device to minimize suffering. A portion of the animals' large intestines, from the cecum to the anus, was then removed.

2.3. Preparation of tissue for aberrant crypt foci count

The removed large intestine was washed in a phosphate buffer solution (PBS) and opened longitudinally along the counter mesenteric margin, placed in paraffin plates, with the mucosa facing the top of the plate and fixed in Carson's formalin for 24 hours. After fixation, the large intestine was then measured and divided into three equal fragments called proximal, medial, and distal, in relation to the cecum. The fragments were then stained in a 0.1% methylene blue (Sigma® Chemical Co., St. Louis, Missouri, USA) solution for 2 minutes and washed in PBS to remove excess dye for further analysis. The counting was performed with the aid of a light microscope (Olympus® America Inc., CBA model, Center Valley, Pennsylvania, USA) at 100x magnification by two double-blind trained observers. The number of aberrant crypts per focus, that is, foci with up to three crypts and foci with more than three crypts stratified the ACF categorization (Bird, 1987).

2.4. Reverse transcriptase and polymerase chain reaction (RT-PCR)

2.4.1 Extraction and quantification of mRNA and cDNA synthesis

The total mRNA of the large intestine was extracted using the TRIZOL reagent (Invitrogen®, Rockville, Maryland, USA), according to the manufacturer's recommendations. To eliminate DNA contamination, the total mRNA was treated with RNase-free DNase (Thermo Fisher®, Waltham,

Massachusetts, USA). After extraction, the RNA was quantified and analyzed on a 1.3% (w/v) denaturing agarose gel (Thermo Fisher®, Waltham, Massachusetts, USA) stained with ethidium bromide (Bio-Rad®, Hercules, California, USA) at 0.1 μg/mL. The synthesis of cDNA was performed using two μg of total RNA, oligodT [18] (Thermo Fisher®, Waltham, Massachusetts, USA) and Superscript II reverse transcriptase (Thermo Fisher®, Waltham, Massachusetts, USA), according to the manufacturer's specifications.

2.4.2 *Quantification of mRNA by real-time polymerase chain reaction (q-PCR)*

The entire real-time PCR procedure was conducted according to the Applied Biosystems® Waltham, Massachusetts, EUA manuals. Real-time PCR reactions were performed using the 7500 Real Time PCR Systems (Applied Biosystems® Waltham, Massachusetts, EUA) specific oligonucleotides, treatment cDNA, and SYBR Green PCR Master Mix (Applied Biosystems® Waltham, Massachusetts, EUA). The amplification conditions were 95 °C for 10 min, 40 cycles of 94 °C for 15 seconds, 60 °C for 1 minute. For the quantification of gene expression, the comparative method Ct: 2-ΔC was used as an endogenous control for normalization of *q*RT-PCR data, the Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) gene was used. The oligonucleotides used are shows in Table 1.

2.5. Processing to obtain histological sections and preparation of the countermensteric portion of the proximal colon for image acquisition

Colon fragments were submitted to histological processing to obtain histological sections and make slides. The histological sections obtained were adhered to the slides and stained with hematoxylin (Sigma® Chemical Co., St. Louis, Missouri, USA) for one minute, washed in distilled water for 15 minutes, stained in eosin (Sigma® Chemical Co., St. Louis, Missouri, USA) for one minute and rinsed in water. They were then dried on a hot plate and mounted with Canada balm (Merck®, Darmstadt, Germany). The prepared slides were used for histopathological evaluation. The images were selected and obtained using the AX-70 photomicroscope (Olympus® America Inc., model CBA, Center Valley, Pennsylvania, USA). To obtain images of the contra mesenteric margin, the colon was washed in phosphate buffer solution (PBS), stained with 1% methylene blue in Petri dishes and placed on glass slides with the contra mesenteric margin facing up. The ACF images were obtained under a microscope (Olympus® America Inc., model CBA, Center Valley, Pennsylvania, USA) at 100X magnification.

2.6. Statistical analysis

Statistical Package for the Social Sciences (SPSS®), version 20.0, was used for the statistical analysis. Descriptive data were presented including frequencies, mean and standard deviation (SD). The type of distribution of quantitative data, normal or non-normal, was verified before each association analysis, using the Shapiro Wilk test. The Kruskal Wallis test was performed to compare three or more groups in independent samples with non-normal distribution. The Mann-Whitney U test was used to investigate the difference between two groups with non-normal distribution and the Tukey test (ANOVA) for difference between two groups with normal distribution. The (p) value was fixed up to 5% in order to obtain 95% confidence.

3. RESULTS AND DISCUSSION

3.1. Aberrant crypt foci count

The number of ACF in the proximal portion of the colon after the use of aqueous extract of the pulp of sour coconut was lower in Treatment IV when compared to Treatment III, which received only DMH. A lower incidence of ACF was observed with foci up to three crypts, $p<0.05$ (Table 2). No differences were observed in the medial and distal portions in any other treatment (data not shown).

Research involving sour coconut is still in an early stage. This study is unprecedented and evaluated whether the aqueous extract of sour coconut pulp prevents the appearance of ACF in the colon of rats. The CRC starts from ACF identified as pre-neoplastic lesions, which precede the evolution of adenomatous polyps. The assumption is that the greater the number of ACF, the greater will be the probability that the tumor will appear (Varago et al., 2017).

DMH is a drug widely used to induce ACF in rodents. These ACF can be distributed in different portions of the proximal, medial or distal colon, depending on the type of study (Gomides et al., 2015, 2014; Marcon et al., 2020; Reis et al., 2019; Rosa et al., 2015). In this study, there was a lower prevalence of ACF in the proximal colon of animals in Treatment IV (Table 2).

3.2. Gene expression

The expression analysis of the *P21*, *P53*, *BCL-2*, *BAD*, *BAX*, *IL-1* and *TNF-α* genes was performed, using *GAPDH* as an endogenous control. A difference appeared in the expression of the *BAD*, *BAX* and *IL-1* genes (Figure 1). There was no difference in the expression of the *P21*, *P53*, *BCL-2* and *TNF-α* genes (data not shown). Figure 2 shows some functions of the proteins produced from the genes quantified in this study.

Proteins known as tumor suppressors control these checkpoints. Among them, two stand out: p53 and p21. These proteins are inhibitors of cyclin-dependent kinases (Cdks), which interrupt the cell cycle when DNA damage occurs (Wenjing et al., 2020). When the damage to DNA is irreversible, the p53 protein accumulates in the cell and activates transcription of genes encoding the pro-apoptotic proteins Puma and Noxa.

Table 1
Specific oligonucleotides forreverse transcriptase quantitative

Genes	Primers
GAPDH	Sense: 5'-CAAGTTCAACGGCACAGTCA-3' Antisense: 5'-CCTGGAAGATGGTGATGGGT-3'
P21	Sense: 5'-CTGGATGCTAGAGGTCTGC-3' Antisense: 5'-AGAGTTGTCAGTGTAGATGC-3'
P53	Sense: 5'-TCTGTTTCAAAAAGCAAAAAGATGAC-3' Antisense: 5'-ATAGCAAGGAAAGTCATGAACTGCCA-3'
BCL-2	Sense: 5'-ATCGCTCTGTGGATGACTGAGTAC-3' Antisense: 5'-AGAGACAGCCAGGAGAAATCAAAC-3'
BAD	Sense: 5'-GCCAACAACAGTCATCATGG-3' Antisense: 5'-CCATCCCTTCATCTTCCTCA-3'
BAX	Sense: 5'-AGGGTGGCTGGGAAGGC-3' Antisense: 5'-TGAGCGAGGCGGTGAGG-3'
IL-1	Sense: 5'-AAGACAAGCCTGTGTTGCTGAAGG-3' Antisense: 5'-TCCCAGAAGAAAATGAGGTCGGTC-3'
TNF-a	Sense: 5'- AAATGGGCTCCCTCTCATCAGTTC-3' Antisense: 5'- TCTGCTTGGTGGTTTGCTACGAC-3'

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase gene, *P21*: *p21* protein gene, *P53*: *p53* protein gene, *BCL-2*: B-cell lymphoma protein gene 2, *BAD*: protein X associated with Bcl-2 gene, *BAX*: bak related to Bcl-2 gene, IL-1: interleukin-1 gene, TNF-a: tumor necrosis factor-alpha gene.

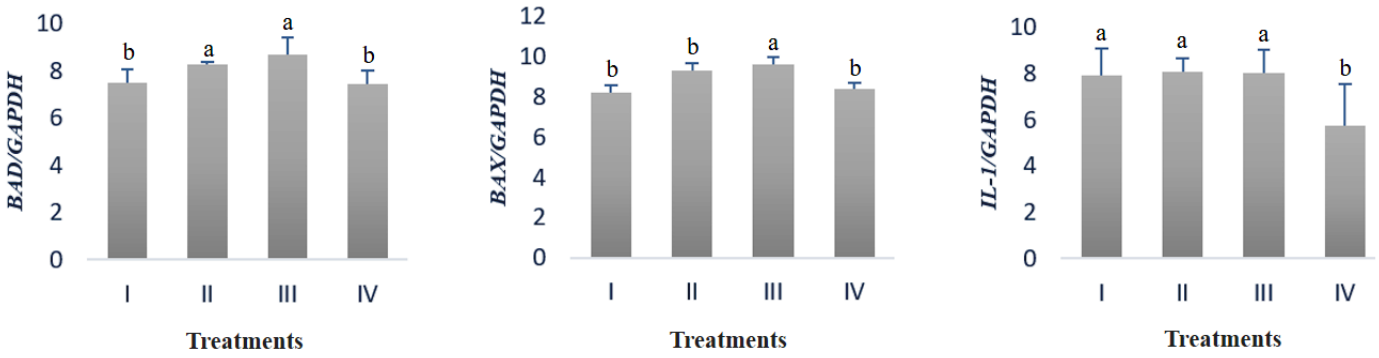

Figure 1. Comparison between treatments of *BAD*, *BAX* and *IL-1* gene expression by RT-PCR of the colon of Wistar rats exposed to 1,2-dimethylhydrazine (DMH), 40mg/kg for nine weeks using as control endogenous the *GAPDH* gene. Data are shown as mean ± SD. ^{a,b} Different letters in the columns indicate statistical significance between treatments ($p < 0.05$), and the same letter does not differ statistically by the Tukey test. I: Control; II: Aqueous extract of the sour coconut pulp; III: DMH; IV: Aqueous extract of the sour coconut pulp + DMH.

Table 2
Number of aberrant cryptfoci (ACF) with up to three crypts and with more than three crypts in the proximal colon of Wistar rats exposed to 1,2-dimethylhydrazine (DMH), 40mg/Kg for nine weeks.

	Up to 3 ACF	Greater than 3 ACF
Treatment	Proximal Colon	Proximal Colon
I (n = 3)	$0,0\pm0,0^{a}$	$0,0\pm0,0^{a}$
II (n = 3)	$0,0\pm0,0^{a}$	$0,0\pm0,0^{a}$
III (n = 7)	$5,79\pm2,40^{b}$	$2,21\pm2,23^{b}$
IV (n = 7)	$3,07\pm1,43^{c}$	$0,21\pm0,39^{c}$

Data are shown as mean ± SD. I: Control; II: Aqueous extract of the sour coconut pulp; III: DMH; IV: Aqueous extract of the sour coconut pulp + DMH. ^{a,b,c}The means followed by the same letter in the column do not differ significantly and the means followed by different letters, differ by Mann Whitney's U test with 5% probability. ACF = aberrant crypt foci, DMH: 1,2-dimethylhydrazine.

These proteins can associate with protein X associated with Bcl-2 (Bax) and bak related to Bcl-2 (Bak), thereby facilitating the formation of oligomeric complexes in the outer mitochondrial membrane and activating the apoptosis process (Akhter et al., 2014; Beckerman & Prives, 2010; Einsele-Scholz et al., 2016). In this study, the increase in *BAX* gene expression in Treatment III did not influence the decrease in ACF in the proximal colon of the animals compared to Treatment IV, indicating that the increase in the respective gene probably does not lead to activation of the mitochondrial pathway of apoptosis.

In the early stages of CRC, the p53 protein is active and inactivation mutations of its gene occur later on because they are rare in polyps, but common in carcinomas (Klumb & Júnior, 2002; Vousden, 2005). To halt the cell cycle, p53 protein it stimulates the expression of the p21 protein gene, an inhibitor of the cycle during the transition from the G1/S phases or during the S phase (Abbas & Dutta, 2009; El-Deiry, 2016). In the present study, no difference appeared in the expression of the *P53* gene and the *P21* gene in any treatment; it was not possible to correlate the expression of these genes with the prevention of ACF incidence in the proximal colon of animals in Treatment IV compared to Treatment III.

A signaling pathway known as phosphatidylinositol-3-kinase-Akt (PI3K-Akt) stimulates animal cell survival and growth by phosphorylation of the Bad protein. Phosphorylated Bad becomes inactive and releases B-cell lymphoma protein 2 (Bcl2) which inhibits the apoptosis process. The balance between Bcl2 and Bad activities can determine whether cells live or die (Willis & Adams, 2005). In the present study, there are no differences in the expression of the *BCL2* gene, but differences in the expression of the *BAD* gene were observed in treatments III and IV ($p<0.05$). The decrease in ACF in IV treatment was probably not influenced by the lower expression of the *BAD*

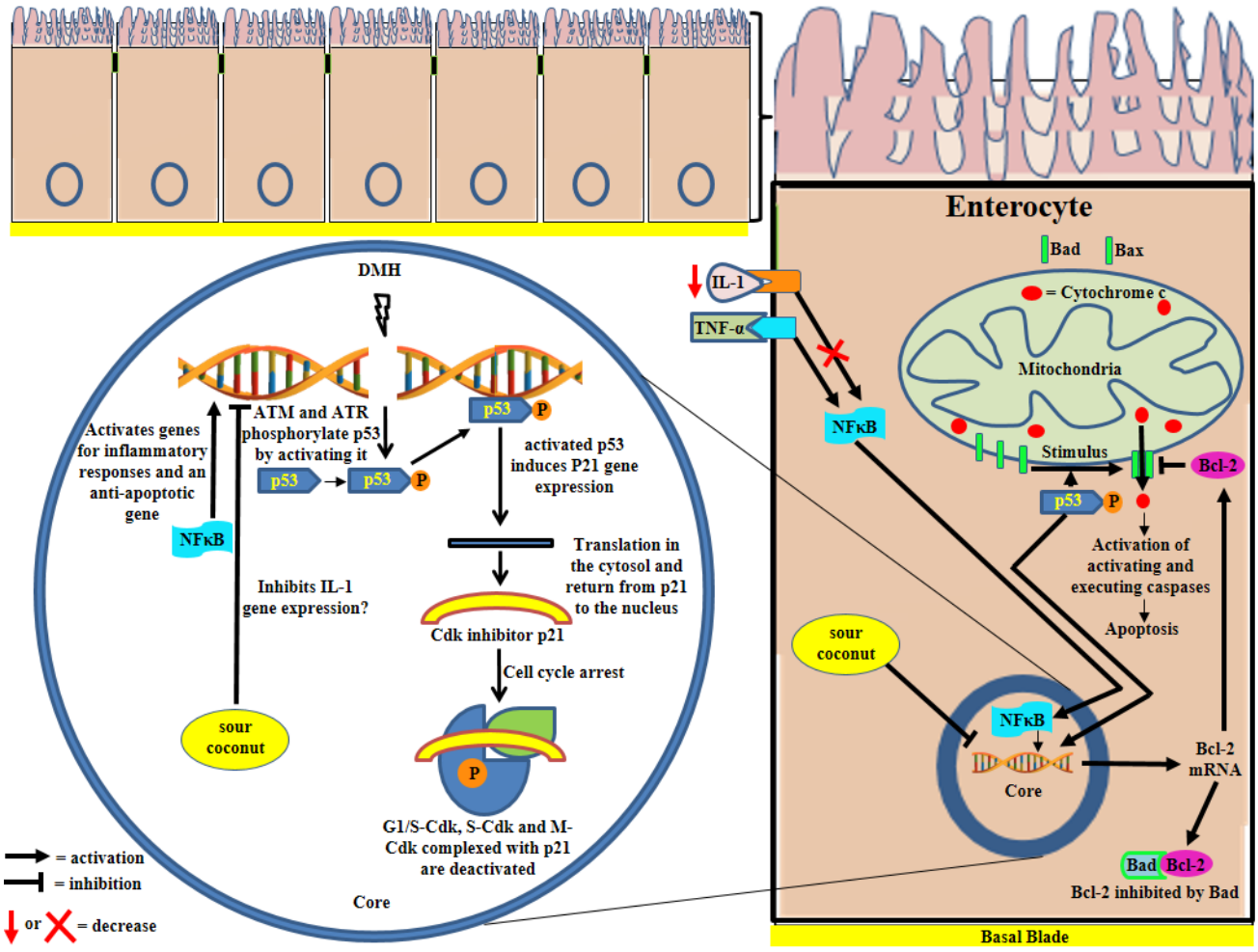

Figure 2. Possible mechanism of action of sour coconut in preventing the appearance of aberrant crypt foci due to the action of 1,2-dimethylhydrazine. In the figure top left, it is possible observe a set of enterocytes from the colon crypts supported on the basal lamina. On the right, a enterocytes is highlighted, showing the action of *IL-1* and TNF-α in signaling and activating the NFκB transcriptional regulator, which moves to the nucleus (in greater magnification on the left of the figure), activating genes from the inflammatory response and one anti-apoptotic gene of Bcl-2 protein.Bcl-2 is an anti-apoptotic protein when activated inhibits the formation of the oligomeric complex that releases cytochrome c from the mitochondria. Bcl-2 is inhibited by the Bad protein. It is believed that sour coconut may act by decreasing the expression of the *IL-1* gene and consequently decreasing the extracellular concentration of this cytokine, leading to a lower incidence of aberrant crypt foci (ACF) in the colon, probably by decreasing the cell signaling that activates NFκB. Two mechanisms of action of the p53 protein canalso be seen. First, after detecting DNA damage, p53 is phosphorylated and activated by ATM and ATR, p53 enters the nucleus where it acts in the expression of the p21 protein gene that will interrupt the cell cycle through the inhibition of two cyclin/Cdk complexes. Second, p53 acts as a promoter of apoptosis after irreversible DNA damage caused by 1,2-dimethylhydrazine (DMH), stimulating the formation of the oligomeric complex of Bax and Bak in the inner mitochondrial membrane with the release of cytochrome c and consequent activation of the cell death by apoptosis.

gene.

The increased expression of genes belonging to the BCL-2 family (example: Bcl-2), through the activity of the inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1), activates at the site nuclear factor kappa B (NFκ-B), a transcriptional regulator, which keeps cells alive and increases tumor mass (Grivennikov et al., 2010; Voronov & Apte, 2015) and leads to inflammation induced by oxidative stress.

This persistent inflammatory and oxidative environment creates a vicious cycle, which can damage healthy epithelial and stromal cells and result in carcinogenesis. Studies have shown that the suppression of TNF and IL-1 decreases the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), inhibiting the proliferation of myeloid leukemia and lymphoma cells. IL-1 can be produced and secreted during colonic inflammation by infiltration of myeloid cells, colonic epithelial cells and other stromal cells. IL-1 can also inhibit apoptotic events initiated in some cells, allowing their proliferation and greater accumulation of mutations and result in a malignant phenotype (Federico et al., 2007; Giri & Aggarwal, 1998; Hussain & Harris, 2007; Voronov & Apte, 2015).

In this study, *IL-1* gene expression was lower in Treatment IV, where a lower incidence of ACF was observed in the proximal colon of the animals. Although there was an increase in the expression of the pro-apoptotic genes *BAD* and *BAX* in Treatment III, the higher incidence of ACF suggests that the increased expression of the *IL-1* gene in this treatment may have exerted pro-inflammatory and anti-apoptotic activity. As earlier mentioned, in Treatment IV there was a lower expression of the *IL-1* gene, which suggests that the aqueous extract of coconut pulp may have had an anti-inflammatory effect and, therefore, influenced the decrease in the incidence of ACF in the colon of the test subjects due to its abundance in phenolic compounds with antioxidant properties.

Several studies have demonstrated the effectiveness of phenolic compounds in inhibiting the proliferation of human CRC cells in culture (Koh et al., 2018; Lee et al., 2020; Mazewski et al., 2018; Riahi-Chebbi et al., 2019; Subramanian et al., 2016). Inhibition of the appearance of tumors and pre-neoplastic lesions in the colon of animals (Gunasekaran et al., 2019; Koh et al., 2018; Lee et al., 2021, 2020). These studies evidenced that phenolic compounds induce apoptosis and activate or inhibit cell signaling pathways that act in the prevention of tumors, promote cell cycle interruption, control the expression of genes of inflammatory markers, cytokines, metastasis and angiogenic markers, preventing cell migration and invasion as well as decreasing the production of reactive oxygen species (ROS).

Similar effects were observed in the present study with the reduction of *IL-1* gene expression with consequent reduction of ACF counts in the proximal colon of animals in Treatment IV. The phenolic compounds in sour coconut may have thus acted to repress expression of the *IL-1* gene with a decrease in the extracellular concentration of this cytokine and consequently a decrease in the cell signaling that activates NFκB, thereby protecting the colon of rats from the deleterious effects of the DMH (Figure 2).

The pulp of sour coconut contains high levels of polyunsaturated fatty acids (PUFAs), a total of 24% in 100g (Lopes et al., 2012; Martínez et al., 2012; Stahl et al., 2002). PUFAs exert growth inhibitory, pro-apoptotic and anti-inflammatory effects on CRC cells. PUFA metabolites like prostaglandins (PGs), leukotrienes (LTs) and lipoxins (LXs) play a significant role in colon cancer (C. Zhang et al., 2015).

In two studies, rats were induced to CRC with DMH and treated with fish oil and corn oil. In both studies, the test subjects treated with fish oil had an improvement in the effects caused by DMH on colon cells with activation of the intrinsic apoptosis pathway, attenuating carcinogenesis, suggesting that this pathway is unregulated by PUFAs (Agnihotri et al., 2016; Sharma et al., 2016). Although no increase in the expression of pro-apoptotic genes was observed in this study, Treatment IV decreased ACF and the expression of the *IL-1* gene, a pro-inflammatory cytokine, which demonstrates that the presence of PUFAs may also have contributed to the colon protection of animals. Similar data were observed by (Rosa et al., 2015) who induced ACF in the colon of rats fed normal lipid diets plus 4% olive, fish, flaxseed or soy oil (control). In the proximal portion of the colon, lower levels of ACF were found in the fish and linseed oil groups. This behavior was not, however, observed in the middle and distal regions, data corroborated in the present study. The fish oil group showed higher expression of transforming growth factor β (TGF-β) and lower expression of interleukin-8 (IL-8) in relation to the other treatments. According to the authors, fish oil demonstrated a protective effect on the precancerous mucosa of the colon in animals treated with DMH with a beneficial effect on inflammatory modulation.

Vitamin E is a generic term which refers to a family of compounds which may be divided into the subgroups tocopherols and tocotrienols. Although all-natural forms of vitamin E have potent antioxidant activity, tocotrienols are significantly more potent than tocopherols in inhibiting tumor cells (Magalhães et al., 2017). Our research group was the first to assess the vitamin E profile in the pulp and nut of the sour coconut. Of the eight vitamin E counterparts, five were identified in the pulp and nut, with the most prevalent being α-tocopherol, totaling 121.07 mcg.100g $^{-1}$.

A study with the bioactive form of vitamin E, δ-tocotrienol, showed beneficial effects on chemically induced colorectal carcinogenesis in animals, with inhibition of tumor growth and development and prevention of colorectal polyps. The same observation in human CRC cell culture showed inhibition of cell proliferation, migration and invasion, in addition to inducing cell death. According to the authors, this effect of δ-tocotrienol on cells in culture occurred through the decrease in the expression of epithelial (E-cadherin) and mesenchymal (vimentin) transition markers, of metastasis (matrix metalloproteinase 9), of angiogenesis [factor of vascular endothelial growth of angiogenesis (VEGF)], inflammation (NF-κB), Wingless/β-catenin-related integration site signaling (Wnt/β-catenin), in addition to apoptosis induction (Husain et al., 2019).

A similar study with human CRC cell culture with another bioactive form of vitamin E, γ-tocotrienol, also demonstrated inhibition of cell growth, induction of apoptosis, cell cycle arrest, and suppression of the Wnt pathway (J.S. Zhang et al., 2013). In the present study, there was a decrease in the incidence of ACF in the proximal colon of the animals treated IV, which suggests a possible anti-tumor activity attributed to this vitamin and its bioactive derivatives, supposedly due to its anti-inflammatory property, since there was a lower expression of *IL-1* gene.

3.3. Histopathological analysis and visualization of aberrant crypt foci in the counter mesenteric margin portion of the colon

Histological sections of the colon of animals from Treatments I (Control) and II (aqueous extract of sour coconut pulp) showed normal crypts with a narrow lumen, showing prismatic enterocytes with nuclei in the basal portion of the cells and marked abundance of goblet cells (Figure 3A and B). Observing

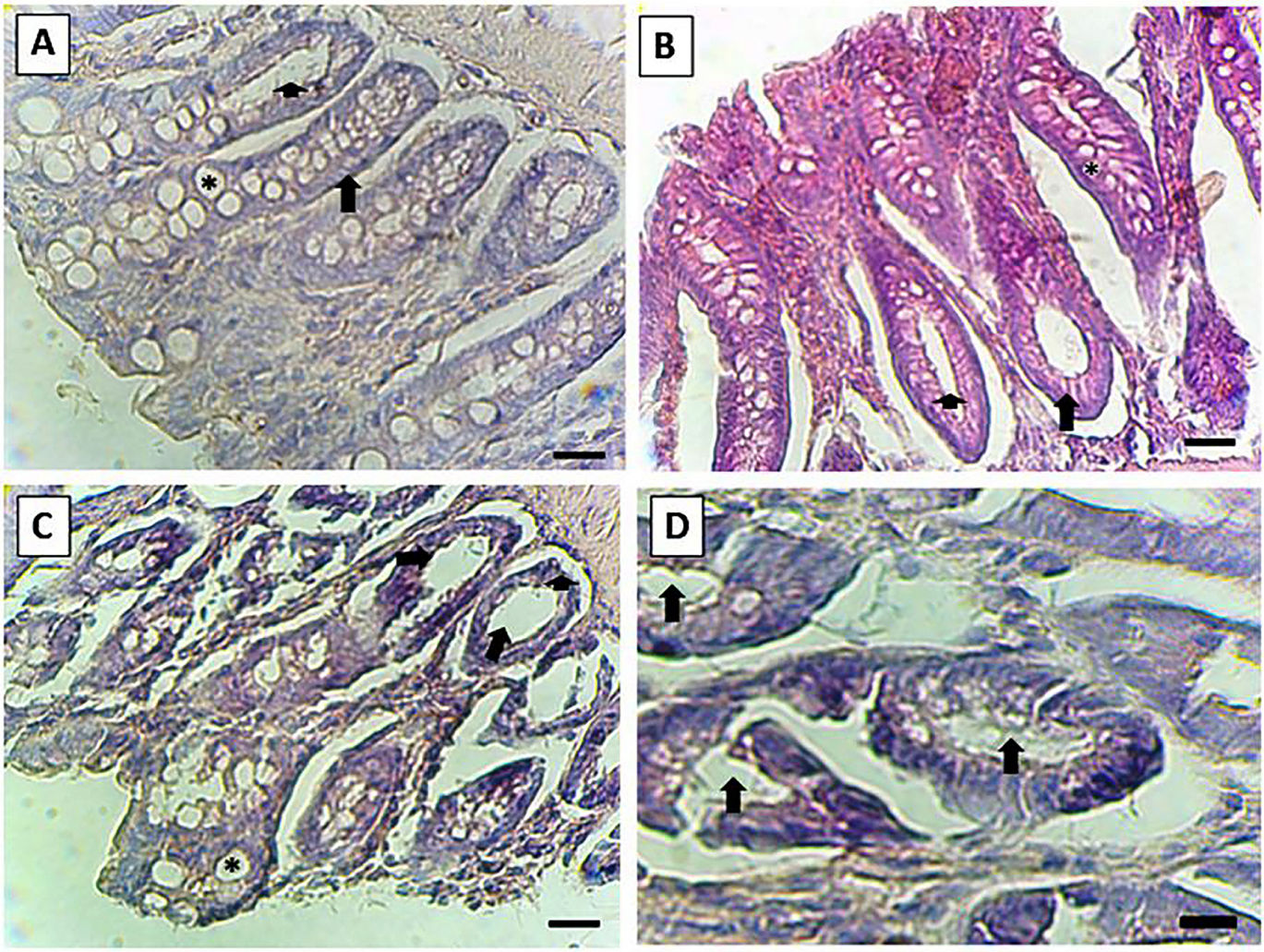

Figure 3. Photomicrographs of histological sections of the colon of animals from Treatment I (Control), in (A) and from Treatment II (Aqueous extract of sour coconut pulp) in (B). There are normal Lieberkühn intestinal crypts, showing a narrow lumen (arrowheads), with prismatic enterocytes (arrows) and numerous goblet cells displaying a clear halo (asterisk). Hematoxylin and Eosin (HE) staining. Bar = 25 μm. Photomicrograph (C) of histological section of the colon of animal from Treatment III (DMH). Note Lieberkühn's intestinal crypts with increased lumen (arrows) and marked reduction of goblet cells (asterisk), with nuclear atypia at the base of the crypt, characterized by process of dysplasia (arrowhead). In the photomicrograph (D), of a histological section from the colon of an animal from treated IV (Aqueous extract of sour coconut pulp + DMH), at higher magnification, less dilatation is observed at the base of the crypts, with a moderately dilated lumen (arrow). Hematoxylin and Eosin (HE) staining. (A, B and C) magnification = 400x and Bar = 25 μm; (D) magnification = 1000x and Bar= 75 μm. DMH: 1,2-dimethylhydrazine.

the superior view of the portion of the counter mesenteric margin of the proximal colon of the animals in Treatments I and II, no ACF was observed (Figure 4A and B).

The histopathological analysis of the histological sections of the colon of the animals of Treatments III (DMH) and IV (aqueous extract of sour coconut pulp + DMH), evidenced a reorganization in the histoarchitecture of the intestinal crypts (Figure 3C and D).

In the histological sections of the colon of the animals of Treatment III (Figure 3C), ACF was characterized by an increase in the diameter of the crypts, with an observed dilation of the lumen and massive decrease in goblet cells. At the base of the crypt, a marked proliferation with intense dysplasia of enterocytes appeared, both elongated and reorganized in the form of pseudostratified epithelium. Observing the superior view of the portion of the counter mesenteric margin of the proximal colon of the animals, one could visualize foci with two (Figure 4C**),** three (Figure 4C1) and eight (Figure 4C2) aberrant crypts.

In the histological sections of the large intestine of the test subjects in Treatment IV, less dilatation was observed at the base of the crypts, with a moderately dilated lumen (Figure 3D). Analyzing the images of the superior view of the contra mesenteric margin, one could visualize foci with two (Figure 4D), three (Figure 4D1) and six (Figure 4D2) aberrant crypts in the colon of the animals. In Figure 4C and D,

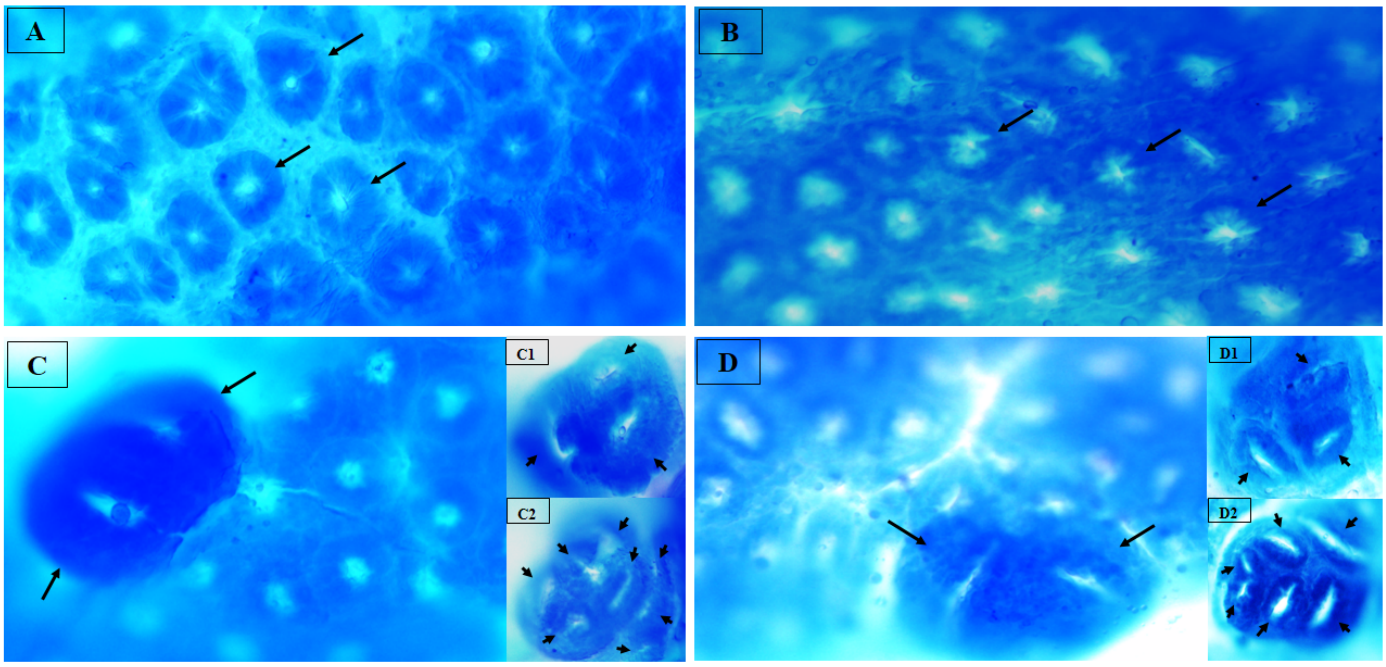

Figure 4. Top view of the counter mesenteric margin portion of the proximal colon of animals from treatments I to IV. In treatments I (figure A) and II (figure B) no aberrant crypt foci (ACF) were observed. Arrows show normal crypt foci in both treatments. In Treatment III, in Figure C one can observe foci with two aberrant crypts (long arrows), in C1 and C2 foci with three crypts and eight aberrant crypts (long arrows) respectively. In Treatment IV, in Figure D we can see foci with two aberrant crypts (long arrows), in D1 and D2 foci with three crypts and six aberrant crypts (long arrows) respectively. In figures C and D, it is also possible to observe a reorganization of the architecture of the contra mesenteric margin of the colon of the animals from treatments III and IV. Staining with methyleneblue, magnification = 100x.

one could also observe a reorganization of the architecture of the contra mesenteric margin of the colon in the animals of Treatments III and IV. The test subjects in Treatment IV had a lower incidence of ACF.

The data observed in the present study corroborate those reported by (Gunasekaran et al., 2019) who evaluated the preventive activity of the phenolic compound p-methoxycinnamic acid in rats chemically induced to CCR with DMH. In the histopathological analysis of the colon, the authors observed that the control group presented normal colonic mucosa, while the group that received DMH presented irregular mucosa, necrosis and neoplastic transformation of the glands. The group that received p-methoxycinnamic acid for a longer period had regular glands without hyperplasia. The test subjects that received the phenolic compound also had a reduced number of ACF.

The same was observed by Hamiza et al. (2012) when evaluating the effect of tannic acid on DMH-induced changes in colon histoarchitecture. The control group presented normal colonic histoarchitecture with glandular structure and normal mucosa, with a slight infiltrate of inflammatory cells. The group that received DMH had massive infiltration of inflammatory cells with formation of crypt abscesses. The groups that received increasing concentrations of tannic acid showed substantial improvement in mucosal histoarchitecture with mild inflammatory cell infiltrate. The data observed in the present study are in agreement with those found by those authors, which suggests that the high amount of phenolic compounds in sour coconut protected the colon mucosa of the animals undergoing Treatment IV, thus preserving its histoarchitecture.

In another study (Husain et al., 2019) using δ-tocotrienol, a bioactive form of vitamin E to inhibit CRC growth and development, the authors observed that the test subjects which received this compound had mild hyperplasia and normal colonic mucosa when compared to the group that received only the carcinogenesis-inducing drug. The findings of the present study are also in agreement with those authors, demonstrating that sour coconut is a source of phenolic compounds and vitamin E, thus acting to prevent the appearance of ACF, as well as to preserve the histoarchitecture of the colon of the animals used in this experiment.

Noteworthy is that Treatment III showed the greatest expression of the *BAD*, *BAX* and *IL-1* genes, which suggests that the increase in the expression of these genes may be related to poor prognosis in the prevention of ACF chemically induced with DMH in the colon of Wistar rats. Furthermore, in Treatment IV, expression of these genes was attenuated (Figure 2), with a lower incidence of ACF and preservation of colon histoarchitecture, thus demonstrating that sour coconut negatively regulates the responses of inflammatory cells and is effective in preventing CRC.

4. CONCLUSION

Sour coconut probably prevents ACF due to numerous bioactive and especially phenolic compounds, PUFAs and vitamin E, which seems to have contributed to high anti-inflammatory potential and control of the expression of the *IL-1* gene. Our results suggest that the consumption of sour coconut should be encouraged in a healthy nutritional diet, mainly due to the presence of bioactive compounds.

CONFLICTS OF INTEREST

All the authors declare that there are no conflicts of interests.

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ETHICAL APPROVAL

The Ethics Committee on the Use of Animals - CEUA / UFV, approved the experiment, process N°. 17/2014.

AUTHOR CONTRIBUTIONS

Antônio Frederico de Freitas Gomides: Conceptualization, Methodology, Research, Writing, Visualization and Supervision; Luana Venuto Santos: Curatorship, Writing; Maria Cristina de Albuquerque Barbosa: Conceptualization, Methodology, Review; Leandro de Morais Cardoso: Conceptualization, Methodology, Review; Maria do Carmo Gouveia Peluzio: Resources, Research, Review; Tiago de Melo Silva: Research; Thaisa Netto Souza Valente: Formal analysis, Curatorship; Pedro Augusto Braga dos Reis: Validation, Formal Analysis, Review; Maria Anete Santana Valente: Conceptualization, Methodology, Validation, Research, Resources, Writing, Supervision and Project Management.

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