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Enhancement of the Antigenotoxic and Antioxidant Actions of Eugenol from Spice Clove and the Stabilizer Gum Arabic on Colorectal Carcinogenesis

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Abstract

Spices are defined as any aromatic condiment of plant origin used to alter the flavor and aroma of foods. Besides flavor and aroma, many spices have antioxidant activity, mainly related to the presence in cloves of phenolic compounds, such as flavonoids, terpenoids and eugenol. In turn, the most common uses of gum arabic are in the form of powder for addition to soft drink syrups, cuisine and baked goods, specifically to stabilize the texture of products, increase the viscosity of liquids and promote the leavening of baked products (e.g., cakes). Both eugenol, extracted from cloves, and gum arabic, extracted from the hardened sap of two species of the Acacia tree, are dietary constituents routinely consumed virtually throughout the world. Both of them are also widely used medicinally to inhibit oxidative stress and genotoxicity. The prevention arm of the study included groups: Ia, IIa, IIIa, Iva, V, VI, VII, VIII. Once a week for 20 weeks, the controls received saline s.c. while the experimental groups received DMH at 20 mg/kg s.c. During the same period and for an additional 9 weeks, the animals received either water, 10% GA, EUG, or 10% GA + EUG by gavage. The treatment arm of the study included groups Ib, IIb, IIIb e IVb, IX, X, XI, XII). Once a week for 20 weeks,

the controls received saline s.c. while the experimental groups received DMH at 20 mg/kg s.c. During the subsequent 9 weeks, the animals received either water, 10% GA, EUG or 10% GA + EUG by gavage. The novelty of this study is the investigation of their use alone and together for the prevention and treatment of experimental colorectal carcinogenesis induced by dimethylhydrazine. Our results show that the combined use of 10% gum arabic and eugenol was effective, with antioxidant action in the colon, as well as reducing oxidative stress in all colon segments and preventing and treating genotoxicity in all colon segments. Furthermore, their joint administration reduced the number of aberrant crypts and the number of aberrant crypt foci (ACF) in the distal segment and entire colon, as well as the number of ACF with at least 5 crypts in the entire colon. Thus, our results also demonstrate the synergistic effects of 10% gum arabic together with eugenol (from cloves), with antioxidant, antigenotoxic and anticarcinogenic actions (prevention and treatment) at the doses and durations studied, in the colon of rats submitted to colorectal carcinogenesis induced by dimethylhydrazine.

Keywords

Eugenol, Gum Arabic, Carcinogenesis, Oxidative Stress, Genotoxicity

1. Introduction

Colorectal cancer (CRC) includes tumors that afflict the large intestine, rectum and anus [1]. In the United States, the most recent estimate is that 609,360 deaths occurred in 2022, corresponding to nearly 1700 deaths per day, making CRC the third most deadly type of cancer, in both sexes [2]. In Brazil, the future estimates for the 2023-2025 period are 704 thousand new cases of cancer, of which 21,970 will be colorectal cancer in men and 23,660 among women [1]. In the world, it is estimated 3.2 million new cases of CRC and 1.6 million deaths by 2040 [3]. Epidemiological evidence points to an increase in the incidence of colorectal cancer, making it an important global health problem with a substantial and growing economic impact, with a total cost of the disease in the order of US$ 1.16 trillion [4].

Carcinogenesis is a process that occurs gradually and involves three correlated phenomena: alteration of the molecular messaging of normal cells (irreversible alteration of DNA); promotion of clonal expansion of existing phenotypic and morphological alterations in cells; and continuation of chromosomal abnormalities, which can lead to malignancy [5]. Exogenous and endogenous factors affecting cell metabolism and/or exposure to chemical substances produce stimuli that contribute to the development of carcinogenesis [6].

The colorectal mucosa is frequently exposed to oxidative and carcinogenic nutrients, promoting the formation of reactive oxygen species (ROS), also known as free radicals [7]. From a normal physiological standpoint, the formation of ROS and their elimination by means of antioxidant mechanisms occurs

in balanced form [8], and the low levels of ROS contribute to the tissue repair process and immunity [7].

The carcinogen DMH, after being metabolized in the liver, produces the metabolites azoxymethane and methylazoxymethanol. These are then transported to the colon, where the final metabolite, diazonium, can methylate the DNA bases, generating hydroxyl radicals or hydrogen peroxide, in turn producing oxidative stress, genotoxicity and colorectal carcinogenesis [9].

Oxidative stress refers to the exacerbated production and accumulation of oxidative substances such as ROS as well as hydroxyl radicals, superoxide anions and hydrogen peroxide, among others, overwhelming the intracellular antioxidant activity [10]. The endogenous antioxidant defense system is composed of various molecules: superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, vitamins E and C, among others. These antioxidants can be found in phenolic compounds and flavonoids, present in the normal diet (fruits, leafy vegetables, roots, spices and herbs), with great importance in reducing oxidative damage and reducing the risk of developing malignant neoplasms [11].

Excessive ROS act to oxidize macromolecules (nucleic acids, lipids and proteins), generating an inflammatory process, with secretion of cytokines and chemokines by immune cells, which in turn produces oxidative stress in the affected area. This oxidative stress activates transcription factors (NF-κB, TP53, HIF-1α, PPAR-γ, Nrf2, AP-1), altering the expression of many other genes and proteins [12], and deregulating the oncogenic signaling pathways that are involved in the carcinogenesis of CRC [8].

Evidence suggests that the production of ROS is fundamental at all stages of colorectal carcinogenesis [11]. The tumor cells produce more ROS than normal cells, causing oxidative stress and contributing to the progression of the disease [13]. As the CRC advances, the level of oxidative stress increases [9]. In the case of DNA, the ROS induces damages such as single or double-strand breaks or modification of the nucleotide base. The final products of the lipid peroxidation react with the DNA bases, giving rise to DNA adducts, which can promote colorectal carcinogenesis [7].

In response to the damage to the DNA, a set of responses is activated. These responses include recognition of damages, activation of checkpoints, cell cycle arrest, and possibly final repair, apoptosis and immune clearance [14]. The defective DNA replication and repair of the damages in the cancer cells contribute to the accumulation of genetic alterations, contributing to the development of carcinogenesis and tumor progression [15].

Genotoxicity consists of the ability to induce damage to the cellular genetic material, in turn altering the DNA sequence or its structure by means of mutations, recombinations or chromosomal aberrations [16] in the presence of the action of genotoxins such as DMH, the carcinogen employed in this study [17].

Gum arabic is a natural resin obtained from the trunks and branches of Acacia trees [18] It has many biological properties reported in the literature, among them important antioxidant and anti-inflammatory activities, and can thus fa-

vorably influence the clinical conditions of diseases that involve oxidative stress, such as neoplasms [19]. In rodents with colorectal carcinogenesis induced by azoxymethane, gum arabic was found to reduce aberrant crypt foci [20] and to have antioxidant and antigenotoxic activity [21].

Eugenol is a phenolic compound [22] present in the oil derived from cloves. This oil is the main natural source of the compound (90% to 95% of the total). It has a characteristic aroma [23] and important biological activities described in the literature, such as analgesic [24], anti-inflammatory [25], antiviral [26], antibacterial [27], antioxidant [28], antineoplastic [29] and anti-metastatic [30].

The objective of this study was to assess the effects of gum arabic and eugenol on oxidative stress and genotoxicity in the colon of Wistar rats submitted to colorectal carcinogenesis induced by dimethylhydrazine (DMH).

2. Materials and Methods

2.1. Study Design

The study protocol complied with the guidelines of the National Board for the Control of Animal Testing (CONCEA) and was approved by the Animal Research Ethics Committee (CEUA) of the Federal University of Ceará (UFC) (protocol #1675020519).

Prevention was evaluated using, 4 control groups (Ia, IIa, IIIa and IVa each group with n = 6) and 4 experimental groups (V, VI, VII and VIII, each group with n = 10), the study used 64 female *Wistar* rats. Once a week, for 20 weeks, the control groups received saline solution (s.c), while the experimental groups received DMH at 20 mg/kg s.c. During 29 weeks, the animals received water (groups Ia and V), GA 10% (groups IIa and VI), EUG (groups IIIa and VII) and GA 10% + EUG (groups IVa and VIII) by gavage.

Treatment was evaluated using, 4 control groups (Ib, IIb, IIIb, IVb each group with n = 6) and 4 experimental groups (IX, X, XI and XII, each group with n = 10), the study used 64 female Wistar rats. Once a week for 20 weeks, the control groups received saline s.c., while the experimental groups received DMH at 20 mg/kg, s.c. During the subsequent 9 weeks, the animals received water (groups Ib and IX), 10% GA (groups IIb and X), EUG (groups IIIb and XI) or 10% GA + EUG (groups IVb and XII) (**Figure 1**).

2.2. Substances

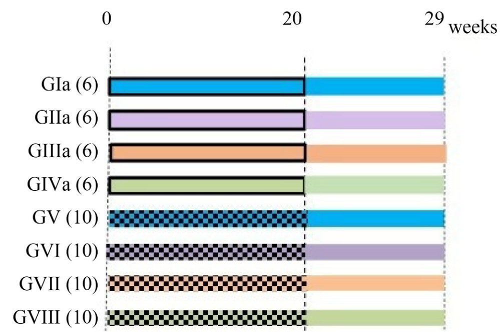
Eugenol (EUG)

EUG (Laboratory Quinari) (**Figure 2**) was administered orally using a pipette at 100 mg/kg body weight 3 times a week [31].

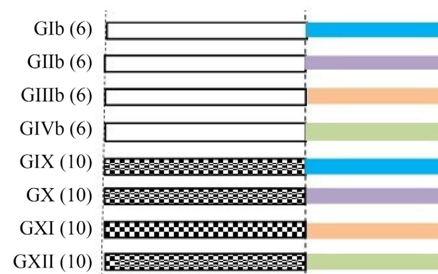
Gum arabic (GA)

Gum arabic (Dinâmica Química Contemporânea Ltda) (**Figure 3**) was diluted in distilled water at 10% [32] and administered by gavage at 5 mL/kg body weight 4 times a week.

Prevention (use of substances concomitantly with the carcinogen)



Treatment (use of substances after carcinogen exposure)



Legend

Water (5 ml/ kg body weight)	Water + DMH
10% Gum arabic (5 ml/ kg body weight)	10% Gum arabic + DMH
Eugenol (100 ml/kg body weight)	Eugenol +DMH
Eugenol + Gum arabic	Eugenol + Gum arabic+ DMH
Euthansia	DMH 0.9% saline

Figure 1. Design experimental.

Figure 2. Clove extract.

Figure 3. Gum arabic.

Carcinogen (DMH)

To induce cancer we used symmetrical 1,2-dimethylhydrazine dihydrochloride (Sigma-Aldrich Brasil Ltda) dissolved in a previously prepared 0.9% NaCl solution containing 1.5% EDTA as the vehicle, adjusted to a final pH of 6.5 using a NaOh solution [33]. The carcinogen was administered s.c at 20 mg/kg body weight once a week for 20 weeks [34].

2.3. Surgical Procedure

By the end of the experiment, the animals were anesthetized with ketamine (100 mg/Kg body weight) and xylazine (10 mg/Kg body weight) i.p. and submitted to longitudinal xyphopubic laparotomy and the complete resection of the colon was performed.

The colon was opened along the antimesenteric border and washed with saline solution, followed by removal of small fragments from the proximal, middle and distal regions for biological examination. The large intestine was rolled up in filter paper and fixed with the 10% buffered formaldehyde for 24 hours.

2.4. Preparation for Biological Assays

To evaluate oxidative stress and genotoxicity colon fragments were individually macerated in a phosphate-buffered saline (PBS) solution at 4˚C and filtered. The cells were obtained from the suspension after filtration.

2.5. Oxidative Stress Measurement

ROS dosage

The cell preparations were placed in contact with 20 μM of 2'7'-dichlorofluorescein diacetate (H2DCFDA) in the absence of light at 37˚C for 30 mins. The H2DCFDA is oxidized when contacting the intracellular ROS, becoming strongly fluorescent. The preparations were then washed, resuspended in a phosphate-buffered saline (PBS) solution, and analyzed by flow cytometry (Guava Technologies, Inc., Hayward, CA, USA®). Among the ROS involved in this process are the radicals hydroxyl (HO•), peroxyl (ROO•), and peroxynitrite (ONOO•), along with hydrogen peroxide (H_2O_2) and singlet oxygen ($1O_2$) [35]

The assays were performed as three independent experiments in triplicate so that ten thousand events were analyzed per sample.

Glutathione dosage (GSH)

After H_2O_2 (150 μM for 1 h) challenge (pre-, co-, and post-treatment protocols described above), the GSH content was determined by a spectrophotometric assay based on the formation of 5-thio-2-nitrobenzoate (TNB) from DTNB, according to Akerboom and Sies [36] (with minor modification. Briefly, treated (100, 500 and 1000 μM) and untreated PBLs (1.5×10^6 cells/mL) were washed with ice-cold PBS, resuspended in 0.1 M sodium phosphate-5 mM EDTA, pH 8.0, and sonicated to obtain the cell homogenate. An equal volume of 2 M HClO4-4 mM EDTA was added to the cell extract, and the precipitated proteins

were pelleted by centrifugation at 8000 g for 15 min at 4˚C. The supernatant was neutralized with 2 M KOH, and the insoluble residue was removed by centrifugation under the same conditions. For spectrophotometric determination, 910 μL of the cell extract supernatant or of a standard GSH solution, in the same phosphate–EDTA buffer, were mixed with 50 μL of 4 mg/mL NADPH in 0.5% (w/v) $NaHCO_3$, 20 μL of 6 U/mL glutathione reductase in phosphate-EDTA buffer, and 20 μL of 1.5 mg/mL DTNB in 0.5% $NaHCO_3$. The increase in absorbance was measured at 412 nm. The results were normalized by protein content [37] and were expressed as μg/mg protein.

2.6. Genotoxicity Tests

Comet assay

The level of DNA damage was determined by comet assay under alkaline and neutral conditions, as described by Hartmann and Speit [38] and Wojewodzka, Buraczewska, Kruszewski [39], respectively.

Modified alkaline comet assay

The modified alkaline comet assay was used to enhance the sensitivity and specificity, the alkaline comet assay was carried out with the addition of the enzyme human 8-oxoguanine DNA-N-glycosylase 1 (hOGG1), which identifies oxidized nitrogen bases such as 8-oxoguanine (8-oxoGua) the test was performed as described by Smith, ÓDonovan and Martin [38]

2.7. Evaluation of the ACF

After fixing for 24 h, each colon was immersed in a 0.1% methylene blue solution buffered with phosphate for 1 min. Then the mucosa of each colon was examined under a stereomicroscope (Vasconcellos M90, DF Vasconcellos S.A.®) with 40× magnification to determine the number of ACF and number of crypts per focus (multiplicity) in each colon segment (proximal, middle and distal).

3. Statistical Analysis

With regard to genotoxicity and oxidative stress, the quantitative variables were also analyzed by the Shapiro-Wilk test to verify their normal distribution. When that requirement was met, analysis of variance for two classification factors was used to evaluate the effects of the treatments (factor 1: AD, GA, EUG and GA + EUG) and of exposure to the carcinogen (factor 2: DMH or SF) on the parameters for quantification of genotoxicity and oxidative stress, considering both the prevention protocol and the treatment protocol. This was complemented by the Tukey multiple comparisons test (comparisons between treatments of the rats exposed and not exposed to the carcinogen, as well as comparisons between those exposed and not exposed to the carcinogen for each treatment).

In the case of aberrant crypts, the quantitative variables (*i.e.*, the counts of aberrant crypts, aberrant crypt foci and number of crypts per focus were initially

analyzed by the Shapiro-Wilk test to verify the normal distribution. Since that requirement was not satisfied for any of the variables, we calculated the median and interquartile range (percentile 25 - percentile 75), and then applied nonparametric tests. In the analysis of these variables, we only considered the groups of animals exposed to the carcinogen (DMH) and treated concomitantly (prevention protocol) and subsequently (treatment protocol) with gum arabic (DMH-GA), eugenol (DMH-EUG), and the association of the two substances (DMH-GA + EUG) or the vehicle (distilled water, DMH-AD), since the measures of those variables were equal to zero in the groups not exposed to the carcinogen. Therefore, pairwise comparisons between the groups DMH-AD, DMH-GA, DMH-EUG and DMH-GA + EUG, both for the prevention protocol and treatment protocol, in relation to the referred variables were carried out by the Kruskal-Wallis test associated with Dunn's multiple comparisons test, to verify pairwise differences between the groups.

In all analyses, we used two-tailed tests with a significance level of 0.05 (5%), so that statistical significance was indicated by P-value < 0.05. All the statistical procedures and preparation of graphs were carried out with GraphPad Prism version 8.0 (GraphPad Software, San Diego, California, USA).

4. Results

Oxidative stress measurement and genotoxicity tests

Prevention (use of substances concomitantly with the carcinogen)

ROS dosage

The groups that received EUG (*P < 0.05) and GA + EUG (*P < 0.05) in the proximal segment(Figure 4), GA (***P < 0.001), EUG (**P < 0.01) and GA + EUG (***P < 0.001) in the middle segment (Figure 5) and GA + EUG (**P < 0.01) in the distal segment (Figure 6) significantly reduced the amount of ROS compared to the control (AD).

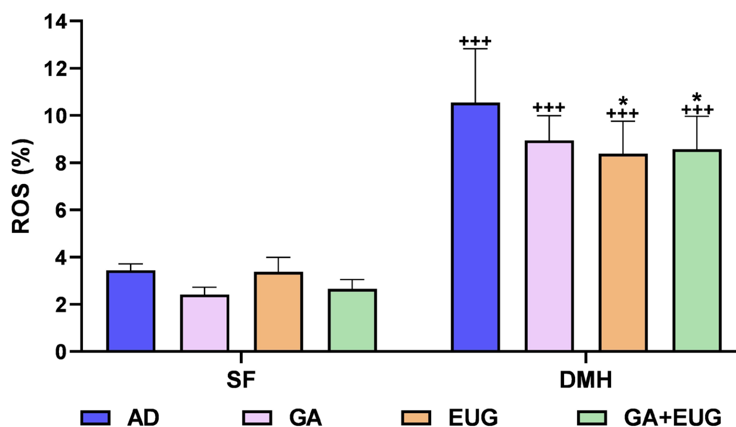


Figure 4. Dosage of reactive oxygen species (ROS) in the proximal segment of the colon. Legend: +++ DMH significantly increased the amount of ROS (P < 0.001). * denotes statistically significant difference EUG, GA + EUG (P < 0.05).

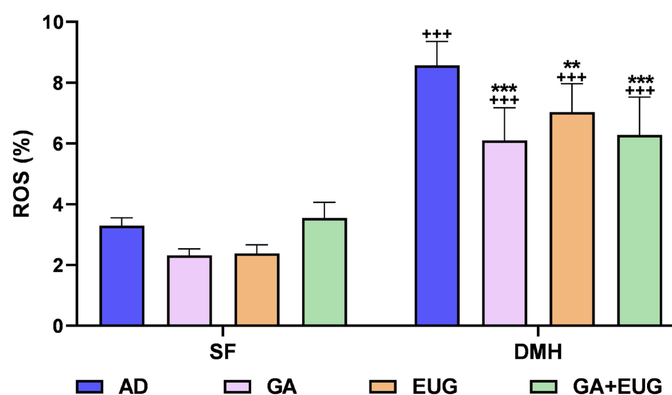


Figure 5. Dosage of reactive oxygen species (ROS) in the middle segment of the colon. Legend: +++ DMH significantly increased the amount of ROS (P < 0.001). *** denotes statistically significant difference GA (P < 0.001), GA + EUG (P < 0.001). ** denotes statistically significant difference EUG (P < 0.01).

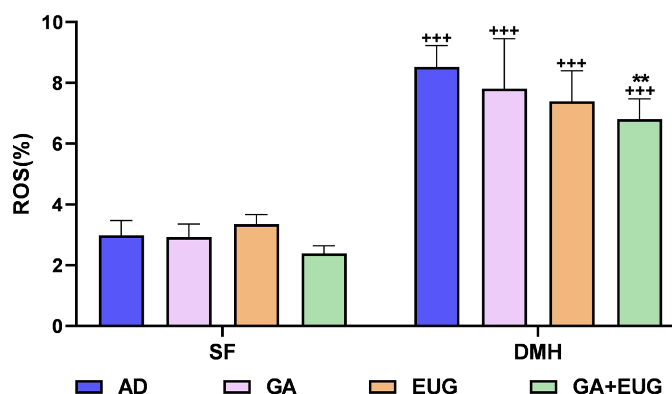


Figure 6. Dosage of reactive oxygen species (ROS) in the distal segment of the colon. Legend: +++ DMH significantly increased the amount of ROS (P < 0.001). ** denotes statistically significant difference GA + EUG (P < 0.01).

Glutathione dosage (GSH)

The groups that received EUG (*P < 0.05) and GA + EUG (**P < 0.01) in the proximal segment significantly increased the amount of GSH compared to the control (AD) (Figure 7). in the middle and distal segments, no statistically significant differences were found (Figure 8) and Figure 9).

Comet assay

The groups that received GA, EUG and GA + EUG significantly reduced the DNA damage index in the segments: proximal (***P < 0.001) without enzyme (Figure 10) and with enzyme (Figure 11); no statistical significance occurred between the groups that received the substances and the carcinogen without enzyme in the middle segment (Figure 12); middle segment (*P < 0.05) with enzyme (Figure 13); distal (**P < 0.001), EUG (**P < 0.01) and GA + EUG (**P < 0.01)) without enzyme (Figure 14). with the enzyme, GA significantly reduced **P < 0.01 (Figure 15) compared to control (AD).

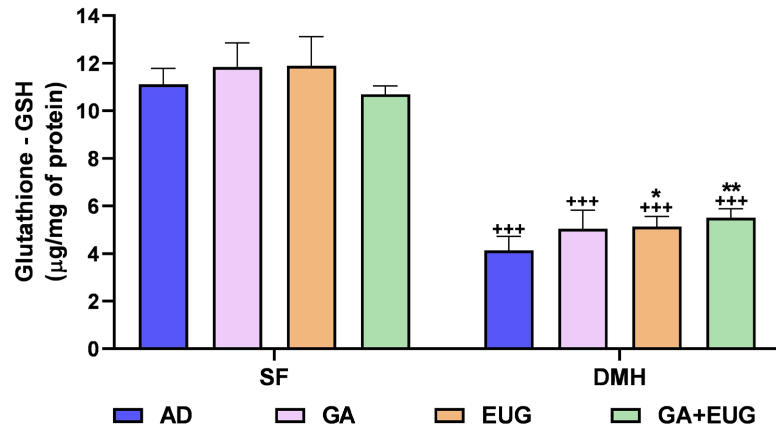


Figure 7. Dosage of glutathione (GSH) in the proximal segment of the colon. Legend: +++ DMH significantly reduced the amount of GSH (P < 0.001). * denotes statistically significant difference EUG (P < 0.05). ** denotes statistically significant difference GA + EUG (P < 0.01).

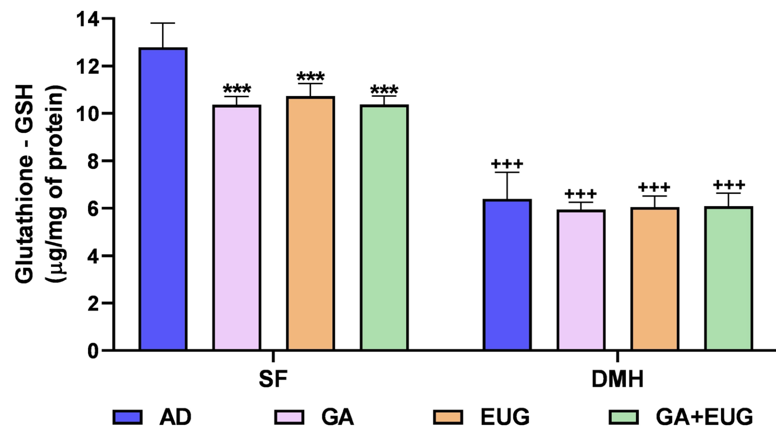


Figure 8. Dosage of glutathione (GSH) in the middle segment of the colon. Legend: +++ DMH significantly increased the amount of ROS (P < 0.001).

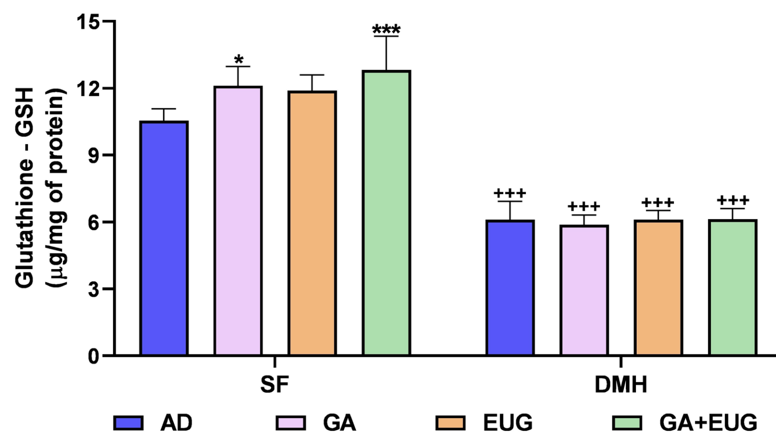


Figure 9. Dosage of glutathione (GSH) in the distal segment of the colon. Legend: +++ DMH significantly increased the amount of ROS (P < 0.001).

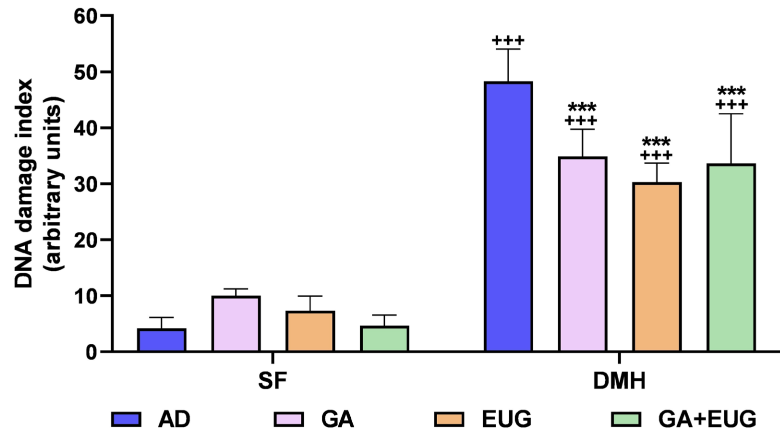


Figure 10. Comet assay in the proximal colon. Legend: +++ DMH significantly increased the DNA damage index (P < 0.001). *** denotes statistically significant difference GA, EUG, GA + EUG (P < 0.001).

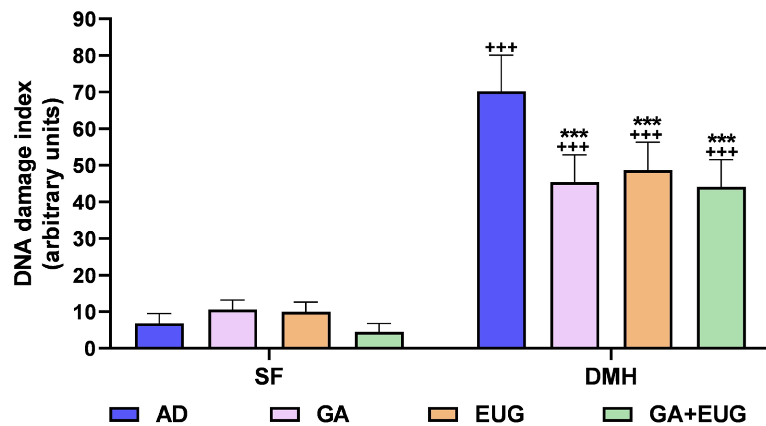


Figure 11. Comet assay in the proximal colon with enzyme FPG. Legend: +++ DMH significantly increased the DNA damage index (P < 0.001). *** denotes statistically significant difference GA, EUG, GA + EUG (P < 0.001).

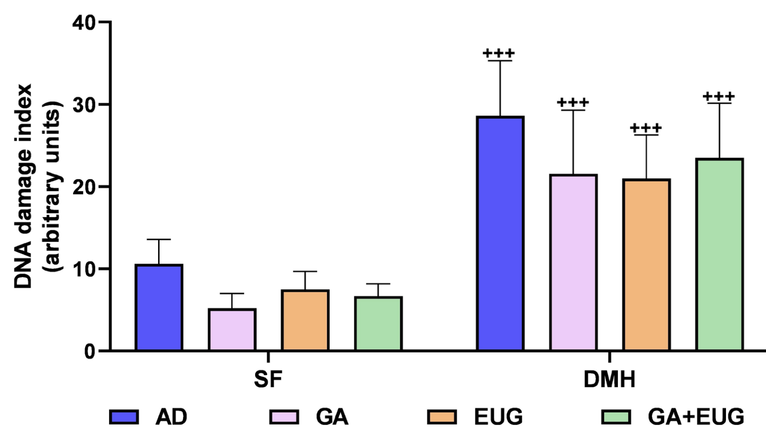


Figure 12. Comet assay in the middle colon. Legend: +++ DMH significantly increased the DNA damage index (P < 0.001).

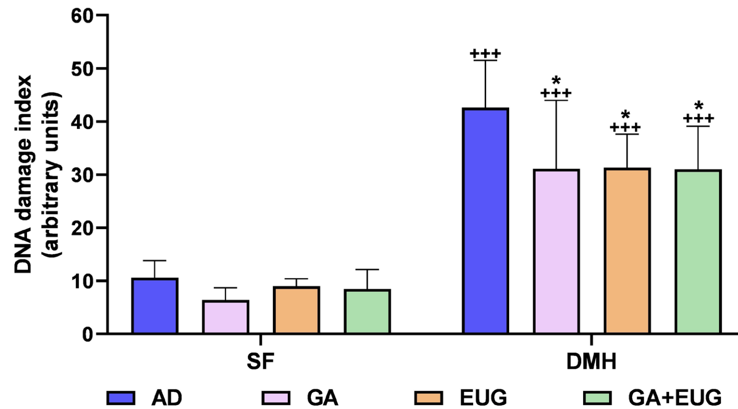


Figure 13. Comet assay in the middle colon with enzyme FPG. Legend: +++ DMH significantly increased the DNA damage index ($P < 0.001$). * denotes statistically significant difference GA, EUG e GA + EUG ($P < 0.05$).

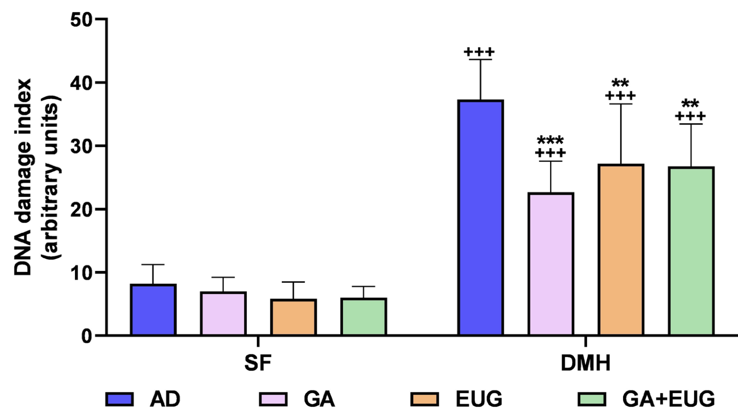


Figure 14. Comet assay in the distal colon. Legend: +++ DMH significantly increased the DNA damage index ($P < 0.001$). ** denotes statistically significant difference EUG, GA + EUG ($P < 0.01$). *** denotes statistically significant difference GA ($P < 0.001$).

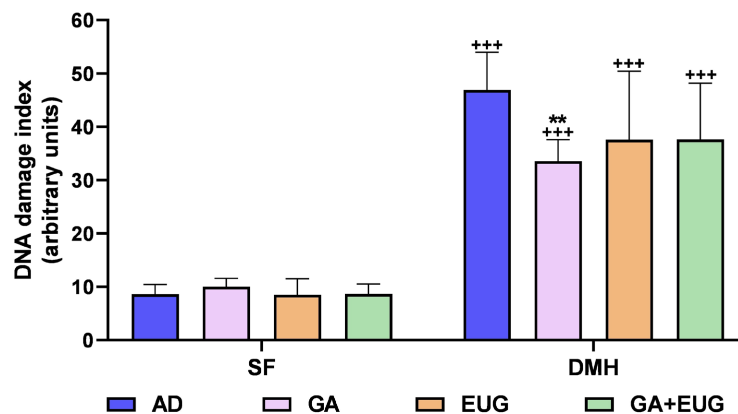


Figure 15. Comet assay in the distal colon with enzyme FPG. Legend: +++ DMH significantly increased the DNA damage index ($P < 0.001$). ** denotes statistically significant difference GA ($P < 0.01$).

Aberrants Crypts

Number of Aberrant Crypts (Total and Per Colon Segment)

Analyzing the aberrant crypts observed in groups V, VI, VII and VIII, by segment of the colon (proximal, middle or distal), a statistically significant reduction of this number was observed in group GVIII (GA + EUG + DMH) in relation to group GVII (EUG + DMH) in the distal segment ($P = 0.0189$) (Figure 14) and in the total colon ($P = 0.0261$).

Aberrant Crypt Foci (ACF)

Analyzing the number of aberrant crypt foci observed in groups V, VI, VII and VIII, by colonic segment (proximal, middle or distal), as well as its entire extension, statistically significant differences were observed between the groups ($P = 0.0495$) in the distal colon (Figure 16), comparisons between groups in pairs, using Dunn's multiple comparison test, showed only a marginally significant difference ($P = 0.0513$) between groups VIII (DMH- GA-EUG) and VII (DMH-EUG). However, there were statistically significant differences in group VIII (DMH-GA-EUG) when compared to group VII (DMH-EUG) in the total colon.

Multiplicity of Crypts (Foci with up to five aberrant crypts)

Analyzing the number of aberrant crypt foci with less than 5 crypts observed in groups V, VI, VII and VIII, by segment of the colon (proximal, middle and distal), as well as its entire extension, a statistically significant reduction was observed. significant difference of this number in group GVIII (GA + EUG + DMH) compared to group GVII (EUG + DMH) in total colon ($P = 0.0309$)

Treatment (use of substances after carcinogen exposure)

ROS dosage

In the proximal segment, treatments with GA, EUG and GA + EUG significantly increased (***$P < 0.001$) the amount of ROS compared to the control (AD), while the amount of ROS verified in the GA + EUG group was significantly higher (###$P < 0.001$) than that observed in the GA group. In the groups not exposed to the carcinogen (SF), treatments with EUG ($$P < 0.01) and GA + EUG ($$P < 0.01) significantly decreased the amount of ROS compared to the control (AD) (Figure 16). In the middle segment, treatments with GA (*$P < 0.05$) and EUG (**$P < 0.01$) significantly increased the amount of ROS compared to control (AD) (Figure 17). Exposure to the DMH carcinogen significantly increased the amount of ROS (+++$P < 0.001$), regardless of the treatment used (AD, GA, EUG or GA + EUG) in the distal segment of the colon, with no statistical significance occurring between the groups that received the substances and the carcinogen (Figure 18)

Glutathione dosage (GSH)

In the proximal segment, treatments with GA (*$P < 0.05$), EUG (***$P < 0.001$) and GA + EUG (***$P < 0.001$) significantly increased the amount of GSH compared to control (AD), while the amount of GSH verified in the GA + EUG group was significantly higher (#$P < 0.05$) than that observed in the GA group. In the carcinogen-unexposed (SF) groups, treatment with GA + EUG ($P < 0.05) significantly decreased the amount of GSH compared to the EUG group (Figure 19).

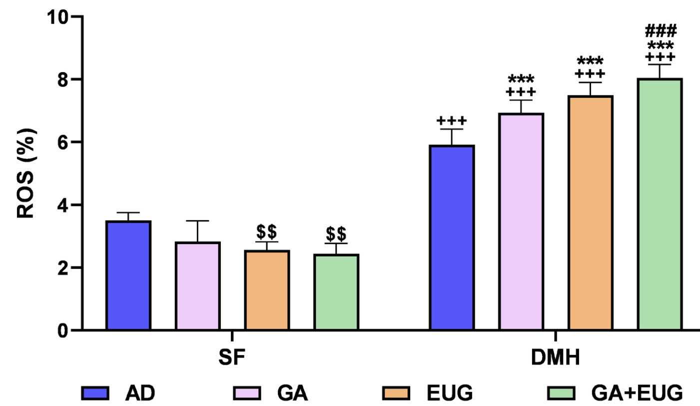


Figure 16. Dosage of reactive oxygen species (ROS) in the proximal segment of the colon. Legend: +++ DMH significantly increased the amount of ROS (P < 0.001). *** denotes statistically significant difference GA, EUG, GA + EUG (P < 0.001). ### denotes statistically significant difference GA + EUG (P < 0.001).

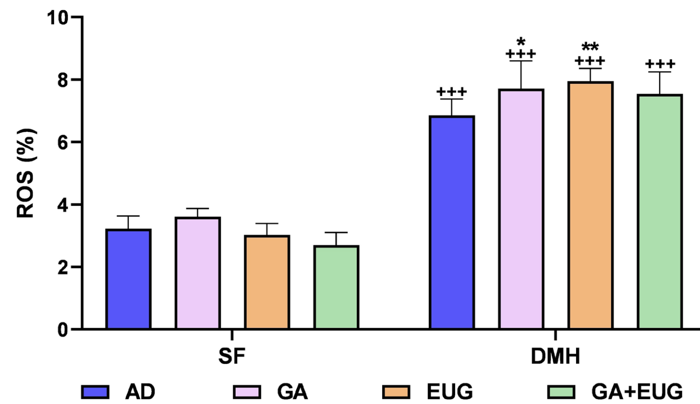


Figure 17. Dosage of reactive oxygen species (ROS) in the middle segment of the colon. Legend: +++ DMH significantly increased the amount of ROS (P < 0.001). * denotes statistically significant difference GA (P < 0.05) e EUG (P < 0.01).

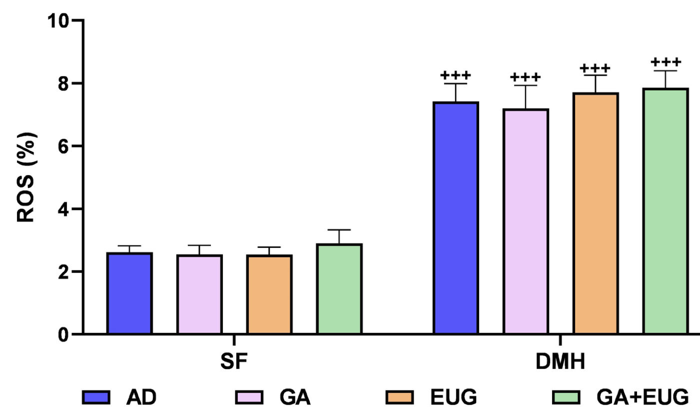


Figure 18. Dosage of reactive oxygen species (ROS) in the distal segment of the colon. Legend: +++ DMH significantly increased the amount of ROS (P < 0.001).

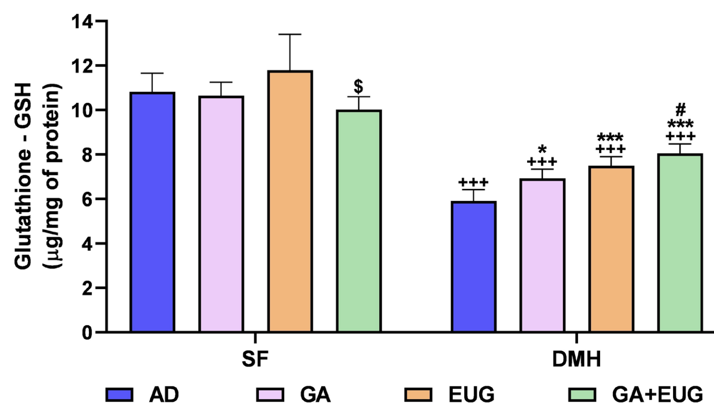


Figure 19. Dosage of glutathione (GSH) in the proximal segment of the colon. Legend: +++ DMH significantly reduced the amount of GSH ($P < 0.001$). * denotes statistically significant difference GA (*$P < 0.05$). *** denotes statistically significant difference EUG, GA + EUG (***$P < 0.001$). # denotes statistically significant difference GA + EUG (#$P < 0.05$).

Exposure to the carcinogen DMH significantly reduced the amount of GSH (+++$P < 0.001$), regardless of the treatment used (AD, GA, EUG or GA + EUG) in the middle (Figure 20) and distal (Figure 21) segment of the colon, with no statistical significance between the groups that received the substances and the carcinogen.

Comet assay

In the proximal segment, treatments with GA (*$P < 0.05$) and GA + EUG (***$P < 0.001$) without enzyme (Figure 22); GA (***$P < 0.001$), EUG (**$P < 0.01$) and GA + EUG (***$P < 0.001$) with enzyme (Figure 23), in the middle segment GA (*$P < 0.05$), EUG (**$P < 0.01$) and GA + EUG (***$P < 0.001$) without enzyme (Figure 24) GA, EUG and GA + EUG (***$P < 0.001$) with enzyme (Figure 25), in the distal segment GA (**$P < 0.01$), EUG (***$P < 0.001$) and GA + EUG (***$P < 0.001$) without enzyme (Figure 26), GA, EUG and GA+ EUG (***$P < 0.001$) with enzyme (Figure 27) significantly reduced the DNA damage index compared to control (AD).

Aberrants Crypts, Number of Aberrant Crypts (Total and Per Colon Segment), Aberrant Crypt Foci (ACF) and Multiplicity of Crypts

Analyzing the number of aberrant crypts, number of foci and multiplicity of crypts observed in groups IX, X, XI and XII, by colon segment (proximal, middle or distal) and total colon, it was observed that there was no significant difference between the different colonic segments and neither in the total colon.

5. Discussion

The use of spices as functional foods dates back at least to 4500 BC. Currently, many studies describe the biological activities of different spices, from the regulation of blood glucose levels [39] to prevention of dental caries[40] neuroprotection [41] and even against the pandemic virus SARS-CoV-2 [42] being considered an important source of bioactive and nutraceuticals[43]

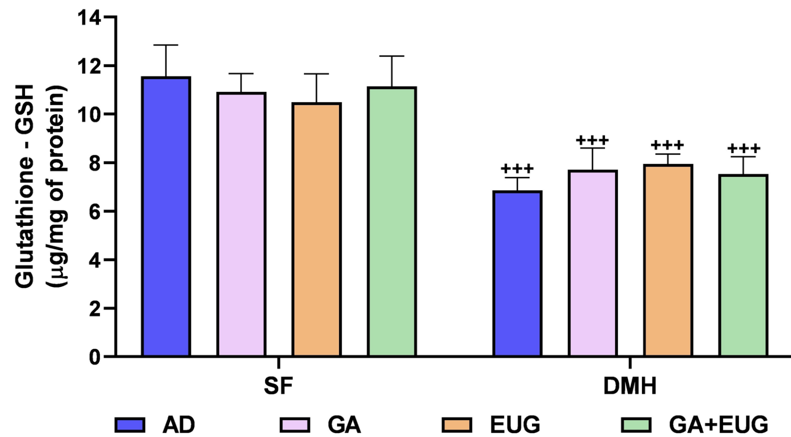


Figure 20. Dosage of glutathione (GSH) in the middle segment of the colon. Legend: +++ DMH significantly reduced the amount of GSH ($P < 0.001$).

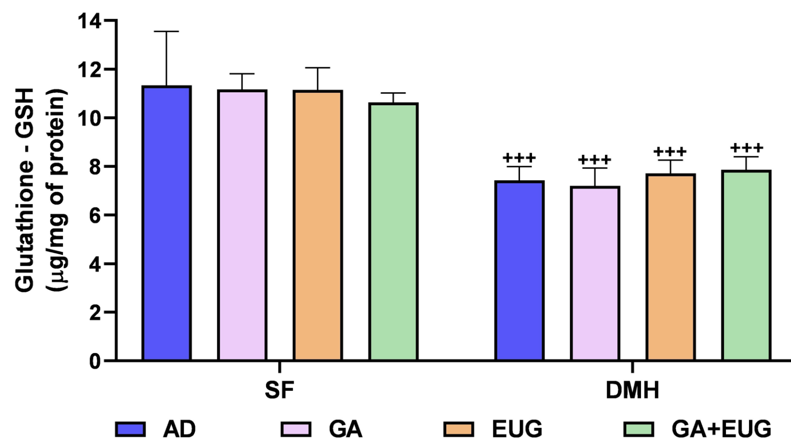


Figure 21. Dosage of glutathione (GSH) in the distal segment of the colon. Legend: +++ DMH significantly reduced the amount of GSH ($P < 0.001$).

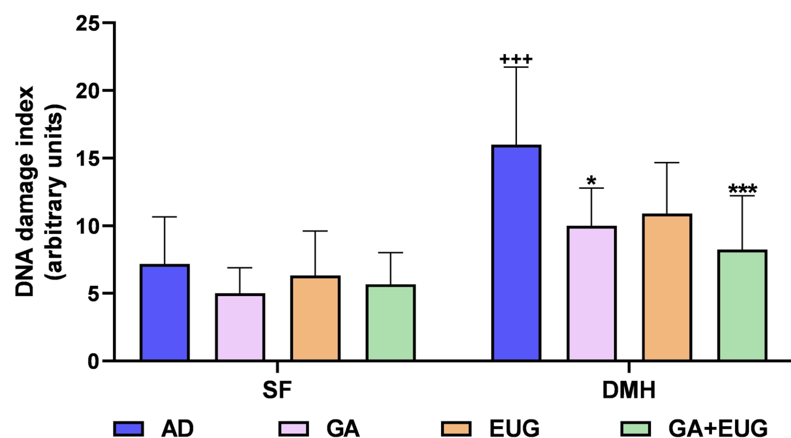


Figure 22. Comet assay in the proximal colon. Legend: +++ DMH significantly increased the DNA damage index only in the AD-treated group ($P < 0.001$). * denotes statistically significant difference GA (*$P < 0.05$). *** denotes statistically significant difference GA + EUG (***$P < 0.001$).

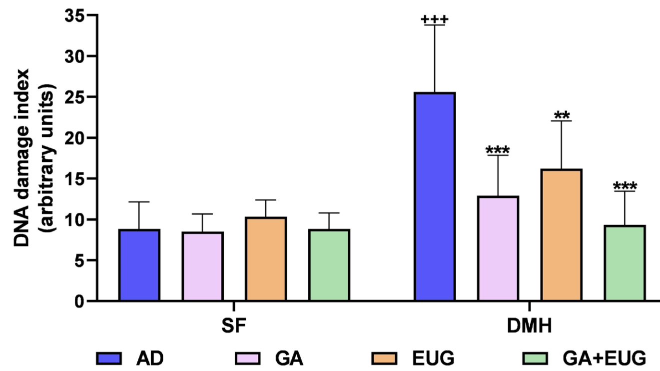


Figure 23. Comet assay in the proximal colon with enzyme FPG. Legend: +++ DMH significantly increased the DNA damage index only in the AD-treated group (P < 0.001). ** denotes statistically significant difference EUG (**P < 0.01). *** denotes statistically significant difference GA, GA + EUG (***P < 0.001).

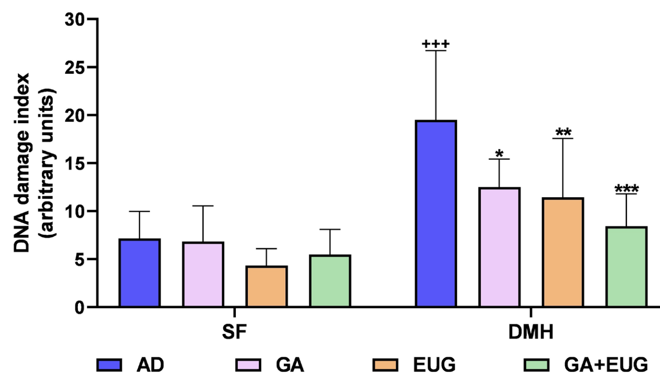


Figure 24. Comet assay in the middle colon. Legend: +++ DMH significantly increased the DNA damage index only in the AD-treated group (P < 0.001). * denotes statistically significant difference GA (*P < 0.05). ** denotes statistically significant difference EUG (**P < 0.01). *** denotes statistically significant difference GA + EUG (***P < 0.001).

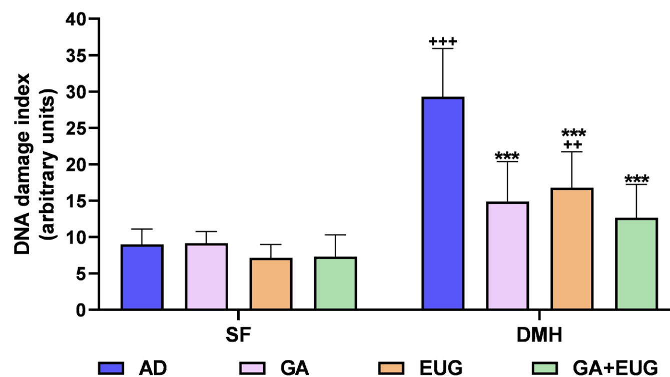


Figure 25. Comet assay in the middle colon with enzyme FPG. Legend: +++ DMH significantly increased the DNA damage index only in the AD-treated group (P < 0.001). ++ (P < 0.01) DMH significantly increased the DNA damage index in the EUG group. *** denotes statistically significant difference GA, EUG e GA + EUG (***P < 0.001).

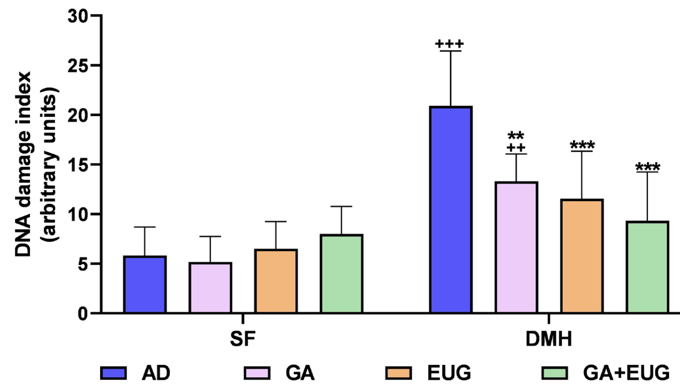


Figure 26. Comet assay in the distal colon. Legend: +++ DMH significantly increased the DNA damage index only in the AD-treated group (P < 0.001). ++ (P < 0.01) DMH significantly increased the DNA damage index in the GA group. **denotes statistically significant difference GA (**P < 0.01). *** denotes statistically significant difference EUG, GA + EUG (***P < 0.001).

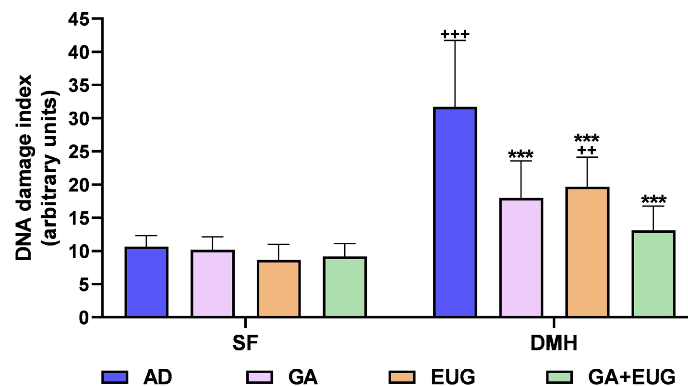


Figure 27. Comet assay in the distal colon with enzyme FPG. Legend: +++ DMH significantly increased the DNA damage index only in the AD-treated group (P < 0.001). ++ (P < 0.01) DMH significantly increased the DNA damage index in the EUG group. *** denotes statistically significant difference GA, EUG e GA + EUG (***P < 0.001).

These spices were (and are) obtained from the entire range of plant parts (bark, roots, leaves, fruits, seeds, etc.), and compose a diversified arsenal of bioactive compounds with different applications [44]. Clove (Syzygium aromaticum) is an aromatic plant containing both volatile (eugenol, isoeugenol and eugenol acetate) and non-volatile substances (flavonoids, chromones, tannins, triterpenoids, coumarin and phenolic ester). Eugenol represents more than 50% of the clove volatile oil and is responsible for the characteristic aroma [44]. The remaining 10% - 40% consists of eugenyl acetate, β-caryophyllene and α-humulin [45]. The chemical composition of clove oil varies according to the agroecological conditions and extraction method [46]. Besides the oil, the plant has varied nutritional components, consisting of proteins, fatty acids, minerals, amino acids and vitamins, conferring high biological and economic value [44].

Substances from cloves can be found in cosmetics, pharmaceuticals [47] and especially in the food industry, in the composition of biodegradable packaging, preparations for postharvest washing of horticultural products [48], as seasoning, dye and natural preservative of baked goods, chicken and beef, among others, without affecting the organoleptic characteristics of foods. Clove is classified as a safe dietary supplement by the FDA, and has recognized antioxidant, antibacterial and antifungal properties [45].

These substances' use in foods provides health benefits by modifying the intestinal microbiota and increasing the production of mucus by the internal layer of the colon, leading to protection of the mucosa against microorganisms and improving immunity to infections [49]. They also prevent metabolic alterations arising from obesity and hepatic steatosis, by reducing the accumulation of lipids, an effect mediated in part by the transient vanilloid receptor 1 (TRPV1) expressed in the functional regulation of the liver and adipose tissue. The activation of signaling of the TRPV1 promotes the accumulation of fat, while the inhibition of this signaling protects against this buildup [50].

The other substance investigated here, gum arabic, is a resin extracted from Acacia trees, originally native to Africa. It is composed of glycoproteins (a class of proteins that contain carbohydrate groups linked to the polypeptide chain), polysaccharides (carbohydrates containing various sugar molecules linked together), and oligosaccharides (another class of carbohydrates). Besides these substances, the gum is a source of natural sugar compounds called arabinose and ribose, which were among the first concentrated sugars derived from plants and trees. The exact chemical composition of gum arabic varies depending on the climate and soil conditions where the trees grow [51].

Gum arabic can be found in the composition of desserts and other sweets, such a fruit syrups, marshmallows, confectionary sugar, icings, chewing gum, chocolate candies and comestible decorative ingredients for baked goods and soft candies, such as glitter. The use of gum arabic not only helps baked goods to rise, it also adds naturally soluble fibers to recipes [51].

Gum arabic is also considered to be a natural prebiotic and source of soluble dietary fiber (a complex polysaccharide), meaning that humans cannot digest its carbohydrates. This promotes benefits to intestinal health, digestion and even cardiovascular health since the soluble fiber helps link to cholesterol. After ingestion, gum arabic ferments in the colon with the help of bacteria and other microorganisms. This essentially helps to "feed" beneficial probiotic bacteria in the gut, which have many important roles in the body. One study found that supplementation for four months with 10 grams per day of gum arabic led to significant increases of the bacteria Bifidobacteria, Lactobacteria and Bacteriodes, indicating a prebiotic effect [51].

As can be gathered from the descriptions above, both eugenol extracted from cloves and gum arabic extracted from acacia gum are common dietary constituents that can inhibit oxidative stress and genotoxicity. The novelty of this study is the examination of their administration alone and jointly on the prevention

and treatment of experimental colorectal carcinogenesis.

The colorectal carcinogenesis was induced by administration of the chemical carcinogen dimethylhydrazine (DMH), which when metabolized in the liver, results in the metabolites azoxymethane (AOM) and methylazoxymethanol [what abbreviation?]. In the colon, the final active metabolite, in the form of diazonium [9], triggers oxidative stress and the inflammatory process, increasing the cell proliferation in the colonic mucosa and causing injury to DNA and mutations of genes such as β-catenin and KRAS, inducing colorectal carcinogenesis [52].

The DMH dose used, 20 mg/kg of animal weight, is well established as a parameter for induction of colorectal carcinogenesis [53]. DMH is inexpensive and widely used to induce biological alterations and progression of cancer in laboratory animals that are similar to the results in humans [54], making its employment useful in studies of substances for prevention or reversion of colorectal carcinogenesis, such as gum arabic and eugenol.

Therefore, based on the intrinsic relationships among oxidative stress, [55] genotoxicity and genetic mutations in the pathophysiology of colorectal cancer [56], it is useful to investigate how this interplay can be affected by natural substances such as gum arabic and eugenol, for the prevention and/or treatment of carcinogenesis, due to their antioxidant and antimutagenic properties [55].

The metabolism of DMH results in the release of free radicals [57], causing a surfeit of these radicals in relation to their elimination via the antioxidant systems [58], culminating in their accumulation, [55], hence resulting in excessive oxidative stress, inducing mutations and consequent genotoxicity [53]. To evaluate oxidative stress, we quantified the reactive oxygen species (ROS) and reduced glutathione (GSH).

The data showed that exposure to the carcinogen DMH significantly increased the quantity of ROS in all colon segments, enabling measurement of the prevention and treatment. In the evaluation of oxidative stress in relation to the level of ROS in the colon for prevention, determined in the G VIII group (DMH + GA 10% + EUG), there was a significant reduction in the quantity of ROS in all the colon segments: proximal (*$P < 0.05$), middle ($P < 0.001$) and distal ($P < 0.01$). The same effect was observed regarding treatment in G XII (DMH + GA 10% + EUG), but only in the proximal segment, since in the middle segment, there was a significant increase in the amount of ROS. This finding casts doubt on whether or not in the treatment protocol the substances were sufficient to reduce the oxidative stress triggered by DMH.

GSH is a tripeptide compound formed by glutamic acid, cysteine and glycine. It is present in various organs, where it is responsible for maintaining the immunologic function by eliminating free radicals, with consequent antitumor activity [59].

In the prevention analysis, in groups G VII (DMH + EUG) and G VIII (DMH + GA 10% + EUG), the quantity of GSH increased significantly in comparison with the control group. However, regarding treatment, in the proximal segment,

the combined use of gum arabic and eugenol in G XII (DMH + GA 10% + EUG) caused a significant increase in the quantity of GSH in comparison with the group only treated with gum arabic, GX (DMH + GA 10%).

To evaluate genotoxicity, we employed the comet assay, which is widely used to detect DNA damages caused by exposure to chemical carcinogens, besides being easy to perform and inexpensive [60]. The test consists of measuring the DNA strand breaks (single and double strand breakage and breakage of alkaline-labile sites) in individual cells, where the fragmented DNA migrates faster than the integral DNA through an agarose matrix under electrophoresis. It can be carried out with alkaline or neutral electrophoresis, to detect different types of injury. Genotoxic substances react with the DNA bases, causing injury to the DNA, and leading to irreversible mutations that in turn trigger carcinogenesis [61]. The comet assay can be modified by adding various enzymes that detect DNA lesions, thus increasing the variety of lesions detected. We used the comet assay modified with the use of the enzyme formamidopyrimidine DNA glycosylase (FPG) [62]. The comet assay can be used both to characterize the mode of action of chemical carcinogens and to identify substances to be tested for their antigenotoxic potential.

The exposure to the carcinogen DMH significantly increased the DNA damage index of the colon (+++$P < 0.001$), irrespective of the treatment used (AD, GA, EUG or GA + EUG), in all the segments. There was a significant reduction in damage in the groups given gum arabic and eugenol with regard to prevention in all segments: proximal (***$P < 0.001$), middle (*$P < 0.05$) and distal (**$P < 0.01$), as well as regarding treatment, also in all segments ($P < 0.001$).

In another study, pending publication, our research group evaluated oxidative stress, lipid peroxidation and genotoxicity of these same groups and observed that gum arabic and eugenol used together were effective for prevention and treatment, by reducing the oxidative stress, lipid peroxidation and genotoxicity in the liver.

The anticarcinogenic effect of gum arabic alone has previously been reported in the literature [20] [21] [32]. Other studies have reported beneficial effects of gum arabic together with other substances against cancer cells in the colon (HT29) [63]. This can be explained by the antioxidant activity of phenol compounds and amino acids [64], as well as the anti-inflammatory activity [65].

The antioxidant potential of gum arabic can be attributed to its ability to reduce free radicals and increase the activity of the endogenous antioxidant systems [66], while the anti-inflammatory property is due to the production of short-chain fatty acids, which alter the production of inflammatory cytokines and promote chemotaxis in immune cells [67]. Besides this, it causes modifications in the expression of mRNA of genes related to cell proliferation and/or tumor growth [68], culminating in the anticarcinogenic effect.

In turn, the anticarcinogenic effect of eugenol has also been described in the literature, involving various cancer types: lungs, skin, gastric system [69], liver, ovary, prostate, breast and oral squamous cell carcinomas [70]. Its antioxidant

activity can be attributed to the induction of apoptosis, inflammation, proliferation, cell cycle arrest, angiogenesis and metastasis [71] [72], through modulation of the genes involved in these mechanisms [70]. In HT-29 colorectal adenocarcinoma cells, eugenol increased the expression of tumor suppressor genes and inhibited the expression of KRAS oncogenes, exhibiting cytotoxic activity [70]. Of these, KRAS p53, among other genes, mutates in colorectal carcinogenesis [73]. The apoptosis induced by eugenol occurs by inhibition of phosphorylation of the PI3K/AKT/Mtor signaling pathway, contributing to its antitumor effect [74]. Finally, eugenol also prevents inflammation and oxidative stress, which are intrinsic conditions of carcinogenesis [75].

In colorectal cancer, the adenoma-carcinoma pathway is one of the most common molecular pathways, leading to the formation of the tumor itself and altering the proliferation pattern of epithelial cells of the colon, forming aberrant crypts and/or aberrant crypt foci (ACF), which are called pre-neoplastic lesions of CRC [53] and are used as biomarkers of the initial stage of the disease [9].

All the groups that received DMH developed numerous ACF in the colon mucosa. Our results corroborate previous findings in the literature of the formation of numerous aberrant crypt foci in response to the administration of DMH [76]. Those authors reported a significant reduction in the number of aberrant crypts and of ACF in the distal portion and entire colon in the groups treated preventively with gum arabic and eugenol together. Our research group previously found a reduction in the distal segment with the use of gum arabic at 1%, 2.5% and 5%, and a reduction of the number of ACF with ≤4 crypts in rats, while reporting a reduction in the number of ACF with ≤5 crypts in mice treated with 5% GA [21]. With regard to prevention, the reduction of the number of aberrant crypts and of ACF observed in the group treated with gum arabic and eugenol after administration of the carcinogen can be explained by the protective effects of these two substances mentioned previously, producing a synergistic anticarcinogenic effect.

The study reveals the unprecedented discovery of the synergistic effects of gum arabic and eugenol in the prevention of colorectal carcinogenesis. This paves the way for further investigation into the combined use of natural substances and their potential synergistic interactions in cancer prevention and treatment.

In addition, other conditions where oxidative stress and genotoxicity are fundamental for the development of diseases, benefit from the biological activity of eugenol such as diabetes [77] multidrug-resistant bacterial infections by Klebsiella[78] stress-induced reproductive system disorders [79] kidney damage induced by CCl4- [80], as well as the biological activity of gum arabic, such as in dyslipidemia that occurs as a result of sickle cell anemia, chronic kidney disease [81] protection hepatica after exposure to aflatoxins [66] (and cardiac protection in ischemia and reperfusion injuries [82]

It is important to stress that the antioxidant and anticarcinogenic effects of

gum arabic and eugenol described previously in the literature only involved the isolated use of the substances. Here we describe the novel synergistic effects of the two substances administered together.

6. Conclusion

The joint use of the dietary constituents gum arabic at 10% and eugenol (from cloves) at the doses and time intervals described had a synergistic effect, with antioxidant action (reduction of ROS) in all colon segments (prevention), an increase of the levels of GSH in the proximal colon (prevention and treatment) and antigenotoxic action (in all colon segments regarding prevention and treatment), besides being effective in reducing the number of aberrant crypts and aberrant crypt foci in the distal colon segment and entire colon (prevention) of rats submitted to colorectal carcinogenesis induced by dimethylhydrazine.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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