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Effect of eugenol and gum arabic on oxidative stress and genotoxicity in rat spleen, kidney and lung tissue following colorectal carcinogenesis

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Abstract
Genetic mutations are linked to cancer. We evaluated gum arabic (GA) and eugenol (EUG) on systemic genotoxicity (spleen, kidney, lung) in rats with DMH-induced colorectal carcinogenesis. The prevention arm, once a week for 20 weeks, the controls received saline while the experimental groups received DMH at 20 mg/kg. During the same period and for an additional 9 weeks, the animals received water, GA, EUG or GA + EUG. The treatment arm, once a week for 20 weeks, the controls received saline while the experimental groups received DMH at 20 mg/kg. During the subsequent 9 weeks, the animals received water, GA, EUG or GA + EUG. The spleen, kidneys and lungs were harvested for genotoxicity evaluation. Genotoxicity was significantly less severe in spleen and kidney tissue in Group VIII and in Group XII, reflecting the synergistic antioxidant and antigenotoxic effect of GA and EUG administered in rats with DMH-induced colorectal carcinogenesis.

Keywords: Eugenol, gum arabic, carcinogenesis, antimutagenic agents

1. Introduction
Colorectal carcinogenesis involves the progressive accumulation of multiple genetic mutations. Mutations involving the genes p53 and Kras [1] cause loss of function in tumor suppressor genes and gain of function in oncogenes (antagonists) [2], which in turn contribute directly to the development of colorectal cancer [3].
DMH (1, 2-dimethylhydrazine) is widely used in animal colorectal carcinogenesis models. Metabolically activated in the liver via cytochrome P450, DMH initially oxidizes into azomethane, which is converted into azoxymethane (AOM). AOM is then hydroxylated into methylazoxymethanol (MAM) and excreted to the colon via the bile. MAM is in turn converted by bacterial β-glucuronidase into the methyldiazonium ion-a powerful DNA alkylating agent. The process induces oxidative stress, resulting in genetic damage and mutations in colon cells [4]. Since the active carcinogen is transported by the blood stream, other organs in the body may be directly affected [5].
The mutagenic activity of DMH and AOM is initiated by the methylation of guanine at the N-7 position. By donating a proton, the alkylated guanine is paired with thymidine rather than with cytosine, inducing changes in the bases. This is followed by further replication, with mispairing of guanine to thymine and cytosine to adenine, favoring the emergence of mutations [5].
The oxidative stress resulting from exposure to DMH has been shown to damage the DNA in rodent colon cells and in several other tissues, such as the liver, kidneys and heart [4] and the stomach and lungs [6].
DMH-induced colon carcinogenesis triggers an insufficient immune response [7], followed by inhibition of the antioxidant system from oxidative stress, increased oxidative damage to proteins and lipids in the spleen [8], lung metastasis [9], and the formation of a large number of alkylated DNA adducts in the kidneys [10].
Much research has been conducted to explore the genotoxic mechanisms involved in colorectal carcinogenesis [11], in some cases in order to identify natural compounds, such as gum arabic (GA) and eugenol (EUG), capable of preventing or treating carcinogenesis and its systemic repercussions. Natural compounds often have fewer side effects and lower toxicity than allopathic treatments, without detriment to their biological properties, making them an important field of study [12-13].

GA is an important input in food processing but is also renowned for its pharmacological uses as an antibacterial, antifungal, hypoglycemiant, antioxidant and antimalarial agent. Of special interest here is the antigenotoxic and antineoplastic properties of GA [14] which have been evaluated in a number of scenarios with the purpose of, among other things, reducing genotoxicity in the colon and liver, or systemically, in mice with AOM-induced colorectal carcinogenesis [15], preventing genetic damage in rats with adenine-induced chronic renal failure [16], and alleviating ioxitalamate-induced oxidative stress and genotoxicity in rats [17]. *GA* is also protective against preneoplastic colorectal lesions [18] and can significantly reduce the number of colon carcinomas [19].

EUG is a light yellow, translucid, volatile aromatic phenylpropanoid found mainly in clove oil but also in certain herbs [20]. The compound has a range of pharmacological properties, with emphasis on its use as an anti-inflammatory, analgesic, antimicrobial, antimutagenic and antineoplastic agent [21].

EUG has been shown to inhibit genotoxicity induced by 7,12-dimethylbenz[a]anthracene in MCF-7 cells [22], to have antigenotoxic activity against compounds such as cyclophosphamide, procarbazine and urethane [23], and to suppress mutagenicity induced by furylfuramide and aflatoxin B in Salmonella typhimurium [24]. The antineoplastic effect of *EUG* is associated with its antiproliferative and cytotoxic properties [13] and has been documented for different types of cancer (leukemia, lung, breast, colorectal) [25].

In this study we evaluated the effects of 10% *GA* and *EUG* on systemic genotoxicity in spleen, kidney and lung tissue of rats with DMH-induced colorectal carcinogenesis.

2. Materials and Methods

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).

The study used 128 female Wistar rats randomly assigned to 8 control groups (Ia, IIa, IIIa, IVa, Ib, IIb, IIIb, IVb) with 6 animals each and 8 experimental groups (V, VI, VII, VIII, IX, X, XI, XII) with 10 animals each.

Prevention was evaluated using 4 control groups (Ia, IIa, IIIa, IVa) and 4 experimental groups (V, VI, VII, VIII). 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 same period and for an additional 9 weeks, the animals were given either water (Ia, V), 10% *GA* (IIa, VI), *EUG* (IIIa, VII) or 10% *GA* + *EUG* (IVa, VIII) by gavage.

Treatment was evaluated using 4 control groups (Ib, IIb, IIIb, IVb) and 4 experimental groups (IX, X, XI, XII). 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 either water (Ib, IX), 10% *GA* (IIb, X), *EUG* (IIIb, XI) or 10% *GA* + *EUG* (IVb, XII) by gavage (Figure 1).

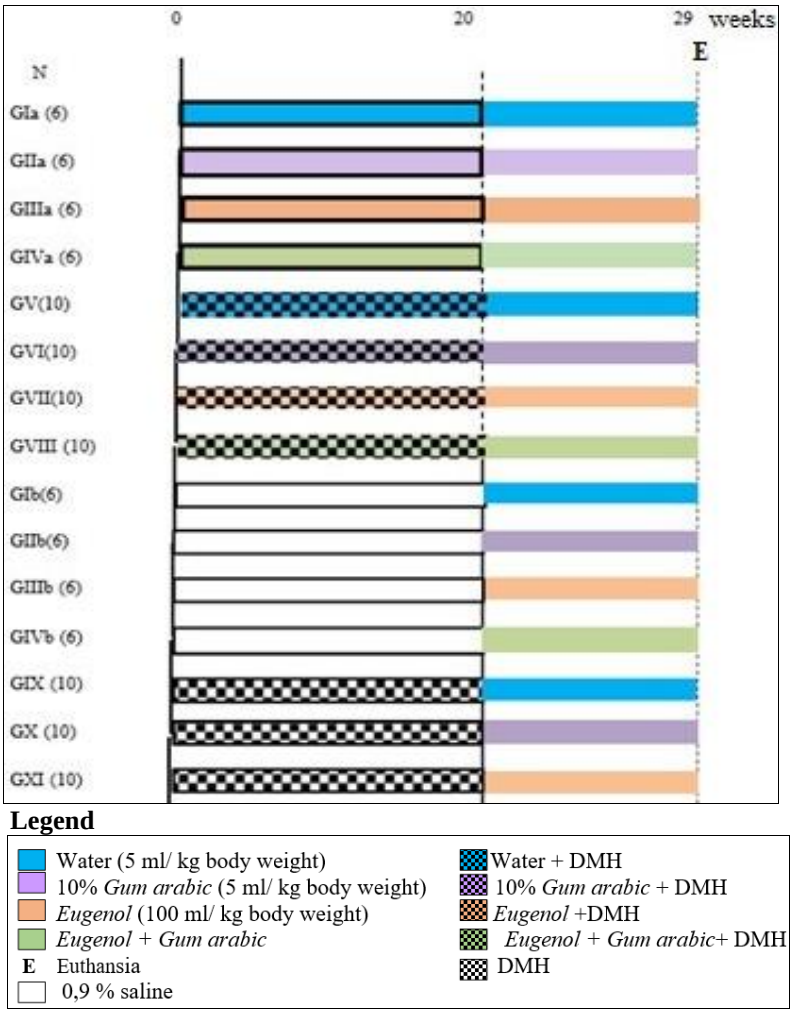

Fig 1: Study design

2.1 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 vehicle, adjusted to a final pH of 6.5 using a NaOh solution [26]. The carcinogen was administered S.C. at 20 mg/kg body weight once a week for 20 weeks [27].

2.2 Gum arabic (GA)
Gum arabic (GA) (Dinâmica Química Contemporânea Ltda) was diluted in distilled water at 10% [19] and administered by gavage at 5 mL/kg body weight 4 times a week.

2.3 Eugenol (EUG)
Eugenol (EUG) (Laboratório Quinari) was administered orally using a pipette at 100 mg/kg body weight 3 times a week [28].

2.4 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 for the harvesting of the spleen, kidneys and lungs.

2.5 Evaluation of genotoxicity
To test for genotoxicity, we macerated spleen, kidney and lung fragments individually in phosphate buffered saline (PBS) at 4 ºC. Cells were obtained by filtering the suspension.

2.6 Comet assay
The level of DNA damage was determined by comet assay under alkaline and neutral conditions, as described by Hartmann and Speit (1997) [29] and Wojewodzka, Buraczewska and Kruszewski (2002) [30], respectively.

2.7 Modified alkaline comet assay
The modified alkaline comet assay was used to increase the sensitivity and specificity of the comet assay. The method consists of adding the enzyme DNA-formamidopyrimidine glycosylase (FPG) which recognizes oxidized nitrogenated bases, as described for the alkaline comet assay with minor modifications

2.8 Statistical analysis
All statistical analyses were performed with the software Python, using the package scikit-learn. Differences between groups were analyzed with the Conover-Iman test (the nonparametric equivalent of the Tukey HSD test).

3. Results
The research is part of PhD thesis, Postgraduate Program in Medical-Surgical Sciences.
Prevention study: test compounds administered concomitantly with the carcinogen In the prevention arm of the study (Groups V, VI, VII and VIII), spleen, kidney and lung fragments were submitted to the comet assay for genotoxicity. The table 1 shows the sample median of the prevention groups and the respective 95% confidence intervals for the population median obtained by bootstrapping.

Table 1: Sample median of the prevention groups and the respective 95% confidence intervals for the population median obtained by bootstrapping

	Prevention							
	Control groups				**DMH groups**			
	IA	**IIA**	**IIIA**	**IVA**	**V**	**VI**	**VII**	**VIII**
Spleen	5.0	7.0	8.0	7.5	28.5	18.0	18.5	12.5
	(5.0 - 6.0)	(5.0 - 9.0)	(6.5 - 8.75)	(6.0 - 10.5)	(23.5 - 31.0)	(13.0 - 19.0)	(15.5 - 23.25)	(10.0 - 13.25)
Spleen + FPG	5.0	8.0	9.5	9.0	69.5	39.0	39.5	30.5
	(4.0 - 6.0)	(7.0 - 9.0)	(7.5 - 10.75)	(7.5 - 9.75)	(63.25 - 73.75)	(34.0 - 44.0)	(37.5 - 42.0)	(27.75 - 35.0)
Kidney	8.0	9.0	9.0	6.5	34.5	19.0	16.5	10.5
	(5.0 - 10.0)	(8.0 - 10.0)	(8.25 - 11.25)	(5.25 - 10.0)	(30.25 - 37.5)	(17.0 - 21.0)	(15.0 - 19.75)	(9.5 - 13.75)
Kidney + FPG	6.0	8.0	9.5	8.5	59.0	34.0	28.5	19.5
	(6.0 - 8.0)	(7.0 - 10.0)	(8.25 - 10.0)	(6.25 - 10.0)	(55.5 - 63.25)	(31.0 - 36.0)	(25.0 - 32.0)	(16.5 - 20.25)
Lung	10.0	9.0	8.5	8.5	3.5	5.0	3.5	5.0
	(10.0 - 11.0)	(6.0 - 11.0)	(7.25 - 9.0)	(5.0 - 9.0)	(1.25 - 6.25)	(2.0 - 7.0)	(2.0 - 5.0)	(2.0 - 6.0)
Lung + FPG	12.0	9.0	10.0	8.5	5.5	5.0	6.5	5.0
	(10.0 - 13.0)	(8.0 - 12.0)	(9.25 - 11.5)	(6.25 - 10.75)	(4.25 - 6.75)	3.0 - 7.0)	(5.0 - 8.0)	(4.75 - 7.25)

Spleen tissue displayed less DNA damage in Group VI (DMH + 10% *GA*) and Group VII (DMH + *EUG*) than in Group V (DMH + water). Group VIII (DMH + 10% *GA* + *EUG*) had significantly lower levels of DNA damage than Group V (DMH + water) and Group VII (DMH + *EUG*) (Figure 2). Similar results were found in the modified alkaline comet assay (Figure 3).

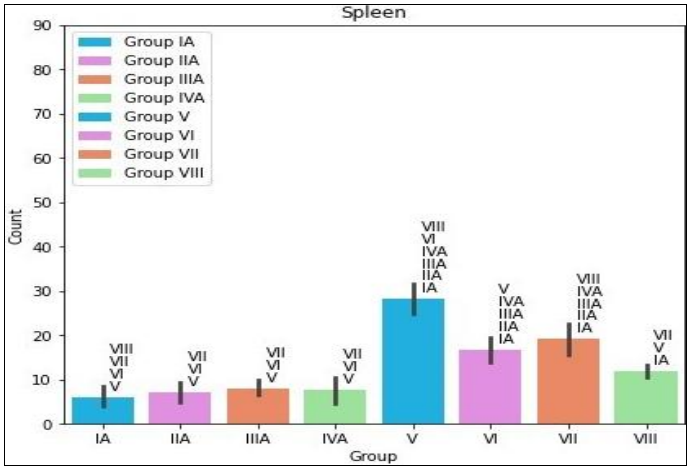

Fig 2: Levels of DNA damage in spleen tissue in the comet assay

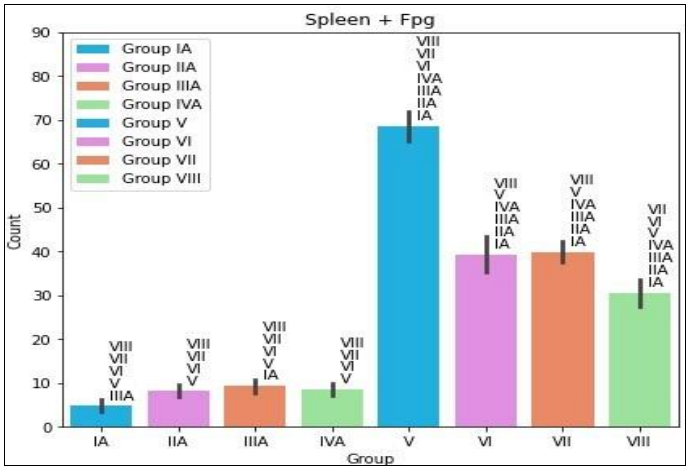

Fig 3: Levels of DNA damage in spleen tissue in the modified alkaline comet assay

DNA damage in kidney tissue was less severe in Group VI (DMH + 10% *GA*) and Group VII (DMH + *EUG*) than in Group V (DMH + water). Group VIII (DMH + 10% *GA* + *EUG*) had significantly lower levels of DNA damage than

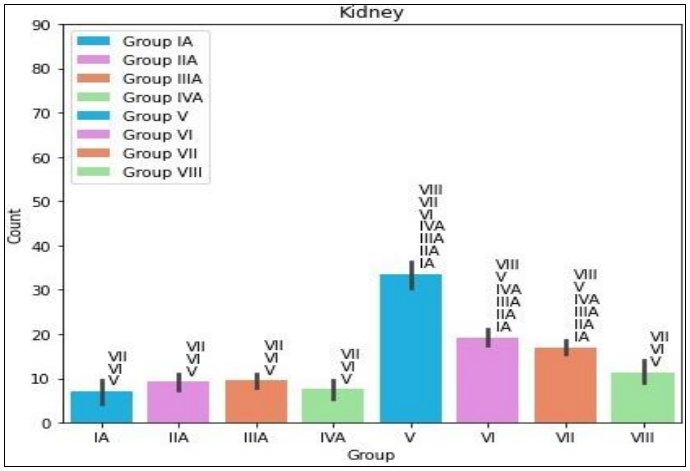

Fig 4: Levels of DNA damage in kidney tissue in the comet assay

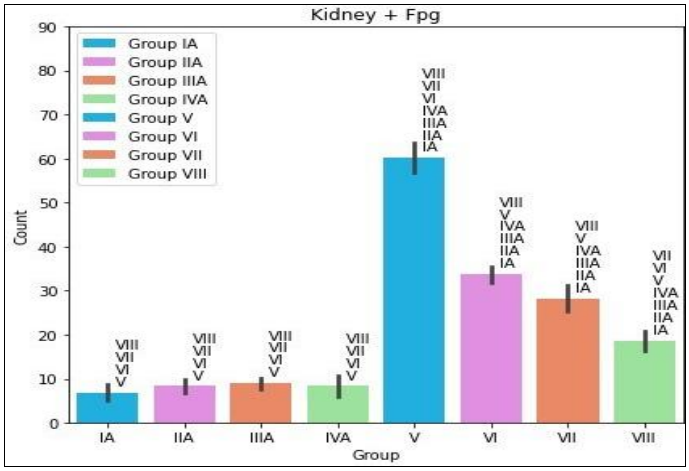

Fig 5: Levels of DNA damage in kidney tissue in the modified alkaline comet assay

Group V (DMH + water), Group VI (DMH + 10% *GA*) and Group VII (DMH + *EUG*) (Figure 4). Similar results were observed in the modified alkaline comet assay (Figure 5).

As for lung tissue, no significant difference was found between the groups treated with DMH and the control groups,

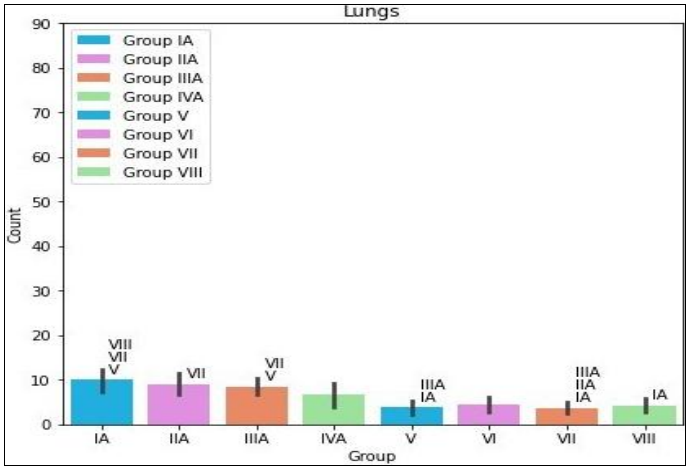

Fig 6: Levels of DNA damage in lung tissue in the comet assay

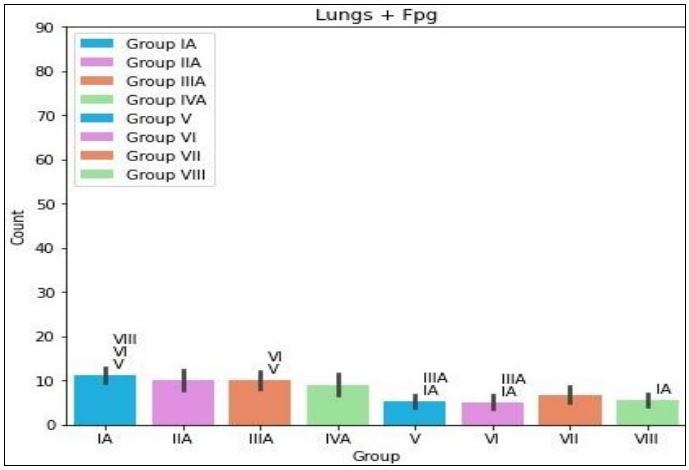

Fig 7: Levels of DNA damage in lung tissue in the modified alkaline comet assay

regardless of which assay was used (Figures 6 and 7).

Treatment study: carcinogen administered before the test compounds.

In the treatment arm of the study (Groups IX, X, XI and XII), spleen, kidney and lung fragments were submitted to the

comet assay for the evaluation of genotoxicity. The table 2 shows the sample median of the treatment groups and the respective 95% confidence intervals for the population median obtained by bootstrapping.

Table 2: Sample median of the treatment groups and the respective 95% confidence intervals for the population median obtained by bootstrapping.

Treatment								
	Control Groups				DMH groups			
	IB	IIB	IIIB	IVB	IX	X	XI	XII
Spleen	6.5	9.0	6.0	10.0	24.0	13.0	10.0	11.0
	(4.25 - 8.0)	(8.0 - 10.0)	(5.0 - 7.0)	(10.0 - 10.75)	(22.25 - 28.5)	(9.75 - 17.5)	(7.0 - 11.0)	(7.0 - 15.0)
Spleen + FPG	8.0	9.5	8.0	10.5	60.5	25.5	20.0	11.0
	(6.25 - 9.75)	(7.5 - 10.0)	(6.25 - 9.75)	(10.0 - 12.5)	(56.5 - 65.75)	(21.25 - 27.75)	(18.0 - 25.0)	(8.0 - 16.0)
Kidney	5.0	6.5	6.5	5.0	27.0	9.5	11.0	11.0
	(3.5 - 7.25)	(5.25 - 8.5)	(5.0 - 8.75)	(4.25 - 6.5)	(18.5 - 32.0)	(7.5 - 10.75)	(6.0 - 14.0)	(10.0 - 17.0)
Kidney + FPG	7.0	9.5	6.0	7.0	51.5	17.5	21.0	19.0
	(3.5 - 9.75)	(8.25 - 10.75)	(4.5 - 6.75)	(6.0 - 8.75)	(50.0 - 60.75)	(14.5 - 21.5)	(16.0 - 23.0)	(17.0 - 22.0)
Lung	6.0	4.5	8.5	8.5	10.0	8.5	8.0	7.0
	4.25 - 8.5)	(4.0 - 5.75)	(7.25 - 9.75)	(7.25 - 12.0)	(9.0 - 13.75)	(6.25 - 10.75)	(8.0 - 10.0)	(3.0 - 9.0)
Lung + FPG	8.5	9.0	8.5	9.5	10.5	10.5	10.0	7.0
	(7.25 - 9.0)	(8.25 - 10.5)	7.25 - 9.75)	(8.25 - 10.0)	(8.25 - 14.25)	(9.25 - 13.5)	(7.0 - 14.0)	(5.0 - 10.0)

In the comet assay, spleen tissue displayed less DNA damage in Group X (DMH + 10% *GA*), Group XI (DMH + *EUG*) and Group XII (DMH + 10% *GA* + *EUG*) than in Group IX (DMH + water). The groups treated with DMH did not differ significantly (Figure 8).

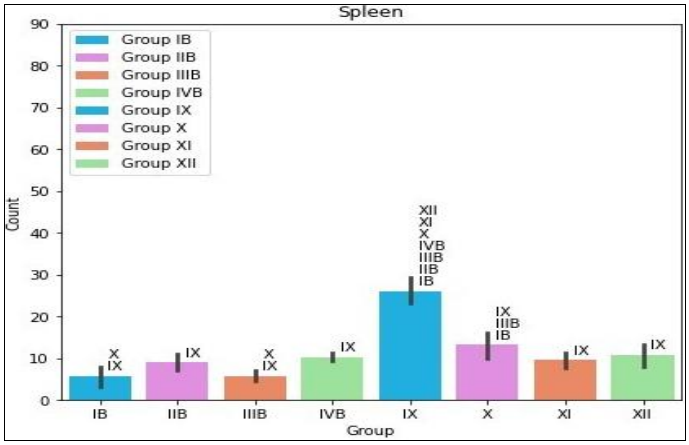

Fig 8: Levels of DNA damage in spleen tissue in the comet assay

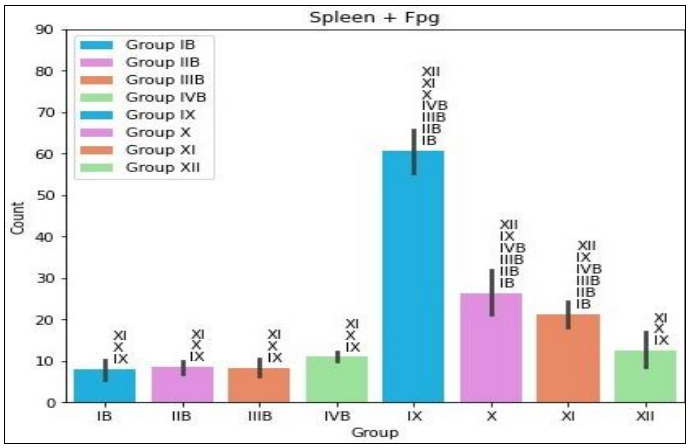

Fig 9: Levels of DNA damage in spleen tissue in the modified alkaline comet assay

In the modified alkaline comet assay, spleen tissue displayed less DNA damage in Group X (DMH + 10% *GA*) and Group XI (DMH + *EUG*) than in Group IX (DMH + water). DNA damage was significantly lower in Group XII (DMH + 10% *GA* + *EUG*) than in Group IX (DMH + water), Group X (DMH + 10% *GA*) and Group XI (DMH + *EUG*) (Figure 9).

In the comet assay, DNA damage in kidney tissue was less severe in Group X (DMH + 10% *GA*), Group XI (DMH + *EUG*) and Group XII (DMH + 10% *GA* + *EUG*) than in Group IX (DMH + water) (Figure 10). The groups treated with DMH did not differ significantly.

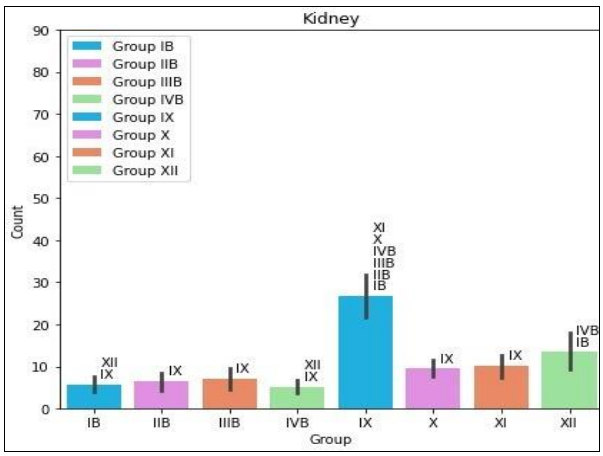

Fig 10: Levels of DNA damage in kidney tissue in the comet assay

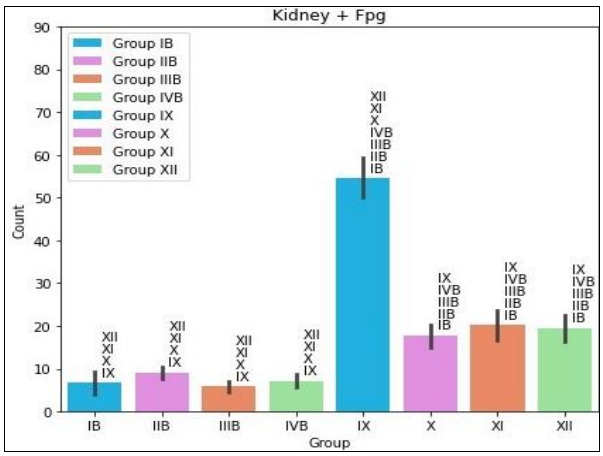

Fig 11: Levels of DNA damage in kidney tissue in the modified alkaline comet assay

In the modified alkaline comet assay, kidney tissue displayed less DNA damage in Group X (DMH + 10% *GA*), Group XI (DMH + *EUG*) and Group XII (DMH + 10% *GA* + *EUG*) than in Group IX (DMH + water). The groups treated with DMH did not differ significantly (Figure 11).

As for lung tissue, the groups treated with DMH did not differ significantly, regardless of which assay was used (Figures 12 and 13). Moreover, the control groups and the DMH groups yielded statistically similar results.

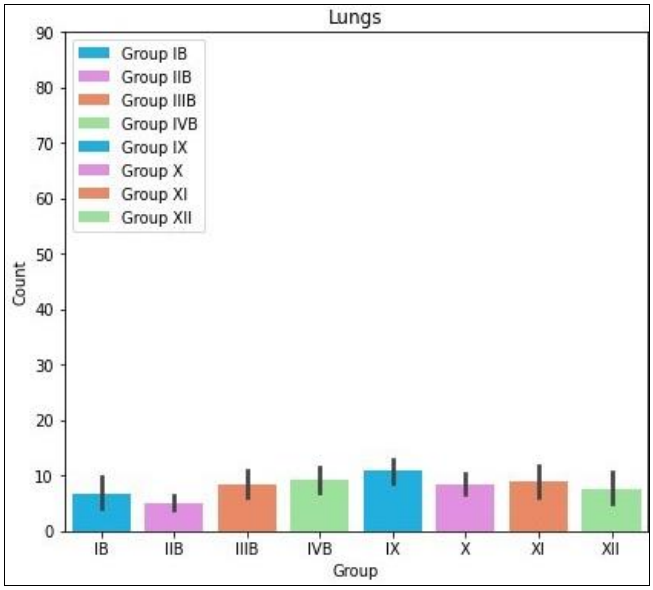

Fig 12: Levels of DNA damage in lung tissue in the comet assay

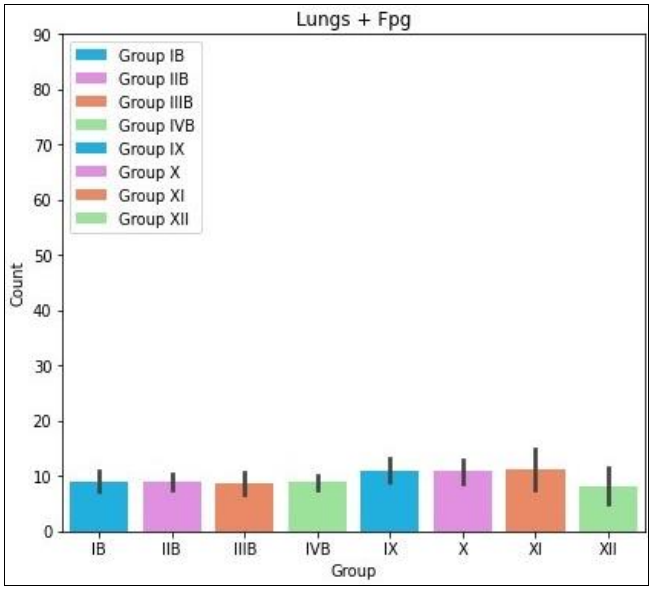

Fig 13: Levels of DNA damage in lung tissue in the modified alkaline comet assay

4. Discussion

The process of colorectal carcinogenesis initiates with oxidative stress mediated by reactive oxygen species (ROS), followed by genetic damage and instability, oncogene activation, mutations and tumor growth [31].

Through a series of chemical reactions, DMH is metabolized into MAM, which decomposes into the methyldiazonium ion- a powerful DNA alkylating agent and inductor of genotoxicity. In this study, DMH was administered at 20 mg/kg body weight, a dosage proven to be efficient at inducing colorectal carcinogenesis [5].

Colorectal carcinogenesis induced by carcinogens like DMH has been used extensively in animal studies because it mimics human disease and allows testing the chemopreventive and therapeutic effect of a number of compounds [10], such as *GA* and *EUG*, on genotoxicity and localized and systemic carcinogenesis involving spleen, kidney and lung tissues.

In this study, DMH efficiently induced genotoxicity in spleen and kidney tissue, as shown by the fact that DMH groups yielded values significantly different (p<0.05) from the control groups. In lung tissue no genotoxicity was observed. The compounds tested in this study (*GA* and *EUG*) have not been associated with mutagenicity or genotoxicity [32 33].

Easy to perform and low-cost, the comet assay is widely used to evaluate DNA damage caused by exposure to chemical carcinogens [34]. The method quantifies breaks of single and double DNA strands and alkali-labile sites in individual cells. Fragmented DNA migrates faster than intact DNA through an agarose matrix under electrophoresis. The assay may be performed under alkaline or neutral conditions, depending on what types of lesions are targeted. Genotoxic compounds damage the DNA, causing irreversible mutations and carcinogenesis [35].

By adding enzymes, the modified alkaline comet assay can detect a wider range of lesions. In this study we used FPG [36]. The comet assay helps clarify the mechanisms involved in chemical carcinogenesis and allows testing compounds with antigenotoxic potential, such as *GA* and *EUG*.

To prevent carcinogenesis, *EUG* and *GA* were administered concomitantly with DMH. As expected, DNA damage in spleen tissue was significantly less severe in Group VIII (DMH + 10% *GA* + *EUG*) than in Group V (DMH + water) and Group VII (DMH + *EUG*). The modified alkaline comet assay yielded similar results. In the treatment arm of the study, the difference was only significant when FPG was added to the assay.

There is evidence that *GA* protects against CCL4-induced toxicity in rodent spleen tissue by reducing oxidative stress. This may be due to the compound's phytochemical composition, with emphasis on the antioxidant properties of the phenolic compounds gallic acid, ellagic acid, benzoic acid and o-coumaric acid [37]. *EUG*, which in this study reduced DNA damage in spleen tissue, is chemoprotective against toxicity induced by TiO2 nanoparticles due to its antioxidant effects [38].

As expected, DNA damage in kidney tissues was significantly less severe in Group VIII (DMH + 10% *GA* + *EUG*) than in Group V (DMH + water), Group VI (DMH + 10% *GA*) and Group VII (DMH + *EUG*), indicating that the combination of *EUG* and *GA* was more effective than each compound alone. The modified alkaline comet assay yielded similar results. In the treatment arm of the study, DNA damage was less severe in Group X (DMH + 10% *GA*), Group XI (DMH + *EUG*) and Group XII (DMH + 10% *GA* + *EUG*) than in Group IX (DMH + water). Interestingly, although *EUG* and *GA* were effective at alleviating genotoxicity, the DMH groups did not differ significantly.

Our study shows that the combination of 10% *GA* and *EUG* efficiently reduced genotoxicity in spleen and kidney tissue when used for prevention and treatment. It should be pointed out that, to our knowledge, no earlier study evaluating the ability of *GA* and *EUG* to reduce genotoxicity in spleen, and kidney tissue has tested these two compounds in combination. Thus, this is the first study to document the synergistic effect

of *GA* and *EUG* in this scenario.

5. Conclusion

GA and *EUG*, alone or in combination, are efficient at preventing and treating genotoxicity in rat spleen and kidney tissue. This is the first study to document the synergistic effect of *GA* and *EUG* on genotoxicity in spleen and kidney tissue in rats with DMH-induced colorectal carcinogenesis.

6. Acknowledgments

Research performed at Laboratory of Experimental Surgery and Nucleus of Research and Development of Medicines, and Laboratory of Pharmacology and Preclinical Research, Medical School, Universidade Federal do Ceará (UFC), Fortaleza-CE, Brazil. This study is part of PhD thesis Postgraduate Program in Medical Surgical Sciences, UFC.

7. Disclosure Statement

The authors report no conflict of interest.

8. Funding

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