Detection of P53 Protein Changes in the Tissues of 1,2 Dimethyl Hydrazine and Zingiber Officinale Root Extract Treated Syrian Hamster Using Real-Time Polymerase Chain Reaction and Immunohistochemistry Staining

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(Received 19 / 4 / 2024. Accepted 30 / 6 /2024)

□ ABSTRACT □

Colon cancer is the third common type of cancer in the world, that is characterized by the emergence of malignant cells in the mucous membrane and the loss of the mechanism of natural control over growth. Because of the high cost and long-term side effects of chemical and immune treatments, we aimed to highlight the possibility of using the Zingiber in the treatment of cancer. Zingiber is one of the most important medical plants and plays a protective role against this disease. Our study contained 110 individuals of Syrian hamster divided into six groups: the first group witness (10 individuals) was given food and distilled, the second group (20 individuals) was dosed daily with Zingiber (80 mg/kg) for 8 Weeks, the third group (20 individuals) was dosed daily with Zingiber (220 mg/kg) for 8 weeks, the fourth group (20 individuals) was injected with a compound 1,2 Dimethyl Hydrazine (40 mg/kg) in the abdomen at a rate of 4 times/week For 8 weeks, the fifth group (20 individuals) was dosed for a week with ginger extract (80 mg/kg) in conjunction with 1,2 Dimethyl Hydrazine (40 mg/kg) 4 times/week for 8 weeks, the sixth group (20 individuals) has been fed for a week with vegetable extract, then with Zingiber extract (220 mg/kg) in conjunction with 1,2 Dimethyl Hydrazine injection (40 mg/kg) 4 times/week for 8 weeks. At the end of the experiment, the colon tissue was obtained from the previous six groups animals, nucleic acids extraction, Real-Time Polymerase chain reaction and the immunohistochemistry staining were performed to detect the P53 protein changes.

The results of our current study showed that 1,2 Dimethyl Hydrazine is capable of causing a mutation in the P53 protein. This was demonstrated by an increase in its gene expression within colon tissue, which led to neoplastic transformation. On the other hand, the aqueous extract of Zingiber roots prevented neoplastic transformation in the colon tissue of experimental animals, and this was evident through reducing the sinusoidal expression of the P53 protein and maintaining its normal level within the tissue.

Keywords: Zingiber officinale - P53 - 1,2 Dimethyl Hydrazine - Real-Time Polymerase chain reaction (q PCR)- Immunohistochemistry Staining

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الكشف عن تغيرات بروتين P53 في أنسجة الهامستر السوري المعالج بـ 1,2 ثنائي ميثيل هيدرازين ومستخلص جذر الزنجبيل باستخدام تفاعل البوليميراز المتسلسل في الوقت الحقيقي والتلوين المناعي النسيجي

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(تاريخ الإيداع 19 / 4 / 2024. قبل للنشر في 30 / 6 / 2024)

□ ملخّص □

يعتبر سرطان القولون ثالث أنواع السرطان شيوعاً في العالم، ويتميز بظهور خلايا خبيثة في الغشاء المخاطي وفقدان آلية التحكم الطبيعي في النمو. ونظراً للتكلفة العالية والآثار الجانبية طويلة الأمد للعلاجات الكيميائية والمناعية، فقد هدفنا إلى تسليط الضوء على إمكانية استخدام نبات الزنجبيل في علاج السرطان. ويعتبر نبات الزنجبيل من أهم النباتات الطبية ويلعب دوراً وقائياً ضد هذا المرض. شملت دراستنا 110 فرد من الهامستر السوري مقسمة إلى ست مجموعات: المجموعة الأولى شاهدة (10 أفراد) أعطيت الطعام والماء المقطر، المجموعة الثانية (20 فردا) أعطيت جرعة يومية من الزنجبيل (80 ملغ / كغ) لمدة 8 أسابيع، المجموعة الثالثة (20 فردا) أعطيت جرعة يومية من الزنجبيل (220 ملغ / كغ) لمدة 8 أسابيع، المجموعة الرابعة (20 فردا) أعطيت جرعة من مركب 1,2 ثنائي ميثيل هيدرازين (40 ملغ / كغ) في البطن بمعدل 4 مرات / أسبوع لمدة 8 أسابيع، المجموعة الخامسة (20 فردا) أعطيت جرعة لمدة أسبوع من مستخلص الزنجبيل (80 ملغ / كغ) بالتزامن مع 1,2 ثنائي ميثيل هيدرازين (40 ملغ / كغ) 4 مرات / أسبوع لمدة 8 أسابيع، المجموعة السادسة (20 فردا) أعطيت لمدة أسبوع من مستخلص الخضار، ثم أعطيت مستخلص الزنجبيل (220 ملغ / كغ) بالتزامن مع حقن 1,2 ثنائي ميثيل هيدرازين (40 ملغ/كغ) 4 مرات/اسبوعيا لمدة 8 اسابيع وفي نهاية التجربة تم الحصول على انسجة القولون من حيوانات المجموعات الست السابقة وتم استخلاص الاحماض النووية وتفاعل البوليميراز المتسلسل في الوقت الحقيقي والتلوين المناعي النسيجي للكشف عن التغيرات في بروتين P53.

وقد اظهرت نتائج دراستنا الحالية ان 1,2 ثنائي ميثيل هيدرازين قادر على التسبب في حدوث طفرة في بروتين P53 وقد ثبت ذلك من خلال زيادة التعبير الجيني له داخل انسجة القولون مما ادى الى التحول السرطاني ومن ناحية اخرى منع المستخلص المائي لجذور الزنجبيل التحول السرطاني في انسجة القولون للحيوانات التجريبية وكان ذلك واضحا من خلال تقليل التعبير الجيبي لبروتين P53 والحفاظ على مستواه الطبيعي داخل الانسجة.

الكلمات المفتاحية:خلاصة الزنجبيل – 1,2 ثنائي ميثيل هيدرازين –P53 – تفاعل البوليميراز المتسلسل في الوقت الحقيقي (q PCR) – التلوين المناعي النسيجي.

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Introduction:

Many studies have shown the importance of plant extracts in protecting living tissues from the harmful effects of free radicals resulting from exposure of the organism to certain conditions. Therefore, they have been used as therapeutic or preventive means for many diseases. [1) ,2)] Plants work to reduce the level of acid phosphatase and fat oxidation. [3)] Therefore, recent studies have used many medicinal plants and their extracts as natural antioxidants and growth stimulants that work to raise the body's immunity. Among these plants is the Zingiber plant, which is used in folk medical treatment to treat many conditions. Pathogenesis [4)] because it contains antioxidants such as flavonoids, saponins, terpenes, iron, manganese, gingerols, and shogaols, [5), 6)] and it also possesses anti-inflammatory properties. [7) ,8)]

1,2 Hydrazine Dimethyl (DMH) is used as fuel for jet engines, rocket engines, and explosives, and to produce chemicals for textiles and agriculture, and materials for displaying photographs. [9) ,10)] It works to generate free radicals within the colon tissue [11)] These radicals attack cellular macromolecules such as lipids and DNA, which may result in genetic mutations. [12), 13)]

2. THE IMPORTANCE AND OBJECTIVES OF THE RESEARCH

The research aims to test the role of Zingiber root extract in reducing the negative damage caused by DMH in colon tissue in vivo, by detecting the amount of P53 protein within this tissue.

Materials And Methods

3.1. Preparation of aqueous extract of ginger plant

The Zingiber plant was obtained from where it is found in nature, then the roots were washed with distilled water and dried in the shade for (4-5 days), then ground with an electric blender to obtain a fine powder. After that (500 g) of root powder was added to 1 liter of distilled water at a temperature (35- 40 ℃) and left the mixture for (24 hours) at laboratory temperature. After that, the solution was filtered twice using special filter papers and the filtrate was transferred to the incubator at a temperature of (40 ℃)) until a pure, dry powder was obtained. [14)] The powder was preserved. Placed in light-proof glass tubes in the refrigerator at (4°C) until use. The experimental animals were dosed daily with the following concentrations (80-220) mg/kg.

3.2. Preparation of carcinogen (DMH) solution

20 g of DMH was dissolved in 1 mM EDTA prepared upon use. The pH was adjusted to 6.5 with 1 mM NaOH solution. DMH at a concentration of (40 mg/kg) was injected into the abdominal peritoneum 4 times/week for 8 weeks. [15)]

3.3. Experimental animals

The study was conducted on 110 hamsters, 3-4 months old on average, and weighing between 90-110 g. The animals were placed in glass tanks in the laboratory under appropriate conditions in terms of food, temperature (25 degrees Celsius), and lighting (12 hours light/12 hours darkness). They were left to acclimate to laboratory conditions for a period of not less than two weeks before the experiment began.

3.4. Experiment design

Experimental animals (110 individuals) were divided into 6 groups.

1- The first group (10 individuals): The control group was given food and distilled water only, freely, throughout the experiment.

2- The second group (20 individuals): was dosed daily with zingiber extract (80 mg/kg), via tube feeding for 8 weeks.
3- The third group (20 individuals): was dosed daily with zingiber extract (220 mg/kg) via tube feeding for 8 weeks.
4- The fourth group (20 individuals): They were injected with DMH at a concentration of (40 mg/kg) into the abdominal peritoneum at a rate of 4 times/week for 8 weeks.
5- The fifth group (20 individuals): They were dosed with zingiber extract for a week, then they were dosed with ginger extract (80 mg/kg) in conjunction with DMH injections (40 mg/kg) 4 times/week for 8 weeks.
6. The sixth group (20 individuals): They were dosed for a week with the plant extract, then they were dosed with zingiber extract at a concentration of (220 mg/kg) in conjunction with DMH injections (40 mg/kg) 4 times/week for 8 weeks.

3.5. Collect colon tissue samples to conduct a (R-T PCR) test

To quantitatively compare the gene expression of P53 proteins in the colon tissue of experimental mice, the mice were dissected at the end of the experiment, and then these tissues were isolated and parts of them were preserved in containers containing RNAlater at a temperature of (-20 ℃) before extracting RNA from them.

3.5.1. RNA extraction

RNA was extracted using a Qiagen extraction kit and its concentration in the samples was measured using a spectrophotometer.

3.5.2 Complementary DNA (cDNA) Synthesis

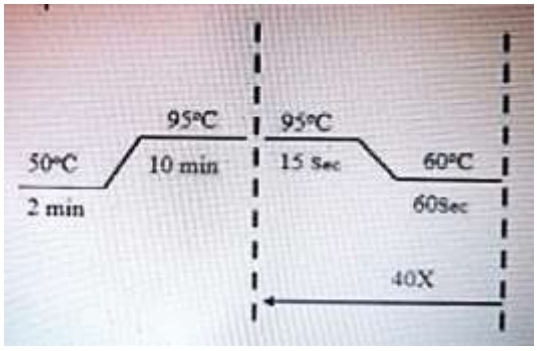
Complementary DNA was synthesized from each RNA sample with a DNA Synthesis kit (M B125) using the following method, Reactive materials:
RNA 2µL (100ng/µL)_ 10X RT Buffer 2Ml_ Oligo-Dt 1µL_ Reverse Transceptase 1µL_ Random Hexamers 1µL_ dNTPs 2µL_ RNase Inhibitor 0.5µL_ DEPC water 10.5µL_ Total 20µL.
After preparing the samples, the samples were placed in the traditional PCR machine and programmed for the following temperatures for one cycle only: 70℃ for ten minutes, 42℃ for an hour, 72℃ for ten minutes.

3.5.3. (q PCR) test

After synthesis of complementary DNA from mice from the six groups, quantitative qPCR was performed to assay the gene expression of P53 gene within these samples. SYBR green master mixture was used to polymerize this gene and the following primers were designed.

Forward Primer: CCCCTCCATCCTTTCTTCTC
Reverse Primer: ATGAGCCAGATCAGGGACTG

Reactive materials (each extract has three samples): SYBR Green 12.5µl_ Forward Primer 1µl_ Reverse Primer 1µl_ Water 8.5µl_ Template (cDNA) 2µl. After preparing the samples, they were placed in the real-time quantitative PCR device and programmed for the following temperatures for 40 cycles



After completing the reaction, the changes in the CT value were calculated and calibrated with a reference CT value for one of the service genes according to the method of. [16)]

3.6. Immunohistochemistry

After taking the sections and fixing them in 10% formalin for a period ranging between (16-24) hours, they were washed with distilled water to remove excess fixative, then dried using varying concentrations of alcohol before being subjected to drying using xylol. The sample was then placed in paraffin in the form of molds, and then we carried out the cutting process via a device. The microtome was 4μm thick and placed on special glass slides, then the slides with the cut preparations were placed in salt for the next day at 60°C. The next day, the paraffin was removed with xylol, then placed in three gradually decreasing concentrations of alcohol and washed with running water. The preparations were then boiled with EDTA in a special bowl in the microwave for (15 minutes), then washed with running water, then placed (Bio SB, Mouse/Rabet Polyclonal Antibody for Proteintech Group) for (5-10 minutes). Then the preparations were washed with running water and distilled water, and placed for (5 minutes) in Wash Buffer After that, the primary antibodies to the colorant used were added for (45 minutes).

After this stage, the preparations were washed with a portable wash buffer for 5 minutes, then the secondary antibody was added (Mouse poly-detector HRP LABEL, Bio SB) for (45 minutes), then washed again with a portable wash buffer. After that, the samples were placed in a portable polydetector chromogen for (10 minutes) and washed with running water. They were dried with alcohol, dried with xyol, and coverslips were placed on them to be ready for examination under the microscope. [17)]

Results And Discussion

4.1. Titration P53 expression in colon tissue of experimental animals using q PCR technology

Table.1 shows the results of comparing P53 gene expression in the six groups of animals. The gene expression rate was normal in the first control group (1.02±0.01) and in the second and third groups, which were dosed with zingiber extract at two different concentrations (1.05±0.04, 1.03±0.02, respectively). While the gene expression in the fourth group that was injected with DMH (40 mg/kg) decreased to (0.26±0.03) compared to the first control group, then the gene expression of the P53 protein increased to (0.65±0.02) in the fifth group as a result of treatment with zingiber extract (80 mg/kg). kg) in conjunction with DMH injections, and it also increased to (0.89±0.05) in the sixth group treated with zingiber extract (220 mg/kg) and DMH.

Table. 1. Titration P53 expression in colon tissue of experimental animals using real time PCR technology.

Group	P53
The first	1.02±0.01
The second	1.05±0.04
The third	1.03±0.02
The fourth	0.26±0.03
The fifth	0.65±0.02
The sixth	0.89±0.05

4.2. Immunohistochemical staining of P53 protein in colon sections of different animal groups

It was revealed through histological study of parts of the colons of animals from the six groups that the first control group showed negative expression of the P53 protein, as the nuclei were not stained with the dyes specific to the P53 protein (brown color) (Fig. 1). This indicates the absence of neoplastic transformation within the colon tissue, as is also the case in animals. The second and third groups were treated with ginger only (Fig. 2) (Fig. 3). As for the fourth group that was injected with DMH, it showed positive expression of the P53 protein, and this was evident through the brown color of the cell nuclei (Fig. 4) (Fig. 5) (Fig. 6). This indicates a mutation in the P53 protein, which led to a defect in its function of protecting the cell. Colon sections of the fifth and sixth groups showed negative expression of the P53 protein (Fig. 7) (Fig. 8) as a result of treatment with zingiber compared to the fourth group. This demonstrates the role of zingiber in preventing harmful effects within the colon tissue resulting from DMH injections.

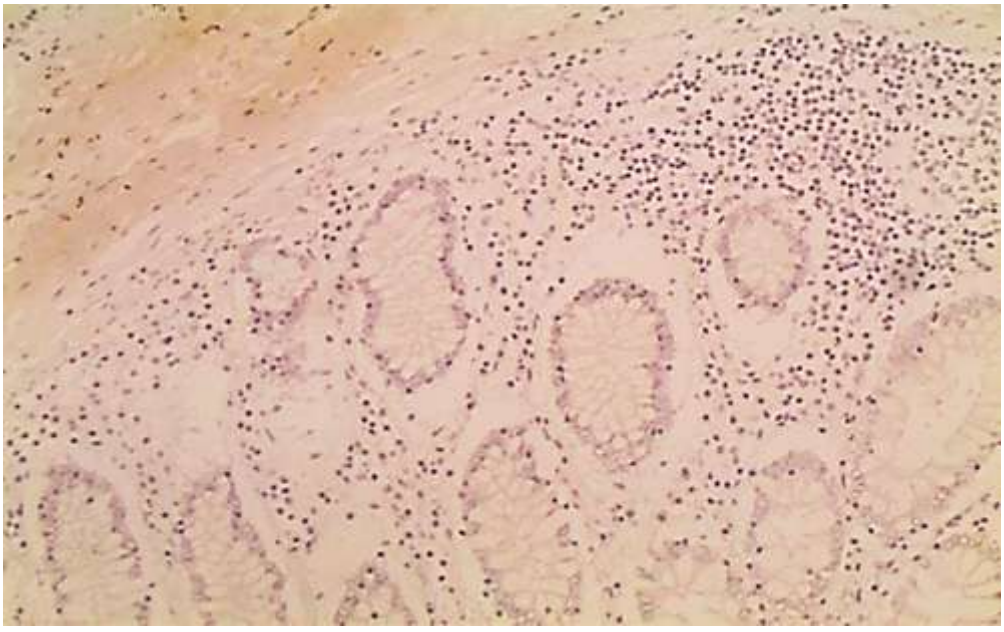


fig. 1. Micrograph showing negative immunohistochemical staining for P53 protein (the first group, magnification 100).

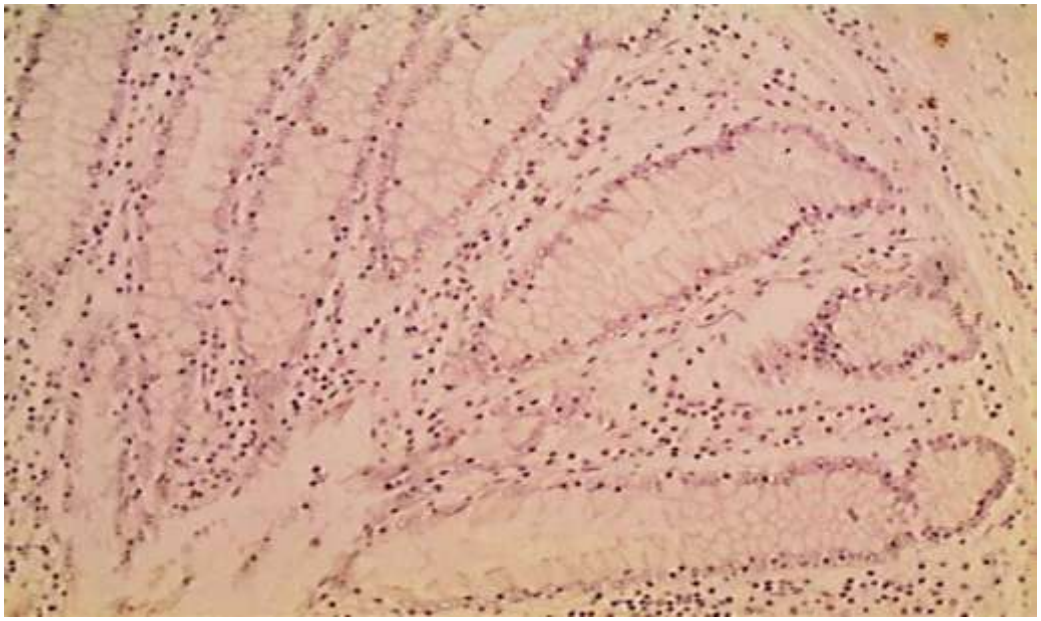


fig. 2. Micrograph showing negative immunohistochemical staining for P53 protein (the second group, magnification 100).

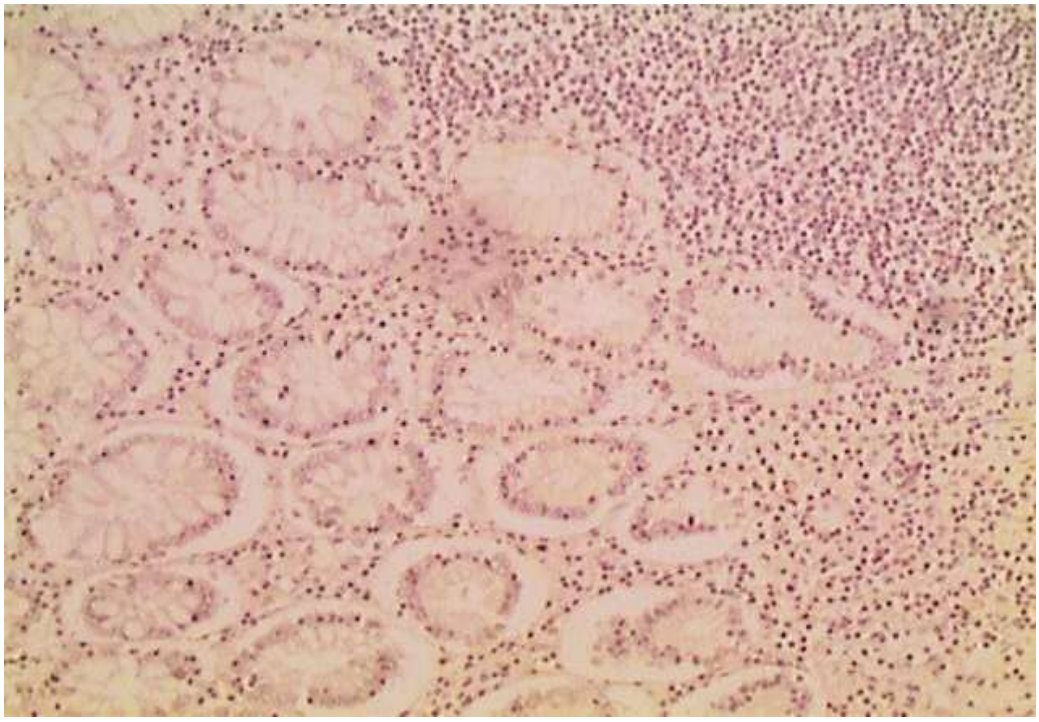


Fig.3Micrograph showing negative immunohistochemical staining for P53 protein (the third groupation 100).

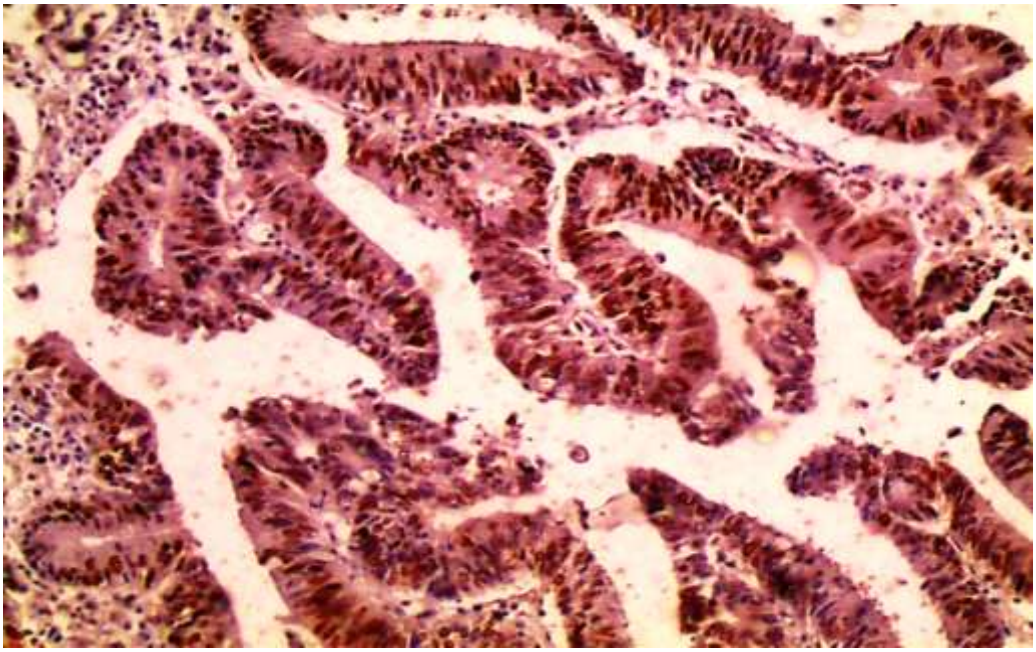


fig. 4. A micrograph showing positive immunohistochemical staining for P53 protein, where the nuclei appear brown in color (the fourth group, magnification 100).

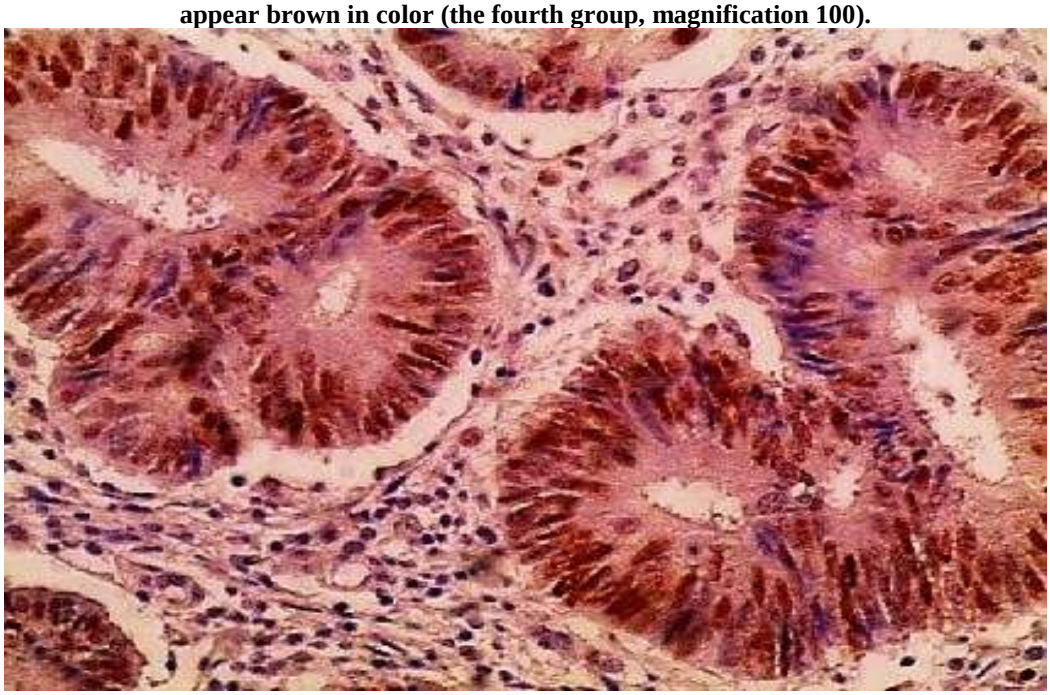


fig. 5. Micrograph showing positive immunohistochemical staining for P53 protein (the fourth group, magnification 400).

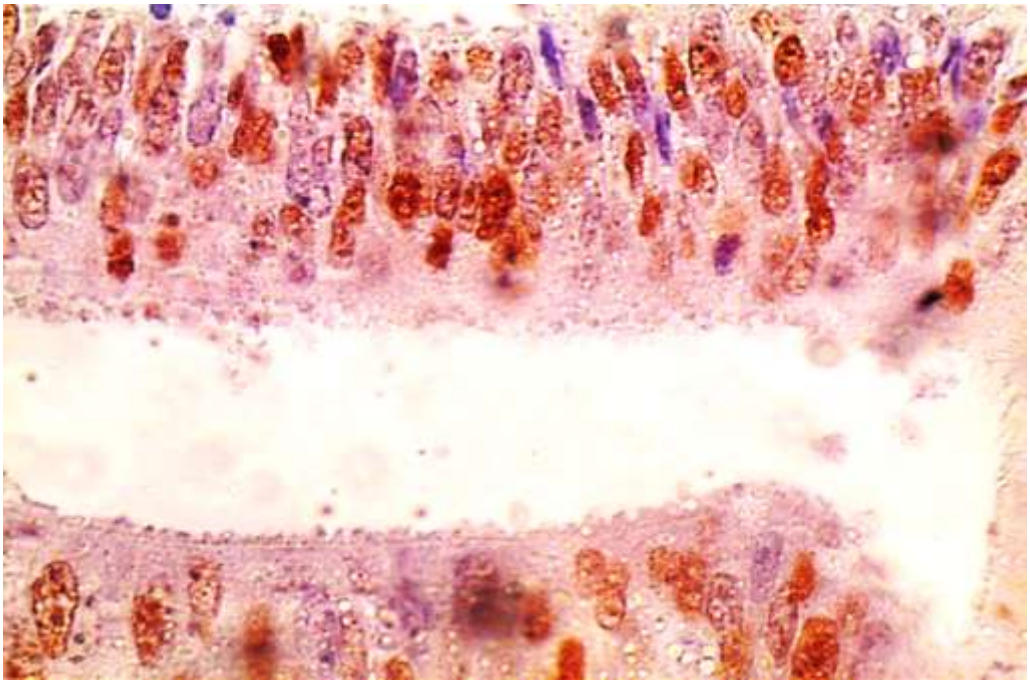


Fig. 6. Micrograph showing positive immunohistochemical staining for P53 protein (the fourth group, magnification 600).

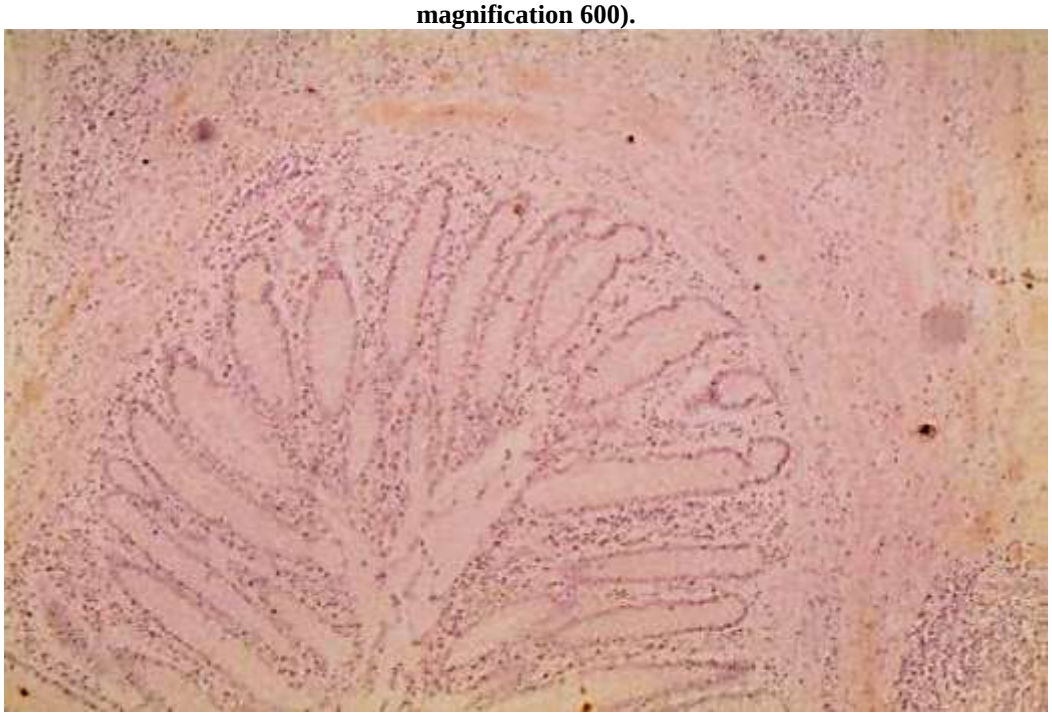


Fig. 7. Micrograph showing negative immunohistochemical staining for P53 protein (the fifth group, magnification 100).

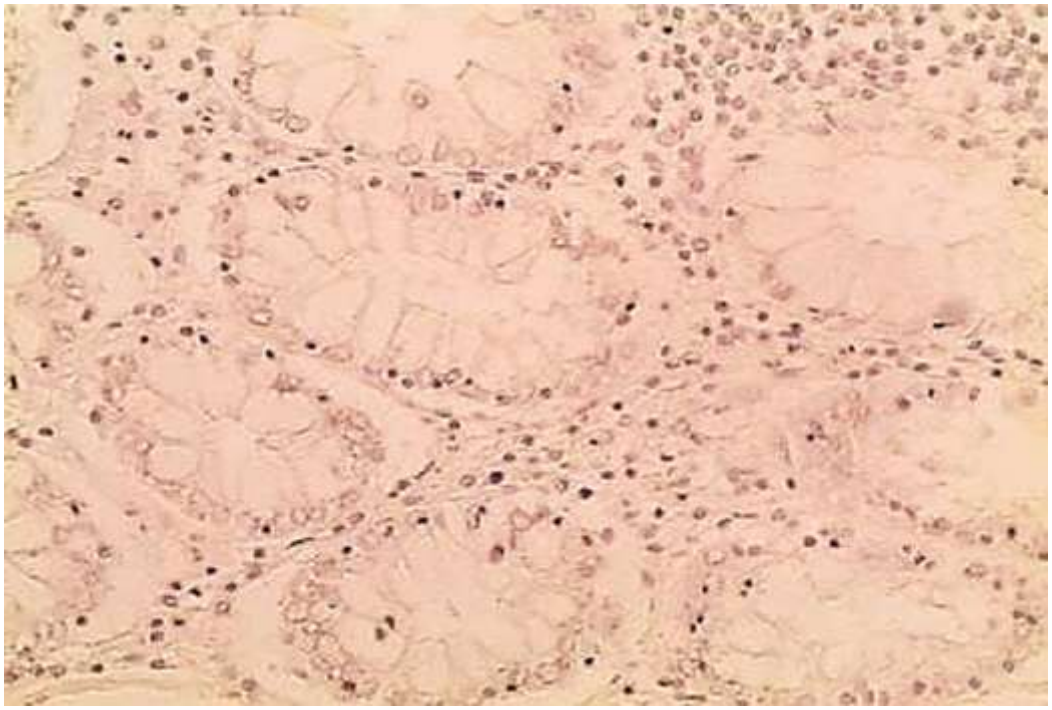


Fig. 8. Micrograph showing negative immunohistochemical staining for P53 protein (the sixth group, magnification 100).

The gene expression of the P53 gene in the control group and the second and third groups reversed the positive expression of the fourth group that was injected with the carcinogen DMH and the return of gene expression in the colon segments in the sixth group when treated with zingiber in conjunction with the injection of DMH compared to the fourth group is consistent with Other studies confirm the negative role of DMH and the positive role of zingiber extract. [18)] This was similar to other similar studies in terms of P53 immunohistochemical staining using plant extracts such as (Ginkgobimoba and ginseng) [19)] as the protein P53 is a tumor suppressor protein that regulates the transcription of genes involved in cell circuitry and DNA repair and contributes to programmed cell death, through which damaged cells are removed during cellular homeostasis of the colon crypts. [20)] Inhibiting cellular transcription is also called the guardian of the genome because of its major role in regulating the cell cycle and inhibiting tumor growth. In normal cells, P53 is not mutant, so its expression is low or absent and when the abnormality occurs, the phosphorylation of the protein increases, so its concentration increases in the nuclei of tumor cells, thus expressing the mutant type. [21)]

The main mechanism by which zingiber mitigates the harmful effects of the carcinogen DMH is believed to be by enhancing detoxification activities and antioxidant enzymes in the circulation. [22)] Previous studies have shown that zingiber reduces oxidative stress resulting from a high-fat diet by increasing antioxidant levels. [23)] The results of the current study can be explained by the fact that the protective properties of zingiber extract in protecting the colon from oxidative stress leading to cancer caused by DMH are due to it containing a number of antioxidant chemical compounds capable of activating some genetic transcription factors that work to suppress the gene expression of genes encoding for the production of proteins that contribute to the release of The meiotic death program in colon cells, such as tumor necrosis factor alpha, caspase enzymes and P53 protein, which work to destroy bioluminescent membranes and release cytochrome c. [24)] This assumption

is supported by the results of many studies that focused on the chemical composition of zingiber extract. [25)]

One study revealed that Zerumbone, a component of Asian zingiber, prevents the growth and spread of pancreatic cancer through various mechanisms. Zerumbone has been reported to induce apoptosis of 1PANC- cells. Induction of apoptosis was associated with upregulation of P53 and p21 proteins as well as ROS production in Zerumbone-treated PANC-1 cells. This result indicated that Zerumbone-induced apoptosis of PANC-1 cells occurs through the P53 signaling pathway. [20)]

Plant extracts work to reduce cancer by introducing cancer cells into the path of programmed cell death, as cancer cells lead to a cellular reaction in many cases of destruction of genetic material which is by increasing their production of the protein factor P53 which is a strong inducer of programmed cell death as it works to Activating the transcription of genes encoding to produce proteins belonging to the Bcl2 family which are concentrated in the outer membrane of the biosensors such as the Bax protein, creates holes in the membrane of the biosensors, causing the leaching and exit of the cytochrome c protein which binds to another protein in the cytoplasm, which is the protein Apaf-1 (activating factor for death-activating proteases) they form a cellular complex called killer bodies or death bodies that bind to the enzyme Caspease-9 and activate caspease-3 and caspease-7 and then activate caspease-8 which accelerates cell death and fragments the cell into small vesicles which facilitates their ingestion by phagocytes. [26), 27)]

Zingiber's anticancer activity is attributed to its ability to modulate several signaling molecules such as NF-B, TNF-α, COX-2, cyclin D1, Bcl-2, caspases and other cell growth regulating proteins. [20)] An in vitro study showed that zingiberol-6 induces apoptosis of gastric cancer cells as it facilitates TNF-associated ligand-inducible (TRAIL-)-induced apoptosis by increasing caspase-3/7 activation.

The current study also hypothesizes that DMH are alkylating agents that are typically injected intraperitoneally or subcutaneously over several weeks to induce tumor development in the distal colon. The majority of these tumors contain mutations in the beta-catenin gene [28)] (Ctnnb1). These mutations affect the N terminus of the amino acids of the β-catenin gene product, making the protein resistant to destruction, and increasing WNT signaling, which drives the process of tumorigenesis. [12)]

Conclusions:

The results of the current study showed that dosing experimental animals with the aqueous extract of zingiber roots contributed to resistance to free radicals and carcinogenic damage to colon tissue resulting from injection of the DMH compound in vivo.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85- 23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

Acknowledgements

Big thanks to Tishreen University and the pathological anatomy laboratories at Tishreen University Hospital in Latakia.

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