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Enhanced effects of ferulic acid against the harmful side effects of chemotherapy in colon cancer: docking and in vivo study

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Abstract

Background: Colorectal cancer is considered a potential causative agent of morbidity and death, making it a particularly dangerous malignancy. The current study aims to assess the efficacy of ferulic acid (FA) to attenuate the harmful side effect of 5-fluorouracil (5FU) in colon cancer tissues induced by 1,2-dimethylhydrazine (DMH).

Results: Regarding the colon tissues of male Wistar-albino rats (*Rattus norvegicus*), combined FA and 5FU showed the approximately normal structure of mucosa. The treated groups showed a remarkable reduction in Ki67, Ck20, and an elevation in caspase-3 and P53. There was significant upregulation of *P53* in both 5FU and combined FA–5FU groups ($p < 0.001$ and $p < 0.00001$, respectively).

Conclusions: The present results revealed a potential role of the combined therapy by 5FU and FA in the suppression of colon cancer induced by DMH by upregulation of apoptosis with the clear effect of FA in attenuating the side effects of 5FU on the normal cells.

Keywords: Ferulic acid, 5-Fluorouracil, Colon cancer, 1,2-Dimethylhydrazine, Caspase-3, P53, Ck20, Docking, Ki67

Background

In 2020, colorectal cancer (CRC) is considered a potential causative agent of morbidity and death, making it a particularly dangerous malignancy (Dai, 2020). The occurrence of colorectal cancer involves the upregulation of oncogenes and the downregulation of tumor suppressor genes. CRC causes are multi-factorial, including age, family history, and inflammatory bowel disease (IBD); these factors had a remarkable role in the triggering, formation, and progression of CRC (El-Khadragy et al., 2018). The early detection of CRC can give better chances of saving lives. Although great progress has been attained in the treatment, the survival rates are still not satisfactory. Thus, it is essential to find novel potential therapeutic drugs for colorectal cancer (Alazzouni et al., 2021).

In the animal models of colon cancer, 1,2-dimethylhydrazine (DMH) is considered the most effective and prevalent. DMH is a pro-carcinogenic chemical inducer for CRC, DMH is a hepatocytes-activated chemical and is transported to the intestine through blood and bile, which induces free radicals formation leading to damage of the DNA of both liver and colon cells. DMH is considered an indirect drug inducer, enhancing DNA hypermethylation of the epithelium of the colon (Alazzouni et al., 2021).

5-Fluorouracil (5FU) is commonly used for a wide range of solid tumors including colon cancer or

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combined with medications. Although the significant role of 5FU, the high cytotoxicity is considered a remarkable disadvantage. 5FU and its metabolites have various pathways leading to inhibition of thymidylate synthase enzyme during DNA replication which leads to inhibition of proliferation during the S-phase (Muthu et al., 2013; Weber, 2015).

5FU is transformed to the active form of 5-fluoroxyuridine monophosphate (F-UMP), which is metabolized into FUMP. This metabolite binds with uridine triphosphate to attach to the RNA strand which inhibits RNA and protein synthesis and by the way inhibits cell growth (Weber, 2015). It not only does this binding mess up the RNA functions, but also has a major effect on DNA when 5FU binds with DNA; it is repaired by its removal by uracil N-glycosylase leading to a single nucleotide polymorphism leaving apyrimidinic sugar for DNA repair (Wu, 2018). 5FU causes clinical complications including myelosuppression, diarrhea, enteritis, and anorexia (Muthu et al., 2013).

Ferulic acid (FA) has plenty of biological effects as antibacterial, antifungal, antiviral, antioxidant, and anti-inflammatory besides its protective effect on the liver, heart, and nerves and against thrombosis and tumors. FA can be found in vegetables, grains, fruits, beans, peanuts, and coffee (Ghosh et al., 2018).

FA also possesses potent antioxidant activity against free radicals by losing a hydrogen atom from the phenolic hydroxyl group (Palani Swamy & Govindaswamy, 2015). The antitumor activity of Ferulic acid comes from its ability to activation of cytoprotective enzymes against reactive oxygen species (ROS) (Barone et al., 2009). Previous in vivo and in vitro experimental models released evidence of the ability of FA the regulation proliferation, cellular growth, and also, reducing cytotoxicity by scavenging free radicals which emphasizes the use of FA in cancer therapy (Mancuso & Santangelo, 2014).

The previous preclinical studies revealed the potential effect of FA accompanied by radiotherapy and chemotherapy. In HeLa, and K562 cell lines FA revealed the ability to reduce the 5-fluorouracil and cisplatin cytotoxic effects (Hemaiswarya & Doble, 2013).

The current study focused on the assessment of the potential effect of FA as a natural product in the attenuation of the harmful side effect of 5FU.

Methods

Animals

Forty mature healthy male Wistar-albino rats (*Rattus norvegicus*) with a mean weight of 180 ± 30 g were sourced from the Experimental Animal Unit at the Ain Shams Research Institute for use in the current investigation. To keep the rats safe, we utilized metal cages with a grid-like floor to contain them. Temperature and humidity levels were maintained at the same level as in their natural habitats (25 ± 2 °C), relative humidity (44–56%), and light/dark cycle of 12:12 h. One week before the study they were fed a standard pellet diet and water ad libitum throughout the course of the experiments. The rats were handled with care according to international guidelines for the use of laboratory animals. The study was approved by the ethical committee at the Faculty of Science, Helwan University (approval number HU2020/ZASG0220/09).

Molecular docking

The molecular operation environment (MOE) program was used for the docking study on TP53-binding protein 1 (PDB code: 6MXZ) (Homosapiens) and Caspase-3 (PDB code: 1RHR) imported from protein data bank (PDB) (Bank, n.d.-a, n.d.-b). With the Merck molecular force field (MMFF94), all minimizations were conducted until the root-mean-square deviation (RMSD) gradient of 0.01 kcal/mol/A was achieved. For ligands, the output data was recorded for the final score function (S) (Kishk et al., 2019).

Colon cancer induction

DMH underwent 15 weeks of subcutaneous administration at 50 mg/kg/week in 1 mM EDTA as a vehicle, and 1 N NaOH was used for adjustment of pH (6.5) (El-Khadragy et al., 2018).

Experimental design

Rats were arbitrarily allocated into group (I): control group ($n = 10$) served as negative control and injected subcutaneously with normal saline (0.9% NaCl), and group (II): induced colon cancer group (DMH group) ($n = 30$) subjected to DMH with subcutaneous injections for 15 weeks (Alazzouni et al., 2021), and then the rats were allocated equally into three groups. DMH group after induction of colon cancer has received diet only. 5FU treated group: 50 mg/kg intraperitoneally administered once a week for four weeks (Alazzouni et al., 2021; Ghosh et al., 2018). Combined FA-5FU group: FA for four weeks, 50 mg/kg was administered intraperitoneally once a week, and FA added to ferulic acid was orally supplied 3 times per week with a dose of 50 mg/kg (Muthu et al., 2013). During the experiment, daily health checks of the rats of all groups were observed. The animals were euthanized under light ether anesthesia one week after the final dose.

Histopathological study

After the end of the treatment, all rats were sacrificed with sharp blades under light ether anesthesia.

The excised colons of all rats were fixed for 24 h in 10% neutral buffered formalin solution, washed in running water then subjected to dehydration with ethanol, then xylene for clearing, paraffin embedding, and sectioning. The sections were then stained with hematoxylin and eosin stains for evaluation through the light microscope (Suvarna et al., 2018).

Immunohistochemical study

Five-μm thickness sections of the processed colon tissues from each rat for all groups were immune-stained using anti-Caspase-3, anti-Ki67, anti-Ck20, and anti-P53 primary antibody for 90 min, and then applied to the secondary antibody using the immunoperoxidase method in order to assess the immunoreactivity of the above-mentioned markers in the study groups (Soliman et al., 2018).

Morphometric study

The assessment was carried out by the "Leica Qwin 500 C" image analyzer computer system present in Pathology Department, National Research Centre. Caspase-3, Ki67, Ck20, and P53 reactions were evaluated in ten non-overlapping fields at magnification × 400 for all specimens.

Real-time PCR

RNeasy Plus Minikit was used for RNA extraction from all samples. The complementary DNA (cDNA) was produced by the RevertAid™ H Minus Reverse Transcriptase. *P53* sequence forward, 5′-ATGTTTTGCCAA CTGGCCAAG-3′ and reverse, 5′-TGAGCAGCGCTC ATGGTG-3′, and *β-actin*, the reference gene used has the following sequence, forward, 5′-GTGACATCCACACCC AGAGG-3′ and reverse, 5′-ACAGGATGTCAAAAC TGCCC-3′. qRT-PCR was carried out using the SYBR Green RT-PCR kit in which the Applied Biosystems 7500 instrument was employed. Regarding typical thermal profile; 95 °C for four minutes, followed by 40 cycles of 94 °C for 60 s and 55 °C for 60 s. (Alazzouni et al., 2021). The delta–delta cycle threshold (Ct) approach was used to measure the fold differences in gene expression between the control and treatment groups by calculation of $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001).

Statistical analysis

The data were presented as the mean ± standard deviation (SD). Multiple variable comparisons were done using a one-way analysis of variance (ANOVA) in the statistical package program (SPSS version 20). Duncan's test was used to make statistical comparisons between the two groups. Statistical significance was defined when the p-value are equal to or lower than 0.05.

Results

Molecular docking

Two compounds were docked with the 3D structure of two proteins Matrix P53 and Caspase-3. The obtained data of Ferulic acid strongly proved the interaction of the target ligand sites: 6-ring, Oxygen (O) 7, and O 8 with the target protein through the arginine (Arg) 1490, lysine (Lys) 1505, and glycine (Gly) 1488 amino acids respectively. However, the hydrogen bond is the most common type of contact bond present, while hydrophobic (Arene-H) bonding is also present. Pose forms with the most binding energy are depicted in this illustration (Fig. 1A, C). In this case, the binding energy was determined to be − 4.38 kcal/mol lower than expected. Only one contact of the target ligand location was found in the docking with 5FU. The amino acid methionine (Met) 1584 connects nitrogen (N) 4 to the target protein. The backbone donor bond was the type of interaction relationship. Pose forms with the most binding energy are depicted in this illustration (Fig. 1B, D). Binding energy was determined to be − 3.95 kcal/mol. (Table 1).

On the other side, the interaction between Ferulic acid with Caspase-3 ligand sites: 6-ring, Oxygen (O) 13, and O 14 with the target protein through the Lys 278, Lys 259, and asparagine (Asn) 263 amino acids, respectively, with hydrophobic interactions and hydrogen bonds as the most prominent. − 4.59 kcal/mol was determined to be the binding energy (Fig. 2A, C).

The interaction between 5-Fluorouracil and Caspase3 target ligand sites: 6-ring and Oxygen (O) 6 with the target protein through the Asparagine (Asn 263) and Lysine (Lys 259) amino acids, respectively (hydrogen bond and hydrogen bond). The binding energy was found as − 378 kcal/mol which is lower than the binding energy of ferulic acid (Fig. 3B, D) (Table 2).

Histopathological analysis

The control group showed a normal pattern of the colon including the mucosa, submucosa, musculosa, and serosa (Fig. 3A). In the DMH treated group, the mucosa of the colon showed marked crowdedness with lymphocytes. Also, there were alterations in the morphology of mucosal glands and decreased number of goblet cells (Fig. 3B). The 5FU treated rats showed a slight improvement in the histological structure of the glands with moderate infiltration with lymphocytes. The mucosa is normally intact. There was no abnormality in the structure of the submucosa or musculosa (Fig. 3C). The treated rats with both ferulic acid and 5-fluorouracil showed the most likely structure of the normal colon histological structure with the fine architecture of mucosa. The mucosa, submucosa, musculosa, and serosa remained intact (Fig. 3D).

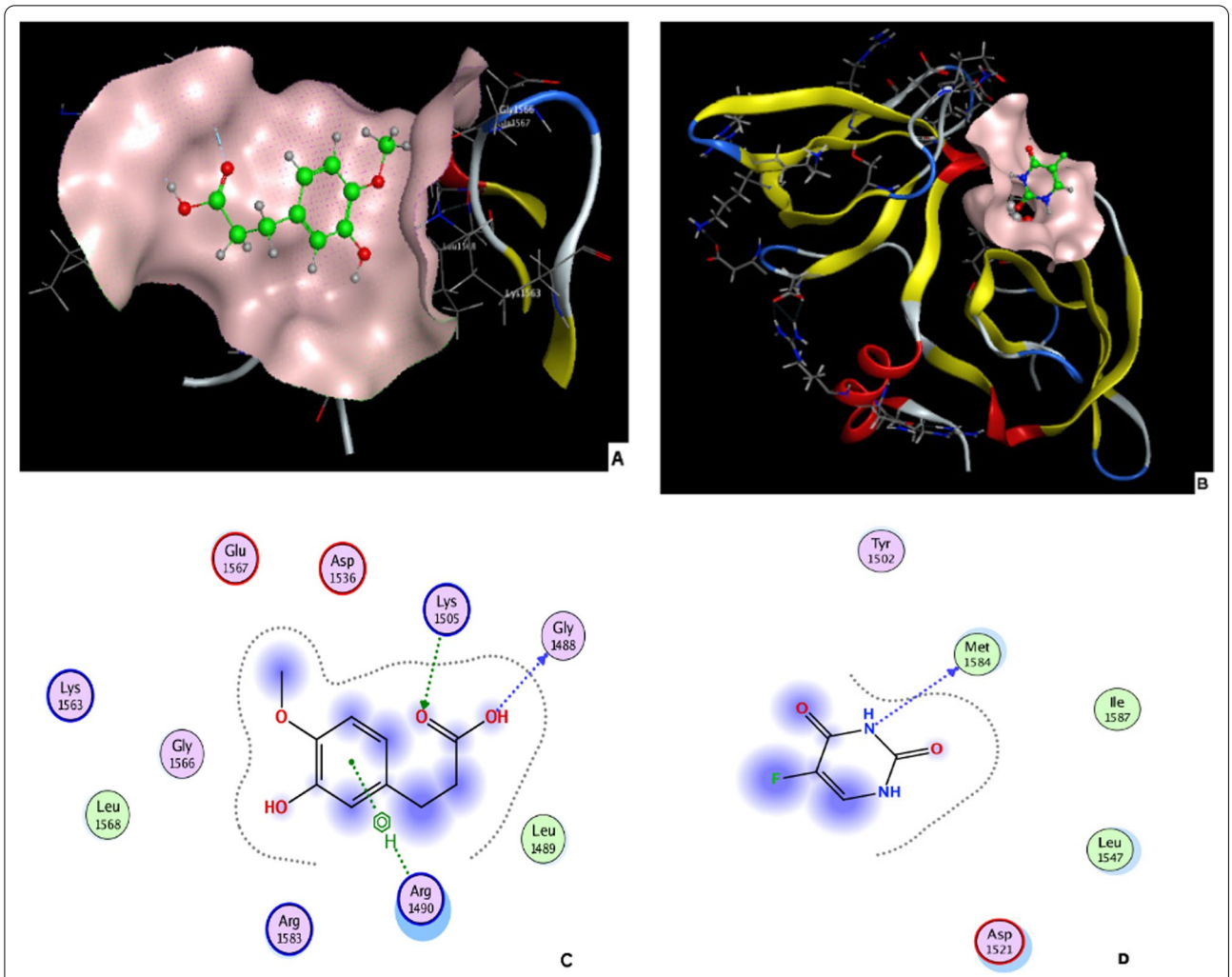

Fig. 1 Molecular docking of P53 (PDB: 6MXZ), **A**: 3D interaction diagram with FA, **B**: 3D interaction diagram with 5FU, **C**: 2D interaction diagram with 5FU, **D**: 2D interaction diagram with 5FU

Table 1 Apparent interaction parameters of the FA and 5FU with P53

	Ligand site	Binding site	Type of interaction	Distance of bond (Å)	Binding energy (Kcal/mol)	Total free binding energy (Kcal/mol)
FA	6-Ring	Arg 1490	Arene-hydrogen (hydrophobic)	3.9	− 0.7	− 4.38
	O (7)	Lys 1505	Sidechain acceptor (hydrogen bond)	3.1	− 3.3	
	O (8)	Gly 1488	Backbone acceptor (hydrogen bond)	2.98	− 3.4	
5FU	N (4)	Met 1584	Back-bone donor	2.98	− 6.4	− 3.95

Arg arginine, *O* oxygen, *Lys* lysine, *Gly* glycine, *Met* methionine

Immunohistochemical analysis

Caspase-3 expression

The control rats showed moderate expression of Caspase-3 (Fig. 4A), while the DMH group showed weak Caspase-3 expression (Fig. 4B). 5FU group showed severe expression of Caspase-3 (Fig. 4C). The combined FA-5FU group revealed moderate Caspase-3 immunoreactivity in the mucosa as the control and with less immunoreactivity than the 5FU group and higher than DMH treated rats (Fig. 4D).

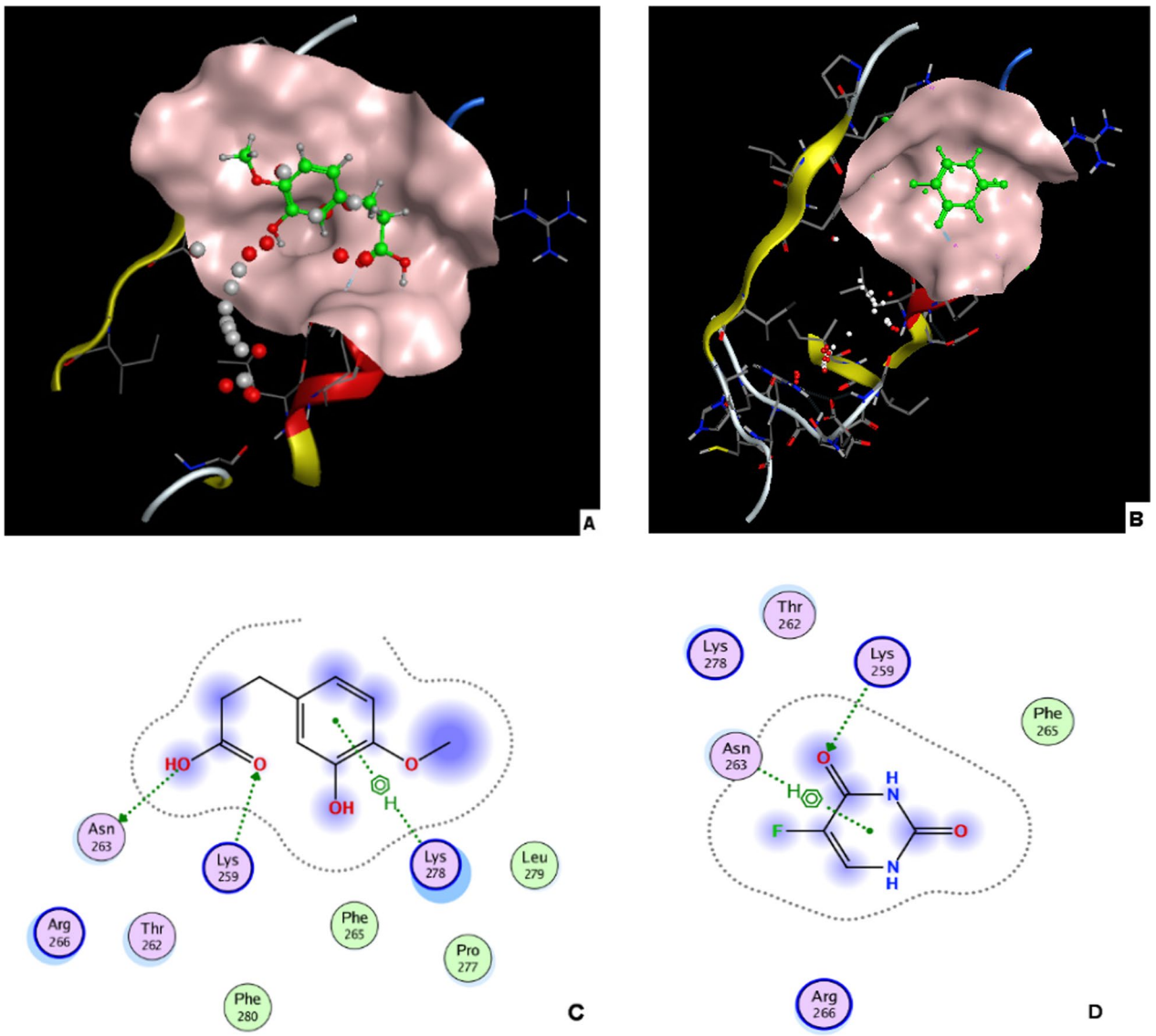

Fig. 2 Molecular docking of Caspase-3 (PDB: 1RHR), **A**: 3D interaction diagram with FA, **B**: 3D interaction diagram with 5FU, **C**: 2D interaction diagram with FA, **D**: 2D interaction diagram with 5FU

Ki67 expression

The mucosa glandular cells of the normal colon tissues showed mild Ki67 immunoreactivity, while the apical region presented weak immunoreactivity (Fig. 5A). The colon tissues of the DMH group revealed severe immunoreactivity with Ki67 (Fig. 5B). 5FU treated group showed lower nuclear Ki67 expression compared with the DMH group through the glandular part (Fig. 5C). The combined FA–5FU group revealed mild Ki67 immunoreactivity (Fig. 5D).

Ck20 expression

The normal colon tissues showed normal Ck20 immunoreactivity (Fig. 6A). The DMH group showed severe cytoplasmic immunoreactivity with CK20 (Fig. 6B). 5FU group presented mild Ck20 immunoreactivity (Fig. 6C), while the combined FA-5FU treated rats showed weak immunoreactivity with Ck20 with irregular distribution throughout the mucosa (Fig. 6D).

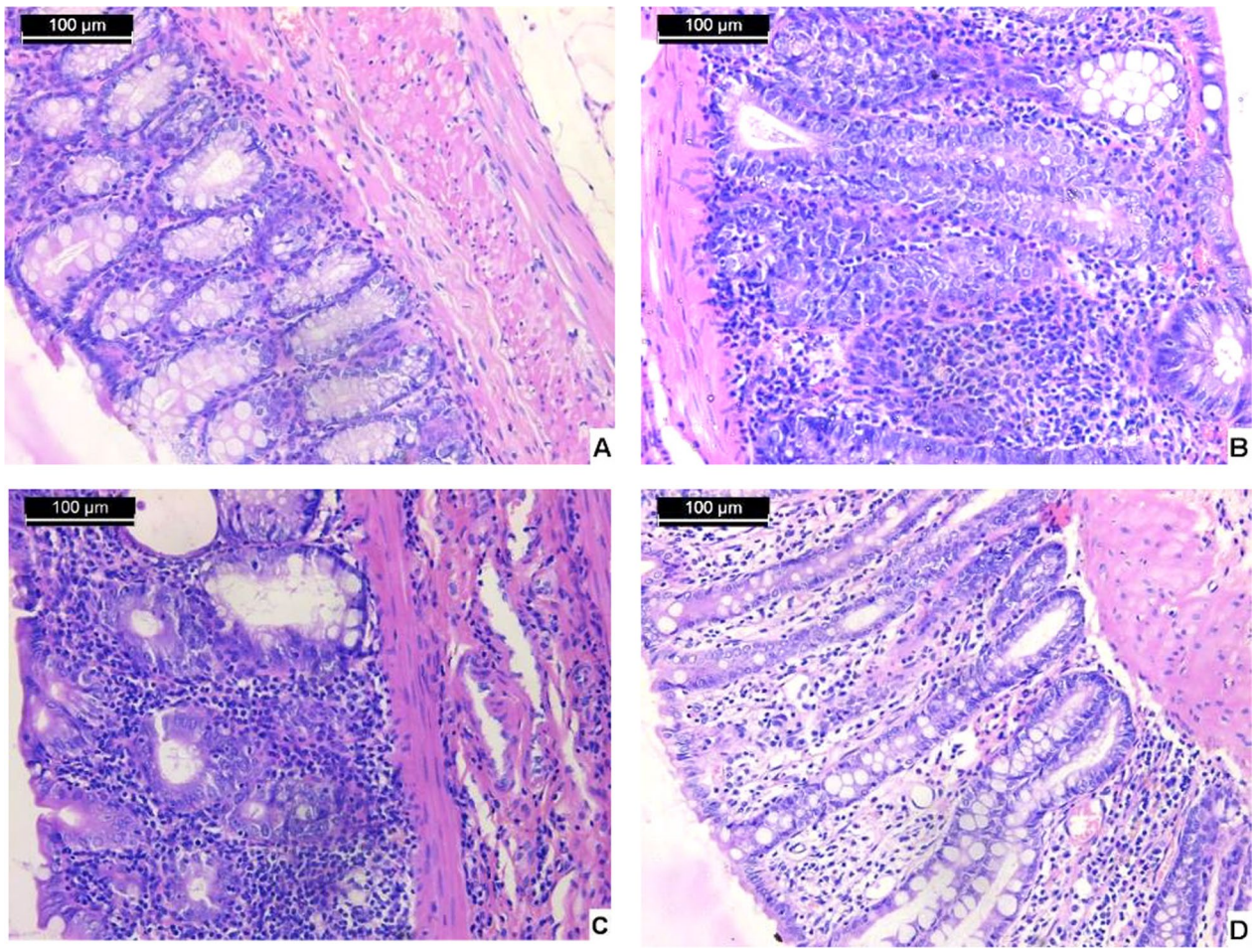

Fig. 3 Photomicrographs of colon specimens of the study groups stained by H&E (×400). **A**: A photomicrograph of the control group showing well-organized glands with normal mucosa. **B**: A photomicrograph of DMH group showing dysplasia, infiltration with mononuclear cells. **C**: A photomicrograph of 5FU treated rat showing infiltration with lymphocytes in the intestinal glands with the improvement of glandular morphology. **D**: A photomicrograph of FA-5FU combined treated rat showing normal intestinal glandular cells and normal covering epithelium

Table 2 Apparent interaction parameters of the FA and 5FU with Caspase-3

	Ligand site	Binding site	Type of interaction	Distance of bond (Å)	Binding energy (Kcal/mol)	Total free binding energy (Kcal/mol)
FA	6-Ring	Lys 278	Arene-hydrogen (hydrophobic)	4.11	− 0.6	− 4.56
	O (13)	Lys 259	Sidechain donor (hydrogen bond)	3.11	− 3.5	
	O (14)	Asn 263	Sidechain acceptor (hydrogen bond)	2.92	− 2.4	
5FU	6-ring	Asn 263	Arene-hydrogen (hydrophobic)	4.22	− 0.8	− 3.78
	O (6)	Lys 259	Sidechain donor (hydrogen bond)	2.94	− 8	

Asn asparagine, *O* oxygen, *N* nitrogen, *Lys* lysine

P53 expression

The control group revealed moderate P53 immunoreactivity (Fig. 7A). The DMH group revealed weak P53 immunoreactivity in the mucosa (Fig. 7B). 5FU group expressed strong P53 mucosal immunoreactivity (Fig. 7C). The FA-5FU combined group showed severe immunoreactivity with P53 which is slightly lower than the 5FU treated group (Fig. 7D).

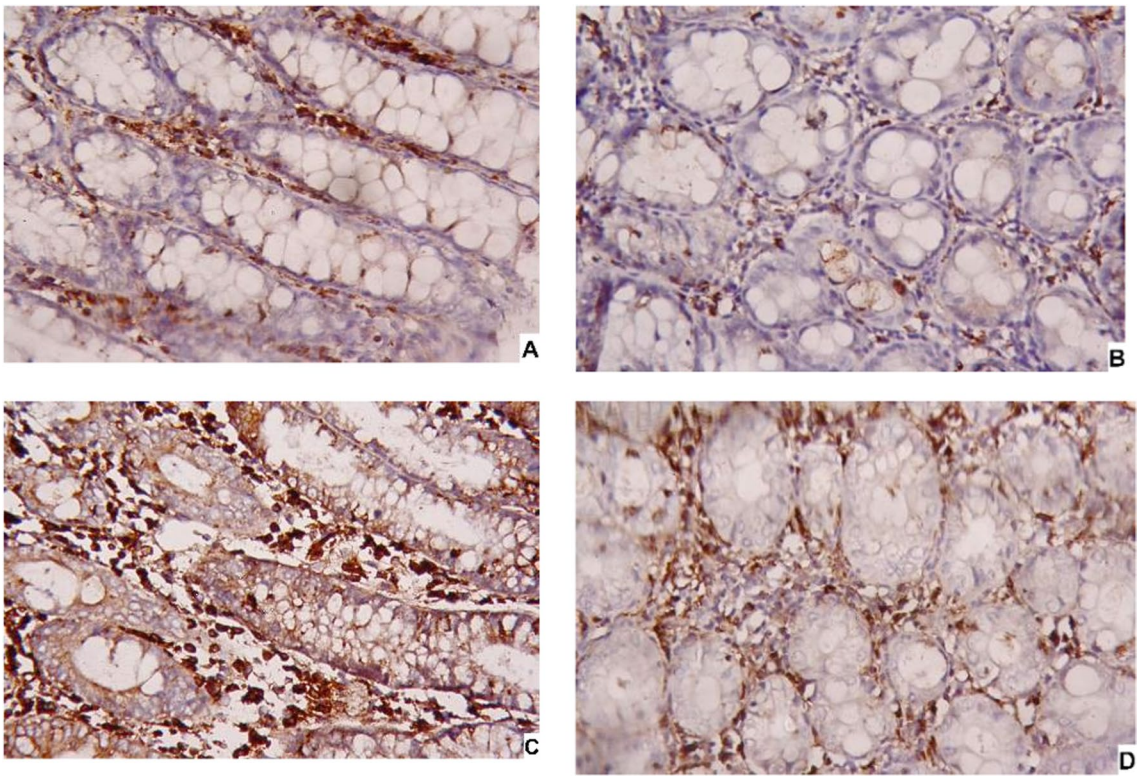

Fig. 4 Photomicrographs of colon specimens of different groups immunostained with Caspase-3 (×400). **A** The control group of normal rats showed normal moderate nuclear Caspase-3 IHC positivity. **B** DMH group showing weak cytoplasmic Caspase-3 IHC positivity. **C** 5FU treated rat showed a severe Caspase-3 immunoreaction in mucosal glands. **D** Ferulic acid and 5-fluorouracil treated rat showing moderate Caspase-3 IHC positivity

Morphometric analysis

The quantitative analysis of IHC for Caspase-3, Ki67, Ck20, and P53. The DMH group revealed a remarkable elevation in Ki67 expression (p 0.01) and a remarkable reduction in Caspase-3 and P53 expression (p 0.007) compared to the control group. 5FU group revealed a remarkable elevation in Caspase-3 and P53 immunoreactivity (p 0.00004 and p 0.00005 respectively) compared with the DMH group. The combined FA-5FU group revealed a marked increase in Caspase-3 and P53 (p 0.01 and p 0.003 respectively) compared with the DMH group (Fig. 8).

P53 gene expression

a. The DMH group revealed a significantly lower *P53* gene than the control group (p 0.0006). 5FU group had a remarkable *P53* upregulation (p 0.001) compared with the DMH group. The combined FA-5FU revealed a significant rise in *P53* expression compared with both DMH and control groups (p 0.001 and p 0.00002 respectively) (Fig. 9).

Discussion

CRC development is considered a pathological complex process and involves in upregulation of specific proteins that regulate and stimulate differentiation and proliferation and downregulates proteins that regulate DNA repair and apoptosis. These proteins are charged for the disarrangement and abnormal structure from normal crypts to aberrant crypt foci, adenoma formation, and progression into CRC (Nabil et al., 2016).

According to the docking work of the FA with P53 (PDB code: 6MXZ), the selected pose gave the interaction of 6-ring, oxygen (O) 7, and O 8 with the target protein through the arginine (Arg) 1490, lysine (Lys) 1505, and glycine (Gly) 1488 amino acids, respectively. FA had more sites of interaction than the 5FU. The binding energy was − 4.38 kcal/mol which is higher than the 5FU (− 3.95 kcal/mol). The number and nature of the interaction sites in FA strongly interpreted the antiproliferative activity of FA which is elevated with 5FU.

The molecular docking of FA with Caspase3 (PDB code: 1RHR) showed interactions of 6-ring, Oxygen (O) 13, and O 14 with the target protein through the Lys 278, Lys 259, and asparagine (Asn) 263 amino

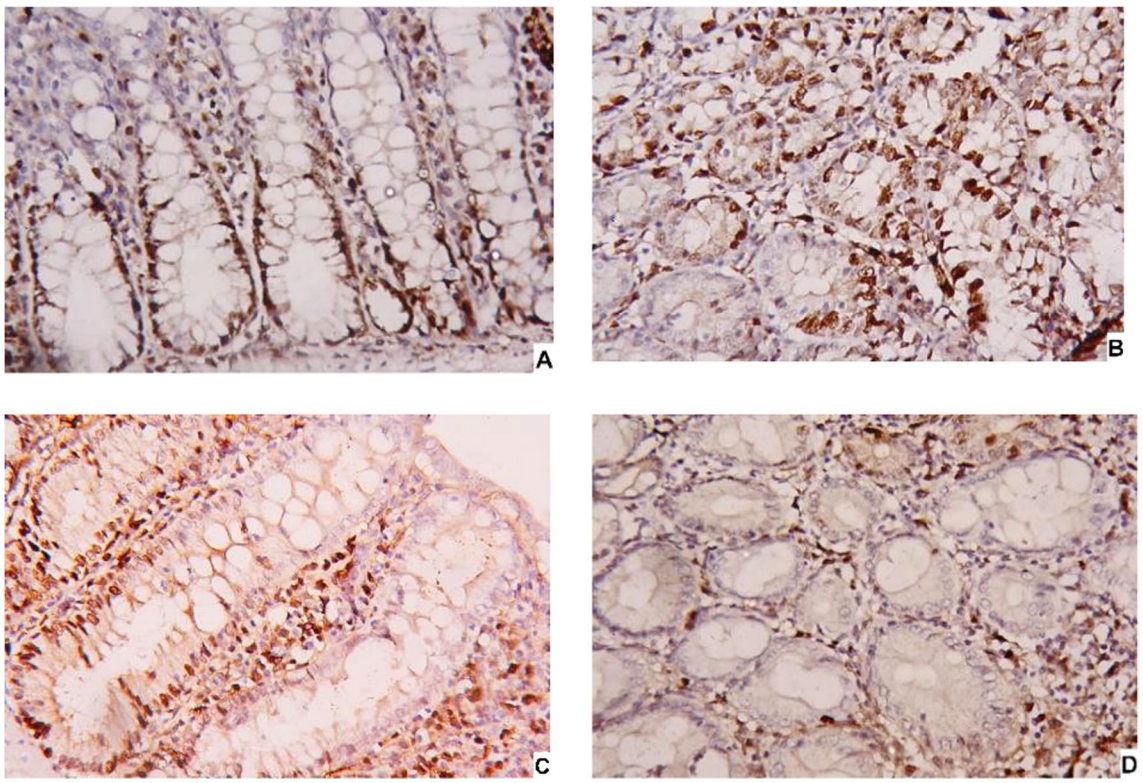

Fig. 5 Photomicrographs of colon sections of different groups immune-stained with Ki67 (×400). **A**: Control group showed normal mild nuclear Ki67 expression. **B**: DMH group showed severe Ki67 immunoreactivity. **C**: 5FU group showed moderate Ki67 expression in mucosal glands. **D** FA and 5FU combined treated group showed an approximately normal anti-Ki67 reaction

acids, respectively. FA had more sites of interaction than 5FU. The binding energy was − 4.56 kcal/mol which is higher than the 5-FU (− 3.78 kcal/mol). The interaction sites' number and nature in FA are potentially interpreted as promoting apoptosis and antitumor activity of FA.

The molecular docking of P53 protein with both ferulic acid and 5-Fluorouracil showed high binding energy, a significant number of hydrogen bonds, and hydrophobic interactions with ferulic acid than 5-Fluorouracil.

The histopathological examination of the colon tissues of the rats injected with 1,2-dimethylhydrazine with a dose of 50 mg/kg/week for 15 weeks showed severe dysplasia and anaplasia with infiltration of the mucosa with mononuclear cells. Mucosa and submucosa showed loss of polarity and prominent hyperchromatic nuclei in the lining epithelium and glandular portion. These histopathological features are similar to the studies of Alazzouni et al., (2021), Jucá et al., (2014). Colon tissues of the 5FU group revealed a slight improvement in the mucosa compared with cancer tissues. The rats treated combination of FA and 5FU showed an almost normal structure of the colon with few mononuclear cells. These findings showed a significant effect of FA in the enhancement of the 5FU effect on colon cancer.

The expression of Ki67 showed a high correlation with proliferation and growth of tumor cells. Ki67 is an established prognostic and predictive biomarker for the evaluation of cancer biopsies (Niotis et al., 2018). The immunohistochemical analysis of Ki67 showed mild expression in normal colon tissues, and 5Fu treated tissues and DMH treated group showed severe Ki67 expression which was supported by a previous study (Alazzouni et al., 2021); on the other hand, the FA–5FU group showed moderate Ki67 expression, and these findings suggest the ability of FA in decreasing proliferation of colon cancer.

Cytokeratin (Ck) is a group of proteins formed from intermediate filaments of the cytoskeleton and are differentially expressed in various sites in the mucosal epithelium (Nabil et al., 2016). Our findings showed significant differences in Ck20 IHC reaction between normal colon tissues and DMH treated rats. The DMH group showed severe CK20 expression in the apical part of the mucosa, while the normal tissues showed mild Ki67 expression.

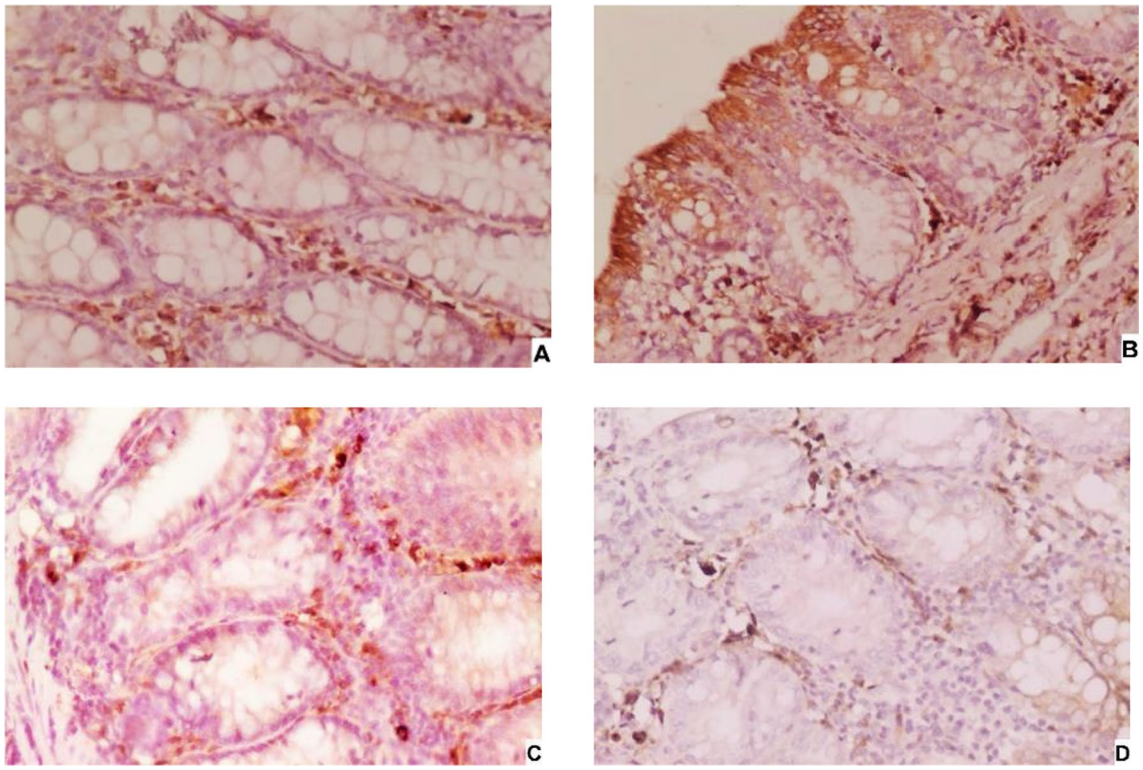

Fig. 6 Photomicrographs of colon sections of different groups immune-stained with Ck20 (×400). **A**: Normal colon of normal rat showing normal moderate cytoplasmic Ck20 IHC positivity. **B**: DMH group showed severe Ck20 expression. **C**: 5FU group presented Ck20 immunoreaction in the mucosal glands. **D** FA-5FU combined treated rats showing mild Ck20 IHC positivity

CRC tissues revealed strong Ck20 immunoreactivity. Recently, the Ck20 protein is a potential marker because of its accurate specificity in gastric cancer (Alazzouni et al., 2021; Bayrak et al., 2012; El-Khadragy et al., 2018; Tunca et al., 2013). The administration of FA combined with 5FU-induced reduction in CK20 immunostaining compared with the 5FU treated group.

In the current study, P53 gene expression showed a significant difference between normal colon tissues and the DMH group. Both 5FU and the combination of FA and 5FU showed upregulated P53 gene expression (P0.0001 and *p* 0.0002, respectively), while DMH showed downregulated expression of P53. P53 immunostaining showed a significant difference between the normal group and the DMH group with weak P53 immunoreactivity in the DMH group and severe expression in the 5FU group and FA and 5FU combined groups. The combination of FA and 5FU decreased the severity of 5FU in promoting apoptosis which affects the normal cells also. These findings give evidence that both 5FU alone and combined with Fa promote DNA repair and apoptosis with decreasing cytotoxicity of normal cells compared with 5FU alone.

Our results found that DMH showed weak Caspase-3 immunoreactivity compared with moderate expression in normal colon tissues. The combination of FA and 5FU showed marked expression of Caspase-3 which is higher than the 5FU treated group. These results support the role of FA enhancement in promoting apoptosis.

A recent study intimated that FA and its derivatives had a remarkable effect on apoptosis proteins, decreased cell growth, caspases, cyclooxygenase, and moderately inhibited colon, lung, breast, and CNS neoplastic cells (Palani Swamy & Govindaswamy, 2015).

Other studies suggest that FA could stop the cell cycle progression due to the down-regulation in genes causing the arrest of the prostate cancer cell cycle (Eroğlu et al., 2015). Inflow cytometry evaluation of ECV304 endothelial cells, FA, enhanced cell cycle arrest in G0/G1 phase. Moreover, FA caused cell cycle arrest by downregulation of CCND1 and upregulation of CDKN1A and phosphorylated RB protein (Hou et al., 2004).

Conclusions

This study concluded that FA with 5FU *is* considered a potential drug against CRC induced by DMH reported by the cellular improvement by histopathological evaluation,

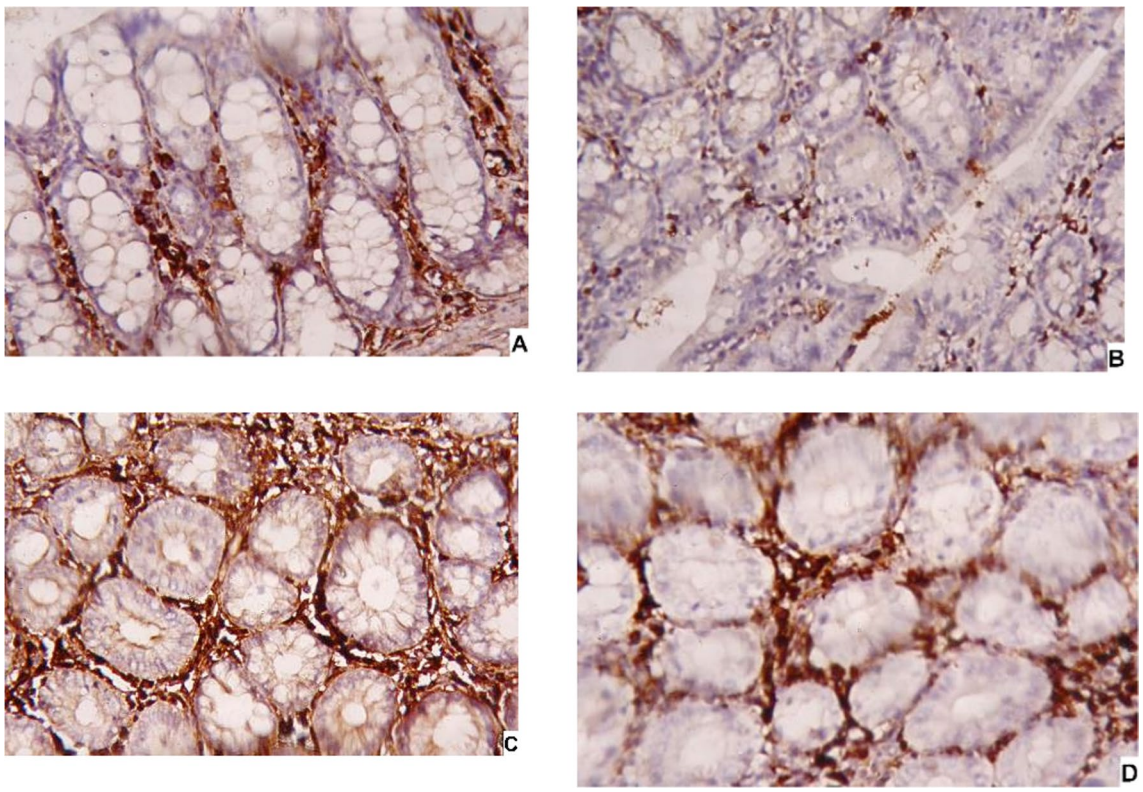

Fig. 7 Photomicrographs of colon sections of different groups immune-stained with P53 (×400). **A**: Control group showed moderate nuclear Caspase-3 IHC positivity. **B**: DMH group showed weak p53 immunoreactivity. **C**: 5FU group showing severe nuclear P53 expression. **D**: FA-5FU group showed severe P53 expression

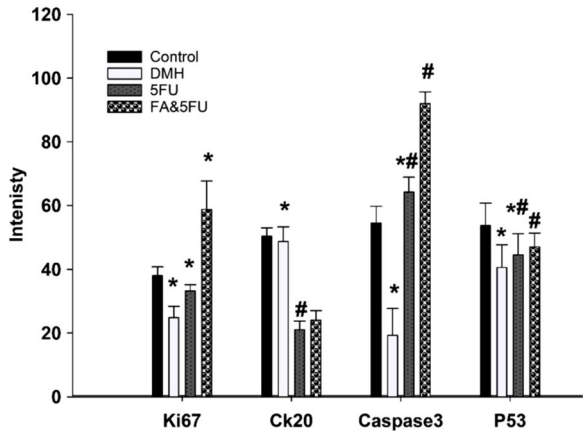

Fig. 8 Intensity percentage quantitative measurement for positive Ki67, Ck20, Caspase 3, and P53 immune reactions. Values are mean ± SD at $p \leq 0.05$. *, significance against control. #, significance against DMH group

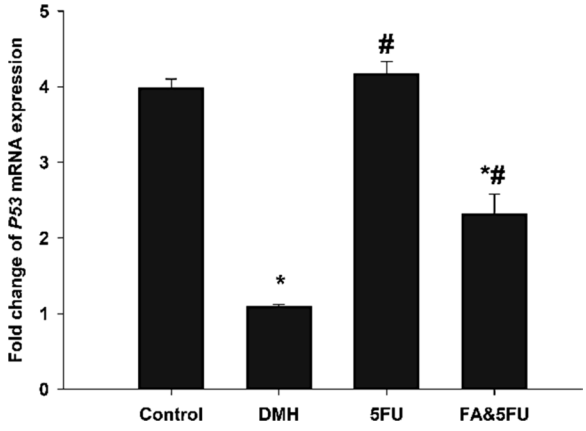

Fig. 9 P53 gene expression among the study groups. Values are mean ± SD at $p \leq 0.05$. *, significance against control. #, significance against DMH group

IHC, and gene expression. These results represented good therapeutic approaches for adjacent chemotherapy with natural products. However, further studies are needed regarding ferulic acid and other natural products' potential effects and decreasing the side effect of chemotherapeutic drugs in CRC.

Abbreviations
CRC: Colorectal cancer; FA: Ferulic acid; 5FU: 5-Fluorouracil; DMH: 1,2-Dimethylhydrazine; P53: Tumor protein p53; Ki67: Proliferation marker protein Ki-67; Ck20: Cytokeratin 20; H&E: Hematoxylin and eosin.

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Not applicable.

Author contributions
Experimental design was done by MHAG and AA. Data interpretation was done by MHAG, AA, AF, MG, and BH. Histological examination was carried out by MHAG and BH. Writing and revision are done by MHAG, AA, and BH. All authors read and approved the final manuscript.

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Availability of data and materials
Data available on request.

Declarations

Ethics approval and consent to participate
The study was approved by the ethical committee at the Faculty of Science, Helwan University (Approval Number HU2020/ZASG0220/09), and according to the National Institutes of Health guide for the care and use of laboratory animals (NIH publications No. 8023, received 1978).

Consent for publication
Not applicable.

Competing interest
The authors declare no competing interest.

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