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Galangin inhibits angiogenesis and induces apoptosis of colon cancer in mice: Molecular estimation of CEA and VEGF

Tarek M. Okda[1*], Mohamed A. Katry[2,3], Noha M. Ragab[4], Mohammad M. Abd-Alhaseeb[2]

[1]*Department of Biochemistry, Faculty of Pharmacy, Damanhour University, Damanhour, Behira, 2251;* [2]*Department of Pharmacology and Toxicology, Faculty of Pharmacy, Damanhour University, Damanhour, Behira, 2251, Arab Republic of Egypt;* [4]*Department of Oral Biology and Diagnostic Sciences, Augusta University, Augusta, GA 30912, USA;* [4]*Department of Pathology, Medical Research Institute, Alexandria University, 21544, Egypt.*

Author for correspondence: Tarek M. Okda, Lecturer of Biochemistry and Molecular Biology, Faculty of Pharmacy, Damanhour University, Damanhour, Egypt.

Abstract:

Background and Aim: *Colon cancer considers common and complicated cancer worldwide. Its treatment remains controversial and complex. 5-fluorouracil (5-FU) is the appropriate chemotherapy for colon cancer; however, it has serious side effects. Therefore, extensive research has been conducted on natural products to prevent cancer and used as adjuncts to chemotherapy to reduce 5-FU toxicity and side effects. Galangin is a natural product found in Alpinia officinarum. It acts as a prophylactic agent and used as an adjunct to chemotherapy in treating colon cancer.* ***Methods****: Sixty male albino mice were used in this study; six groups were designed (10 mice in each group): the control, carcinogen, prophylactic, galangin-treated, 5-FU-treated, and 5-FU- and galangin-treated groups. Reverse transcription-polymerase chain reaction was used to assess the expression of carcinoembryonic antigen (CEA), vascular endothelial growth factor (VEGF), and B-cell leukemia/lymphoma 2 (Bcl-2). Enzyme-linked immunosorbent assay was performed to study the expression of both VEGF and Bcl-2 in colon tissues. Spectrophotometry was used to estimate alanine aminotransferase, aspartate transaminase, malondialdehyde, total nitric oxide, superoxide dismutase, and catalase activity.* ***Results****: The overexpressions of CEA, VEGF, and Bcl-2 were reported in the carcinogenic group, while their expressions were reduced in other treated groups.* ***Conclusion****: These molecular and biochemical results suggest that galangin acts as a prophylactic agent and used as an adjunct to chemotherapy in treating colon cancer. Finally, galangin and 5-FU showed a synergistic effect in treating colon cancer by inhibiting apoptotic signaling pathways. Collectively, this study showed that galangin potentiates the effect of 5-FU as a chemotherapy in colon cancer by inhibiting different signaling pathways.*

Keywords: *CEA, angiogenesis, colon cancer, galangin, 5-FU*

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I. Introduction:

Colon cancer is the third widely spreading cancer and considered the second cause of death worldwide. The most associated risk factors are dietary factors. The quality of nutrition is considered a general index of the important nutrients in the diet (1). Approximately 1.8 million cases of colon cancer were reported in 2018, representing 1/10 of cancer cases and mortality (2). Environmental and genetic factors cause the incidence and advancement of colon cancer, such as chromosomal abnormalities, epigenetic alterations, and lifestyle (3).

The common protocols of colon cancer treatment are chemotherapy, immunotherapy, and radiotherapy; however, these modalities adversely affect normal cells. Therefore, inhibiting the destructive behavior to normal cells of these common treatment modalities encourages finding alternative safe approaches to treat colon cancer. 5-FU is a chemotherapy used in treating colon cancer (4). It acts as an antimetabolite by preventing thymidylate synthase (TS), which causes deoxythymidine monophosphate (dTMP) shortage and inhibits deoxyribonucleic acid (DNA) synthesis. Two main barriers exist to success of 5-FU in treating cancer: first, 5-FU has cytotoxicity to healthy cells, and second, tumor cells have resistance to 5-FU. Therefore, 5-FU is combined with other compounds to produce an effective approach against cancer cells while decreasing the cytotoxicity to healthy cells. 5-FU is one of the most widely used chemotherapeutic agents in treating colon cancer but has a limited therapeutic index due to its toxicity (5).

A new approach has been developed, which puts interest in using natural products as therapeutic alternatives. Natural products are safe, effective, and economic therapeutic alternative agents (6).

Phytocompounds have anticancer effects on different tumors. Polyphenols are a category of these phytocompounds that have potential roles in preventing inflammation and cancer (7). It was reported that the action of indomethacin and vitamin D decreases the risk of several types of cancers (8).

Galangin is a natural flavonoid (trihydroxyflavone) present in *Alpinia officinarum* used as a therapeutic drug. Galangin possesses anticancer properties in different types of cancers. Galangin inhibits epithelial-mesenchymal transition (EMT) and angiogenesis. Furthermore, galangin inhibits the migration, proliferation, and angiogenesis of glioma cells. These results indicate that galangin is a novel product for treating glioblastoma because it suppresses the expression of vascular endothelial growth factor (VEGF) (9).

Galangin stimulates apoptosis via the mitochondrial pathway, decreases Bcl-2 levels, and inhibits G0/G1 cell cycle (10). Galangin enhances apoptosis and autophagy by increasing p53 in HepG2 cell lines (11). Moreover, galangin has several pharmacological effects, including antimutagenic, antioxidant, and scavenging of free radicals. Studies have reported that galangin has antitumor effects in various cancer cells, including hepatocellular carcinoma (HCC), ovarian cancer cells, mammary tumor cells, and prostate cancer (12).

The National Comprehensive Cancer Network of Clinical Oncology and the European Group on Tumor Markers recommended CEA as a marker of colon cancer (13). The expression of CEA is increased in colon cancer associated with metastases and contributes in investigating and staging cancer (14). In addition, it improves the classification of preoperative risk of colon cancer (15). Also, some markers as serum toll-like receptors are clinic-pathological parameters in several types of cancers (16).

In this study, we examined the potential effects of galangin alone and in combination with 5-FU on colon cancer. Furthermore, we studied this effect by determining CEA and VEGF expressions. We further investigated the different possible pathways including apoptosis by estimating Bcl-2, angiogenesis by estimating VEGF, and antioxidation by estimating oxidative stress markers (malondialdehyde (MDA) and nitric oxide (NO)) and antioxidants (superoxide dismutase (SOD) and catalase activity).

II. Materials and methods

Drugs and chemicals

Galangin and 1,2-dimethylhydrazine (1,2-DMH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 5-FU was purchased from Tocris Bioscience (Bristol, UK). All other chemicals were supplied in analytic grades from commercial sources after a quality control test.

Animals

Male Swiss albino mice, weighing 30 g, were purchased from the animal care unit of Vacsera Pharmaceutical Company. The mice were housed in polyethylene cages under controlled laboratory conditions (25 ± 1°C temperature, constant relative humidity, and normal dark–light cycle). Food and water were provided *ad libitum.* All experimental protocols were approved by the Animal Care and Use Committee at the Faculty of Pharmacy, Damanhour University (reference no. 1030PB9).

Experimental design

Two weeks after acclimatization, 60 mice were randomly divided into six groups: Group 1 (control): mice were injected intradermally with 0.9% normal saline (0.1 ml/ mouse) until the last day of the experiment. Group 2 (carcinogenic): mice were injected subcutaneously (sc.) with 1,2-DMH (20 mg/kg) once weekly for 10 weeks (17). The mice in groups 3-6 were injected sc. with 1,2-DMH (20 mg/kg) once weekly for 10 weeks as in group 2. Group 3 (prophylactic): mice were treated for four weeks with galangin (100 mg/kg body weight, daily) suspended in 0.5% (w/v) methylcellulose, administered by oral gavage (4 weeks before induction and 10 weeks during induction) (18). Group 4 (galangin-treated): mice were treated with galangin (100 mg/kg body weight, daily) suspended in 0.5% (w/v) methylcellulose, administered by oral gavage, for 4 weeks after induction with 1,2-DMH. Group 5 (5-FU-treated): mice were treated intraperitoneally with 5-FU (100 mg/kg) once weekly for 4 weeks after induction with 1,2-DMH (19). Group 6 (5-FU- and galangin-treated): mice were treated with a combination of 5-FU (100 mg/kg) once weekly for 4 weeks and galangin (100 mg/kg body weight, daily) suspended in 0.5% (w/v) methylcellulose, administered by oral gavage, for 4 weeks.

At the end of the experimental duration, the mice were fasted overnight and anesthetized using diethyl ether and euthanized by cervical dislocation. For the histopathological investigation, parts of the colon and rectum were used and the remaining tissues were homogenized at 0.1 M Tris-HCl buffer at pH 7.4 and centrifuged for 10 min at 5000 rpm, and the supernatant was used for biochemical and molecular studies.

Tissue and serum samples were collected, aliquotted, and stored at −80°C until use. Reverse transcription-polymerase chain reaction (RT–PCR) was performed to determine the expressions of CEA, VEGF, Bcl-2, and β-actin. ELISA was performed to study the expression of VEGF and Bcl-2 in colon tissues. Spectrophotometry was used to estimate ALT, AST, MDA, total NO, SOD, and catalase activity.

Ribonucleic acid (RNA) was separated from colon tissue using the TRIzol method (Tiangen Biotech Co., Ltd., China). The RNA of each sample was reverse transcribed into cDNA using Taq Master (Jena Bioscience, Jena, Germany) according to the manufacturer's protocol. The forward and reverse primers of CEA, VEGF, Bcl-2, and β-actin are illustrated in Table 1. The thermo-cycling conditions for PCR were as follows: 94°C for 5 min, 30 cycles of 95°C for 1 min, 45°C for 1 min, and 72°C for 1 min. The reaction product (2 µg) was then electrophoresed using 1% agarose gel stained with ethidium bromide and visualized using an ultraviolet lamp. The ladder used was a 100–1,000-kbp DNA ladder (Jena Bioscience, Jena, Germany).

Primer design:

Table 1: NCBI sequence was used for the primer designs for CEA, VEGF, Bcl-2, and β-actin.

Name	Primer (5′–3′)	
CEA	F:	GATGCCGGCGAGTATCAGTG
	R:	TTGACCAGTCTGTCTTTCTGTG
VEGF	F:	CGAGACGCAGCGACAAGGCA
	R:	ACCTCTCCAAACCGTTGGAG
Bcl-2	F:	GACGGAGAACGATGTCCTTA
	R:	GAGCCTTGTCGTACTGAGCA
β-actin	F:	GTAAAGACCTCTATGCCAACA
	R:	GGACTCATCGTACTCCTGCTG

CEA, carcinoembryonic antigen; VEGF, vascular endothelial growth factor; Bcl-2, B-cell leukemia/lymphoma 2; F, forward; R, reverse.

Determination of Bcl-2 in colon tissues using ELISA:

A Bcl-2 Simple Step ELISA Kit (Abcam: ab202441) was used for the estimation of Bcl-2 protein in the tissue extracts. Samples, standards, and reagents were prepared. Phosphate-buffered saline (PBS) was used in preparing the colon homogenate samples. Fifty µl of the sample or standard was added to 50 µl of the antibody in the wells of the ELISA plate, and the plate was incubated for 1 h. Then, the wells were washed with 350 µl of Wash Buffer three times. Tetramethylbenzidine (100 µl) was added to each well, which was then incubated for 10 min. Stop Solution (100 µl) was added, and the absorbance was recorded at 450 nm. A standard curve was used in the calculation of Bcl-2 concentration (ng/mg proteins).

Determination of ALT, AST, MDA, NO, SOD, and catalase activity:

The liver enzymes ALT and AST were estimated using the method defined by Gella et al. (1985). The levels of oxidative stress markers NO and MDA were determined according to the methods described by Van Bezooijen et al. (1998) and (22), respectively. Antioxidants such as SOD and catalase activity were determined. Catalase activity and SOD were determined according to the methods defined by (23) and (24), respectively; these two antioxidants were estimated using commercially available kits according to the manufacturer's instructions (Biodiagnostic, Giza, Egypt). Using the method described by Lowry (1951), the total protein content of colon homogenate was measured using spectrophotometry.

Histopathological study:

The colon was isolated and washed with 0.9% normal saline solution. The colon was cut into small sections, which were fixed in 10% formalin solution. Ethanol (70%) was added to the formalin-fixed sections, which were then embedded in paraffin. The tissue sections were stained with hematoxylin and eosin (HE) and examined to determine any pathological changes using an optical microscope.

Statistical analysis:

Data analysis was performed using Prism (version 6.0; Graph Pad Software, USA). Data were expressed as mean ± standard deviation, and p values of <0.05 were used to denote statistical significance. Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons after ANOVA.

III. Results:

Expression of CEA, VEGF, and Bcl-2 by RT–PCR

Figure 1 shows the expressions of CEA, VEGF, and Bcl-2 by RT–PCR for all experimental groups. CEA, VEGF, and Bcl-2 were overexpressed in the carcinogenic group (G2), whereas their expressions were significantly decreased in other groups (prophylactic, galangin-treated, 5-FU-treated, and 5-FU- and galangin-treated groups). β-actin acted as the housekeeping gene in RT-PCR technique. Figure 2 shows the relative mRNA expression of CEA, VEGF and Bcl-2 in all experimental groups.

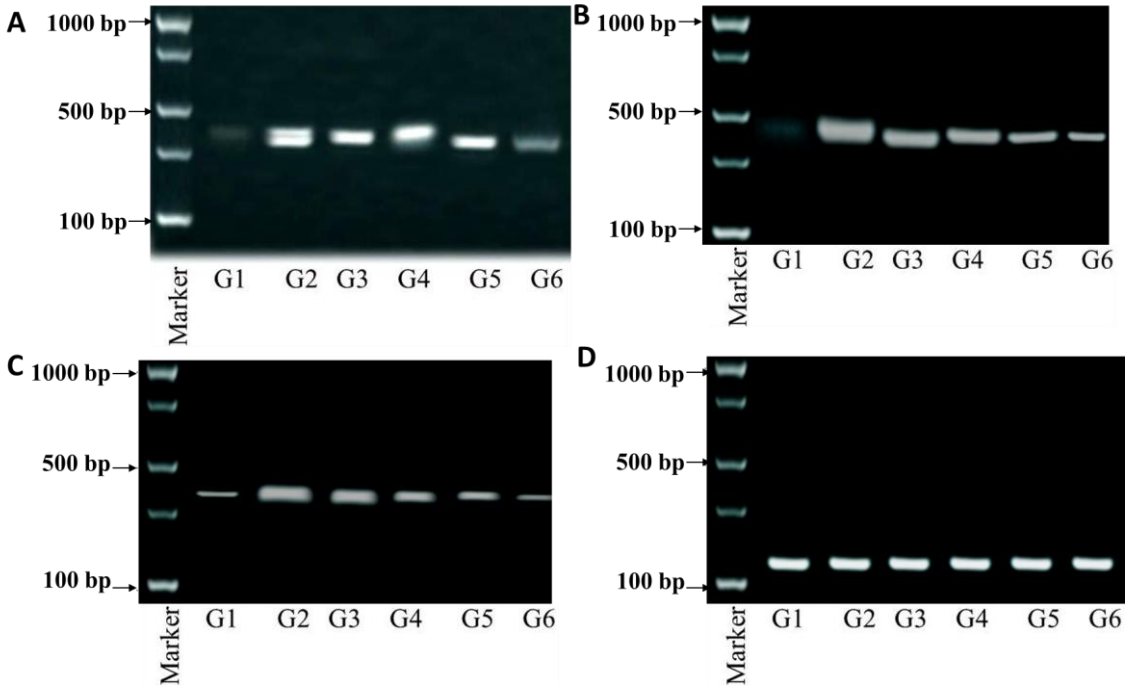

Figure 1: Effect of galangin on CEA, VEGF, Bcl-2 and β-actin levels in all groups of this study. A) The mRNA expression of CEA was determined by RT-PCR technique in all groups. B) The mRNA expression of VEGF was determined by RT-PCR technique in all groups. C) The mRNA expression of Bcl-2 was determined by RT-PCR technique in all groups. D) The mRNA expression of β-actin was determined by RT-PCR technique in all groups.

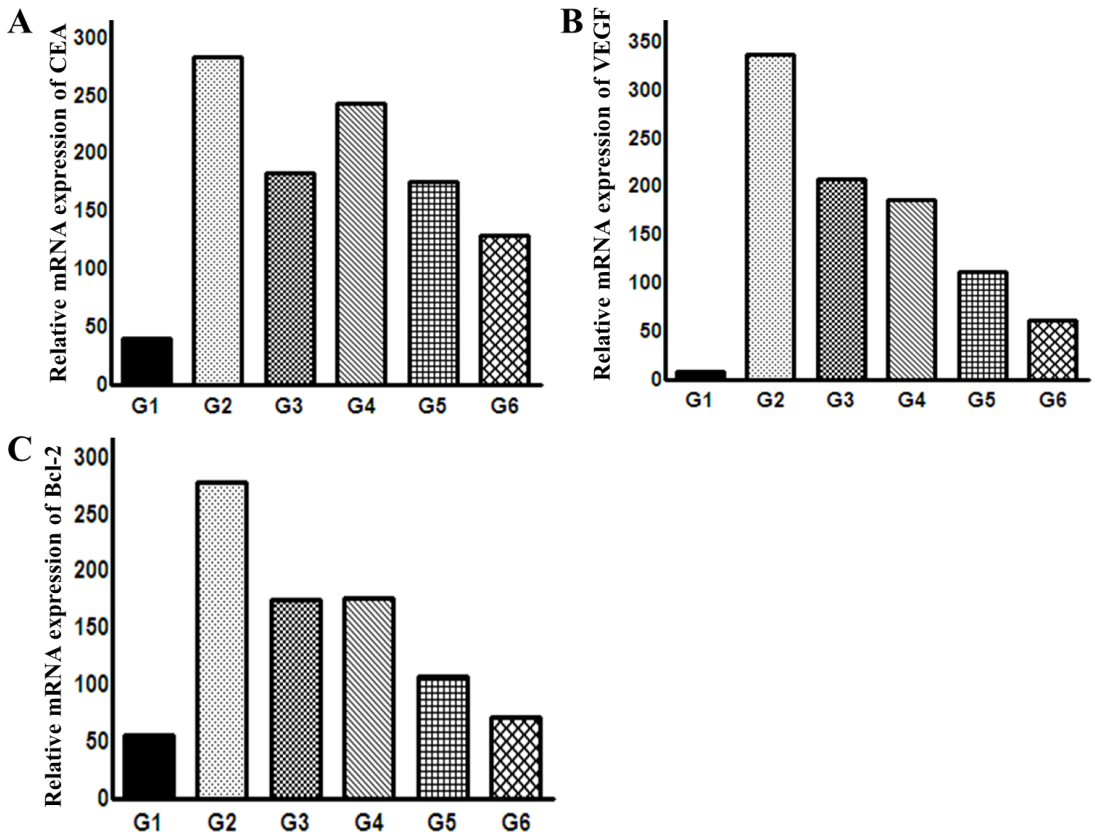

Figure 2: Relative mRNA expression of CEA, VEGF and Bcl-2 in all experimental groups. A) The relative mRNA expression of CEA. B) The relative mRNA expression of VEGF. C) The relative mRNA expression of Bcl-2. Relative expression and peak size was calculated by using image J software (Image J 2x.8) (National Institutes of Health, Bethesda, MD, USA).

Determination of Bcl-2 in colon tissues using ELISA:

Bcl-2 levels were illustrated in all experimental groups. The level of Bcl-2, an anti-apoptotic agent, was markedly increased in the carcinogenic group (G2), whereas its level was reduced in the galangin-treated and 5-FU- and galangin-treated groups (Table 2).

Table 2: Levels of Bcl-2, ALT, AST, Total proteins, MDA, NO, Catalase and SOD.

	G1	G2	G3	G4	G5	G6
Bcl-2 (ng/mg proteins)	2.77±0.83	21.43±1.04*	14.19±0.96*#	12.53±1.47 *#	10.29±1.18*#	6.04±1.78*#
ALT (IU/L)	21.98± 2.71	118.82±3.17 *	87.91±3.41 *#	75.18±2.99 *#	72.88±2.09 *#	46.01± 4.65 *#
AST (IU/L)	41.11±2.48	181.85±4.12 *	135.72±3.18 *#	102.94±6.19 *#	97.84±4.76 *#	63.75±3.92 *#
Total tissue proteins (gm/dl)	2.32±0.82	11.31±1.79 *	9.08±1.12 *#	7.72±0.96 *#	5.45±1.49 *#	3.11±0.53 #
MDA (µM/g wet tissue)	2.81±1.06	19.72±1.88 *	14.12±1.11 *#	10.52±0.78 *#	11.52±1.15 *#	7.16±1.92 *#
NO (µM/g wet tissue)	18.13±2.18	46.32±2.15 *	42.55±3.311 *	37.13±2.925 *#	31.13±2.18 *#	27.13±2.18 *#
Catalase (U/mg protein)	3.04±0.51	0.76±0.07 *	1.64±0.51*#	1.77±0.73 *#	2.15±1.01 *#	2.77±0.61 #
SOD (U/mg protein)	18.54±1.94	7.33±1.17 *	10.54±0.93 *#	9.81±1.31*#	14.27±1.31*#	15.93±1.72 *#

G1: Control group, G2: Carcinogenic group, G3: Prophylactic group, G4: Group treated with galangin, G5: Group treated with 5-FU, and G6: Group treated with 5-FU and galangin. Data were expressed as mean ± SD of Bcl-2: B-cell lymphoma-2, ALT: alanine aminotransferase, AST: Aspartate aminotransferase, MDA: Malondialdehyde and SOD: Super oxide dismutase. Significance: *p<0.001 compared to control group and #p<0.001 compared to carcinogenic group.

Determination of ALT, AST, MDA, NO, SOD, and catalase activity:

Table 2 shows the levels of sALT, sAST, total tissue proteins, MDA, NO, catalase, and SOD activity in the tissue homogenate of the animals under study in all experimental groups. The levels of oxidative stress markers NO and MDA were significantly increased in the carcinogenic group, whereas they were moderately increased in the prophylactic, galangin-treated, and 5-FU- and galangin-treated groups. The levels of MDA and NO in all treated groups were still significantly higher than those in the control group. The levels of some antioxidants in the tissue homogenate of the animals under study in all experimental groups (control, carcinogenic, prophylactic, galangin-treated, 5-FU-treated, and 5-FU- and galangin-treated groups) were determined. A significant decrease in SOD and CAT activities was observed in the carcinogenic group. Meanwhile, they were increased in other treated groups.

Histopathological results:

Colon tissues in all groups were examined to assess the effect of galangin alone or in combination with 5-FU. H&E-stained slides were used in examination of colon cells. Normal histological structures were showed in the colon sections of control group (Figure 3A). Otherwise, the carcinogenic group revealed colon adenocarcinoma with the proliferation of cancer cells from the epithelial layer of the sub-mucosa (arrows). The tumor cells are large and polyhedral cells with severe necrosis (Figure 3B). The prophylactic group showed severe inflammation with some changes in histological appearance (arrows) (Figure 3C). Furthermore, colon cells of the group treated with galangin showed good development in cells with less normal cell structure and apoptosis (arrows) (Figure 3D). Colon cells of the group treated with 5-FU revealed neoplastic cells or apoptosis with sloughing of the tumor cells (arrows) (Figure 3E). Finally, colon cells of the group treated with galangin and 5-FU showed relative normal histological appearance with apoptosis and infiltration of mast cells (Figure 3F).

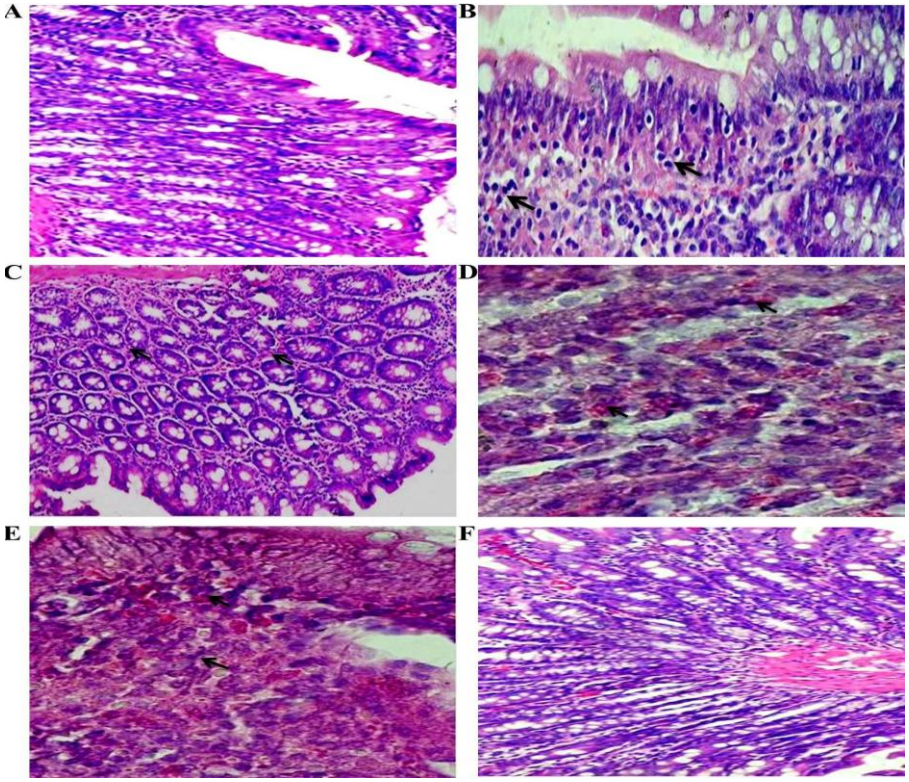

Figure 3: Histopathological investigation of colon tissues; A) Colon of control group showing normal colon cells. B) Colon cells of the carcinogenic group. C) Colon cells of the prophylactic group. D) Colon cells of the group treated with 5-FU. E) Colon cells of the group treated with galangin. F) Colon cells of the group treated with galangin and 5-FU. Slides were stained with H&E, bar = 100 µm.

IV. Discussion:

The third cause of cancer-related deaths is colon cancer. While there are many developments in treatment strategies, deciding to undergo surgery is difficult, and patients may be reluctant to undergo surgery. In patients with colon cancer, studies have attempted to explain the risk factors associated with colon cancer incidence and mortality predictors (26).

Colon cancer treatment strategies are very changeable due to variations in cancer type and stage and general health of patients. Different methods for colon cancer treatment exist, including chemotherapy, radiotherapy, and surgery. Each type of treatment has its possible benefits, side effects, and risks. Therefore, developing therapeutic strategies for treating colon cancer is necessary. 5-FU is a widely used chemotherapeutic agent in treating colon cancer; however, it has a limited therapeutic index due to its toxicity and side effects (5).

In the present study, 5-FU was used in treatment of colon cancer. Due to 5-FU is sorted as an antimetabolite that acts by inhibiting TS, which causes dTMP shortage and inhibits DNA synthesis. Cancer cells have resistance to 5-FU, and the toxicity and side effects of 5-FU are the main barriers to its success as a chemotherapeutic agent. Thus, the combination of 5-FU with other compounds can produce a synergistic effect on cancer cells and reduce the toxicity of 5-FU (27).

A single-chemotherapy approach is less effective in treating cancer. The rationale for using a multi-drug protocol is to damage cancer cells through several pathways and different mechanisms for reducing toxicity leading to enhanced clinical results. So in this study, galangin, a natural product, was used alone and in combination with 5-FU in treating colon cancer. Galangin is a flavonol present in *Alpinia officinarum*. It acts as a prophylactic and adjuvant agent with 5-FU in colon cancer treatment. In addition, galangin inhibits the proliferation of HCC, acts as an inducer of endoplasmic reticulum (ER) stress to inhibit the occurrence of HCC, and can be used as an anticancer agent (28).

To the best of our knowledge, the present study is one of few studies examined different mechanisms of galanin as antioxidants and stimulates apoptosis in colon cancer. Galangin has several activities, such as antiviral and anti-reactive oxygen species (anti-ROS) activity, and stimulates ER stress in cancer cells inhibiting their proliferation (29). **S**tudies have reported that galangin stimulates apoptosis in HCC. It was suggested that galangin acts as a potential antitumor agent (30).

Reports have shown that galangin amplify apoptosis in different types of cancer cells through several pathways. Furthermore, it was demonstrated that galangin promotes apoptosis by inhibiting the G0/G1 cell cycle, adjusting cycline/cdk expression, and decreasing the levels of Bcl-2 (31). Galangin induces apoptosis by activating p38 mitogen-activated protein kinases in melanoma cells (32).

Levels of CEA were estimated in this study. CEA levels were increased significantly in colon cancer induced with 1,2-DMH as a marker for it. In contrast, CEA levels were reduced in animals treated with galangin and 5-FU. These results agree with a previous study which revealed that CEA is a tumor marker widely used for colon cancer diagnosis. The serum CEA level is elevated and considered the main prognostic factor for colon cancer (33). Furthermore, CEA has a predictive role, which received much attention recently. Several studies have reported that the levels of CEA are associated with chemotherapy and chemoradiation responses (34). Molecular mechanisms related to CEA explained how CEA has a predictive and prognostic role in colon cancer (35).

Galangin has antiproliferative activity in different cancer cells. In a study, the anti-angiogenic effect of galangin was investigated *in vivo* and *in vitro*. Galangin prevented angiogenesis induced by OVCAR-3 cells. Several mechanisms were studied through which angiogenesis was suppressed by galangin. Galangin inhibits the synthesis of VEGF and reduced p-p70S6K and p-Akt levels. These results suggest that galangin acts as an anti-angiogenic agent in treating ovarian cancers (36).

Apoptosis was induced by galangin in a dose-dependent manner. Cisplatin effects were potentiated by galangin by inducing apoptosis by inhibiting Bcl-2 in cisplatin-resistant cancer cells in the lung. Bax and Bid are proapoptotic proteins and upregulated and accompanied by cleavage of caspases, producing apoptosis. Furthermore, the combined therapy prevented cancer growth compared with galangin or cisplatin treatment alone. These findings indicate that galangin potentiates the cisplatin effect in lung cancer by inhibiting Bcl-2 and p-STAT3/p65 pathways (37).

Another novel observation of the present study was that NO and MDA levels are significantly elevated in tissue homogenates in carcinogenic groups but their levels were decreased in treated groups. This is in agreement with a previous study by Vukovic *et al*, (38) who reported that galangin was investigated for its toxic effect toward breast and colon cancers. The antioxidative activities of galangin were evaluated by estimating MDA and NO levels.

Moreover, antioxidants as Catalase and SOD activities were significantly decreased in the carcinogenic group and increased in groups treated with galangin alone or in combination with 5-FU. This was in agreement with the study that showed an increased level of ROS plays an important factor in apoptosis of different types of cancer. Galangin stimulates the accumulation of intracellular ROS and MDA and reduces the total antioxidant and superoxide dismutase levels in renal cancer cells. Galangin showed antiproliferative effects and prevented the invasion of renal cancer cells by inhibiting the EMT. Thus, galangin has useful effects by inhibiting renal cancer cell growth, preventing cell invasion through the EMT, and stimulating apoptosis (39).

Galangin and 5-FU had a synergistic effect in treating colon cancer by inhibiting the apoptotic signaling pathways. Collectively, this study reported that galangin potentiates the chemotherapeutic effect of 5-

FU in colon cancer by inhibiting different signaling pathways. These findings conform to those of a study that has reported that some natural products synergize the effect of 5-FU as a chemotherapeutic agent in treating colon cancer by suppressing multiple pathways (5).

In conclusion, galangin exerts its antioxidant and antitumor activities in different types of cancers; however, galangin was not used as an adjunct to 5-FU in treating colon cancer. It was shown that galangin improves the chemotherapeutic effect of 5-FU. In this study, we evaluated the effect of the combined treatment on colon cancer and assessed whether galangin synergizes the antitumor effect of 5-FU. It was found that galangin significantly increased the antitumor effect of 5-FU by increasing the levels of antioxidants and inhibiting apoptosis, angiogenesis, and cell proliferation.

Finally, galangin and 5-FU had a synergistic effect in treating colon cancer by inhibiting apoptotic signaling pathways. Collectively, this study demonstrated that galangin synergized the chemotherapeutic effect of 5-FU on colon cancer by simultaneously suppressing multiple signaling pathways.

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