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Der Pharmacia Lettre, 2015, 7 (4):101-114
(http://scholarsresearchlibrary.com/archive.html)

Der Pharmacia Lettre
Scholars Research Library
ISSN 0975-5071
USA CODEN: DPLEB4

Complementary therapy for colon cancer: Evidences based on pre-clinical study

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ABSTRACT

The present study aimed to investigate the efficacy of Rosmarinus officinalis total methanolic extract on colon cancer- induced in rats. Rats were classified into 5 groups. Group (1) was control. Groups from (2) to (5) were intrarectally injected with N-methylnitrosourea (MNU) for induction of colon cancer then group (2) was left untreated (cancer group); group (3) was treated intraperitoneally with 5-fluorouracil while, groups (4 and 5) were treated orally with 1400 and 2800 mg/kg b.wt. of R. officinalis extract respectively. Cancer group declared significant increase in the expression levels of β-catenin and c-Myc genes in colon tissue. Furthermore, colon cancer induced-rats demonstrated significant increase in colon COX-2, cyclin D1 and survivin expression in colon tissue associated with significant elevation in the circulating levels of all studied biochemical markers. Additionally, histopathological investigation of colon tissue sections in cancer group showed dysplasia and anaplasia in the lining epithelial cells of the glandular structure. In the contrary, treatment of cancer group with 5-fluorouracil or R. officinalis extract showed significant decrease in the expression levels of β-catenin and c-Myc genes. Also, all treated groups exhibited marked decrease in COX-2, cyclin D1 and survivin expression in colon tissue accompanied with a significant reduction in the circulating levels of the all studied biomarkers. As well as, marked improvement in the histological structure of colon tissue was detected. In conclusion, the present study indicated that R. officinalis extract possess promising therapeutic role against colon cancer through their antiinflammatory property, antiproliferative capacity and apoptotic potential.

Key words: Colon cancer, N-methylnitrosourea, 5- fluorouracil, Rosemary, and Rats

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INTRODUCTION

Colorectal cancer (CRC) is the third most diagnosed cancer type in males and the second in females worldwide, and its incidence is increasing even in traditionally low-risk countries. Moreover, mortality rates caused by colorectal cancer remain high, being the fourth and third cause of cancer-related mortality in males and females, respectively [1]. CRC presents with a broad spectrum of neoplasms, ranging from benign growths to invasive cancer. CRC starts in the inner lining of the colon and/or rectum as a growth of tissue called a polyp slowly growing through some or all of its layers. A particular type of polyp called the adenomatous polyp or adenoma is a benign tumor that may undergo malignant transformation to cancer. This malignant transformation is the result of mutation or deletion of major regulator genes, resulting first in hyperplasia moving toward adenoma to carcinoma and then metastasis [2]**.** Rectal bleeding, diarrhea, weight loss, abdominal pain, and anemia are considered to be the common symptoms of this malignancy [3].

Numerous factors are found important in the development of CRC including colonocyte metbolism, high risk luminal environment, inflammation, as well as lifestyle factors such as diet, tobacco, and alchohol consumption [4].

The molecular etiology of CRC revealing that it develops from an accumulation of genomic mutations. The genes more commonly found mutated in CRC include the adenomatous polyposis coli (APC) (approximately 80%), K-ras, SMAD4, and P53, and the cell signaling pathways most commonly impacted by mutations in CRC include the Wnt, Ras, transforming growth factor- β (TGF-β), phosphatidylinositol 3-kinase (PI3K) and P_{53} pathways [5].

Historically, herbs and spices have enjoyed a rich tradition of use for their flavor enhancement characteristics and for their medicinal properties. The rising prevalence of chronic diseases worldwide and the corresponding rise in health care costs is propelling interest among researchers and the public for multiple health benefits related to these food items, including a reduction in cancer risk and modification of tumor behavior. A growing body of epidemiological and preclinical evidence points to culinary herbs and spices as minor dietary constituents with multiple anticancer characteristics [6].

Rosemary (*R. officinalis*) is a powerful herb belonging to the family *Lamiaceae* that originates from the Mediterranean region [7-9]. Owing to its desirable flavor and antimicrobial and antioxidant activities, this plant has been widely employed as a spice and flavoring agent in the food processing and pharmaceutical industries [10-13]**.** *R. officinalis* and many of its components were reported to possess chemopreventive properties in skin [14] and breast [15] cancers *in vivo*, mostly by inhibiting 7, 12-dimethylbenz (a) anthracene (DMBA)-DNA adduct formation. Moreover, they exert antioxidant activity both *in vitro* [16, 17] and *in vivo* [18], thus inhibiting genotoxicity, which is a significant contributory cause of cancer, and protecting from carcinogens or toxic agents. They also were reported to display antiproliferative activity *in vitro* against breast [19-21]**,** leukemia [20, 22], hepatoma [23, 24], colon [25-27], lung [20], prostate [20], ovarian [24, 28], and urinary bladder [21] cancer cells.

Several *R. officinalis* components, such as carnosic acid, carnosol, ursolic acid, as well as some of its essential oil constituents, have been proposed to be responsible for the anticancer effect of *R. officinalis* extracts [14, 22, 24, 28]. Although the concentration ratios of carnosol and carnosic acid were reported to influence the antioxidant and antimicrobial activities [29].

The principal goal of the current study was to explore the potential role *Rosmarinus officinalis* total methanolic extract in alleviating chemically induced colon cancer in rats. This goal was achieved through analysis of genetic markers, investigating colon immunohistochemical indicators, measurement of circulating biochemical indices and examining the histological feature of colon tissue.

MATERIALS AND METHODS

I. Materials

1. Plant material:

R. officinalis was obtained from the Egyptian Herbal Market, Cairo, Egypt. *R. officinalis* was precisely identified and differentiated in the Research Institute for Oily Crops, Cairo, Egypt.

Preparation of *R. officinalis* total methanolic extract (RMTME):

R. officinalis total methanolic extract (RMTME) was prepared by adding 300 ml of methanol (70 %) to 50 g of the plant powder and left for 10-12 hrs. The extract was filtered using filter paper and the solvent was evaporated using rotary evaporator. The resultant extract was dehydrated in an oven at 50 ^{o}C for 24 hours [30].

2. Animals:

Forty adult male Sprague-Dawley rats weighing 150-170 g were obtained from the Animal House Colony of the National Research Centre, Cairo, Egypt and acclimated for one week in a specific pathogen free (SPF) barrier area where the temperature (25±1) and humidity (55%). Rats were controlled constantly with a 12 h light/dark cycle at National Research Centre Animal Facility Breeding Colony. Rats were housed with *ad libitum* access standard laboratory diet consisting of casein 10%, salts mixture 4 %, vitamins mixture 1%, corn oil 10 % and cellulose 5% completed to 100 g with corn starch [31]. Animal cared for according to the guidelines for animal experiments which were approved by the Ethical Committee of Medical Research of the National Research Centre, Cairo, Egypt.

3. Experimental design:

After the acclimatization period, the rats in the current study were classified into 5 groups (8 rats /group). **(1):** Normal healthy animals served as negative control group in which rats received 1 ml of vehicle (Dimethyl sulfoxide DMSO 5% in saline). **(2):** Colon cancer induced group in which rats were intrarectally administered with N-methylnitrosourea in a dose of 2 mg dissolved in 0.5 ml water/rat three times weekly for five weeks [32], **(3):** 5-fluorouracil-treated group in which rats were intrarectally administered with N-methylnitrosourea for five weeks and then intraperitoneally treated with 5-fluorouracil in a dose of 12.5 mg/kg on days 1, 3 and 5 with the cycle being

repeated every four weeks over the duration of the study period (four months) [33]. **(4):** *R. officinalis* total methanolic extract-treated group (RMTME low dose) in which rats were intrarectally administered with N-methylnitrosourea for 5 weeks and then orally treated with low dose (1400 mg/kg) of RMTME daily for 4 months. **(5):** *R. officinalis* total methanolic extract-treated group (RMTME high dose) in which rats were intrarectally administered with N-methylnitrosourea for 5 weeks and the orally treated with high dose (2800 mg/kg) of RMTME daily for 4 months.The selected doses of the medicinal plant extract was calculated accoding to the chronic toxicity study in the current work (unpublished data).

At the end of the experimental period, the rats were fasted overnight and subjected to diethyl ether anaesthesia. The blood samples were immediately collected from the retroorbital venous plexus and divided into two tubes, the first tube contains anticoagulant for separation of plasma and the second tube is free from any anticoagulant for separation of serum for biochemical analyses. Then the rats were sacrificed by cervical dislocation and the colon was dissected, cleaned and washed in saline then divided into two portions, the first portion was collected in liquid nitrogen and stord at -80° C for molecular genetic analyses and the second portion was preserved in formalin saline (10%) for histological investigation and immunohistochemical examination.

II. Methods

1. Molecular genetic analyses:

1.1. Expression of β-catenin and c-Myc genes

(a) Isolation of total RNA

Total RNA was isolated from colon tissue of rats in the different studied groups by the standard TRIzol® Reagent extraction method (Cat#15596-026, Invitrogen, Germany). Briefly, tissue samples were homogenized in 1 ml of TRIzol® Reagent per 50 mg of the tissue. Afterwards, the homogenized sample was incubated for 15 minutes at room temperature. A volume of 0.2 ml of chloroform per 1 ml of TRIzol® Reagent was added. Then the samples were vortexed vigorously for 15 seconds and incubated at room temperature for three minutes. The samples were centrifuged at 12,000 xg for 15 minutes at 4^{o}C. Following centrifugation, the mixture was separated into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA was remained exclusively in the aqueous phase. Therefore, the upper aqueous phase was carefully transferred without disturbing the interphase into a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. A volume of 0.5 ml of isopropyl alcohol was added per one ml of TRIzol® Reagent used for the initial homogenization. Afterwards, the samples were incubated at 15 - 30 ^{o}C for 10 minutes and centrifuged at 12,000 xg for 10 minutes at 4 ^{o}C. The RNA was precipitated forming a gel-like pellet on the side and bottom of the tube. The supernatant was removed completely. The RNA pellet was washed once with 1 ml of 75% ethanol. The samples were mixed by vortex and centrifuged at 7,500 xg for five minutes at 4 ^{o}C. The supernatant was removed and RNA pellet was air-dried for 10 minutes. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by passing solution a few times through a pipette tip .

Total RNA was treated with 1 U of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT).

(b) Reverse transcription (RT) reaction

The complete poly(A)+ RNA isolated from rat colon tissue was reverse transcribed into cDNA in a total volume of 20 µl using RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5µg) was used with a reaction mixture, termed as master mix (MM). The MM was consisted of 50 mM MgCl2, 5x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl (pH 8.3) 10 mM of each dNTP, 50 µM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNAse activity) and 50 U M- MuLV reverse transcriptase. The mixture of each sample was centrifuged for 30 sec at 1000 xg and transferred to the thermocycler (Biometra GmbH, Göttingen, Germany). The RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and the reaction was stopped by heating at 99 °C for 5 min. Afterwards, the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through semi-quantitative real time-polymerase chain reaction (sqRT-PCR).

(c) Semi-quantitative real time-polymerase chain reaction (sqRT-PCR)

An iQ5-BIO-RAD Cycler (Cepheid. USA) was used to determine the rat cDNA copy number. PCR reactions were set up in 25 µL reaction mixtures containing 12.5µL of 1× SYBR® Premix ExTaqTM (TaKaRa, Biotech. Co. Ltd Germany), 0.5 µL 0.2 µM sense primers, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water, and 5 µL of cDNA template. Each experiment included a distilled water control.

Primer sequence for β-catenin- F: 5'-CAAT GGG TCA TAT CAC AGA TTC TT-3', R: 5'-TCT CTT TTC TTC ACC ACA ACA TTT-3' [34] and for c- Myc (GenBank accession number Z38066) were F: 5′-TGA CGA GAC CTT CGT GAA GA-3′ and R: 5′-ATT GAT GTT ATT TAC ACT TAA GGG T-3′ [35]. The semi quantitative values of RT-PCR (sqRT-PCR) of the previous genes were normalized on the expression values of β-actin gene (β-actin-F: 5′- CCC CAT CGA GCA CGG TAT TG -3′, R: ATG GCG GGG GTG TTG AAG GTC [36]. At the end of each sqRT-PCR, a melting curve analysis was performed at 95.0 °C to check the quality of the used primers.

Calculation of Gene Expression

First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formulae [37]:

Ef = 10-1/slope

Efficiency (%) = (Ef – 1) x 100

The relative quantification of the target to the reference was determined by using the ΔCT method if E for the target (β-catenin, K-ras and c-myc) and the reference primers (β-actin) are the same

Ratio (reference/ target gene) = EfCT (reference) – CT (target)

2. Immunohistochemical examination:

One portion of the fixed colon tissue of rats in the different studied groups was washed in tap water then, subjected to serial dilutions of alcohol (methyl, ethyl and absolute ethyl) for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for 24 hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 µm by slidge microtome. The obtained tissue sections were collected on glass positive slides and fixed in a 65^{o}C oven for 1 hr. Then, the slides were placed in a coplin jar filled with 200 ml of triology working solution (Cell Marque, CA-USA. Cat# 920p-06) which is a product that combines the three pretreatment steps: deparaffinization, rehydration and antigen unmasking. After that, the jar is securely positioned in the autoclave. The autoclave was adjusted so that temperature reached 120 ^{o}C and maintained stable for 15 min after which pressure is released and the coplin jar is removed to allow slides to cool for 30 min. Sections were then washed and immersed in Tris buffer saline (TBS) to adjust the PH, this is repeated between each step of the immunohistochemical procedure. Quenching endogenous peroxidase activity was performed by immersing slides in 3% hydrogen peroxide for 10 min. Power stain TM 1.0 Poly HRP DAB Kit Cat# 54-0017 (Genemed Biotechnologies, CA-USA) was used to visualize any antigen-antibody reaction in the tissues. Two to three drops of the rabbit polyclonal primary antibody (COX-2 Cat#RB-9072-R7, Thermoscientific, CA-USA), (cyclin D1 Cat#RB-9041-R7, Thermoscientific, CA-USA) and (survivin Cat#RB-9245-R7, Thermoscientific, CA-USA) were applied, then the slides were incubated in the humidity chamber for overnight at 4^{o}C. Hence forward, poly horse reddish peroxidase (HRP) enzyme conjugate was applied to each slide for 20 mins. 3, 3'-diaminobenzidine (DAB) chromogen was prepared and 2-3 drops were applied on each slide for 2 min. DAB was rinsed, after which counter stained with mayerhematoxylin and cover slipping were performed as the final steps before slides were examined under the light microscope. Image J software (NIH, version v1.45e, USA) was calibrated and the image is opened on the computer screen for image analysis.

3. Biochemical analyses:

Plasma B-cell lymphoma 2 (Bcl-2), serum matrix metalloproteinase-7 (MMP-7), matrix metalloproteinase-9 (MMP-9), epidermal growth factor (EGF) and plasma tumor necrosis factor-α (TNF-α) levels were assayed by ELIZA technique using assay kits purchased from Glory Science Co., Ltd, TX, USA according to the instructions provided.

4. Histological investigation:

After fixation of the other portion of colon tissues of rats in the different studied groups in formalin saline (10%) for 24 hours, these portion were washed in tap water then subjected to serial dilutions of alcohol (methyl, ethyl and absolute ethyl) for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 °C in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 µm by slidge microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained by hematoxylin and eosin stain and examined through the electric light microscope [38].

Statistical analysis:

In the present study, the results were expressed as Mean ± S.E of the mean. Data were analyzed by one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 11 [39].

Percentage difference representing the percent of variation with respect to corresponding control group was also calculated using the following formula:

$$\% \text{ difference} = \frac{\text{Treated value} - \text{Control value}}{\text{Control value}} \times 100$$

RESULTS

1.Molecular genetic results:

The results of gene expression levels assessment of β-catenin and c-Myc in colon tissue of the different studied groups were summarized in Table (1). Our data revealed that β-catenin and c-Myc genes were down-regulated in the colon tissue of control group. However, the expression level of β-catenin and c-Myc genes were significantly up-regulated in colon cancer- induced group (309% and 212.5% respectively) as compared with the control group. In contrast, cancer- induced rats treated with 5- fluorouracil exhibited significant down-regulation in the expression levels of β-catenin and c-Myc genes (-71.85% and -64.66% respectively) as compered with untreated cancer-induced group. Treatment of cancer- induced rats with low or high dose of RMTME reversed the alteration of the expression levels of the two genes. Thus, the expression levels of β-catenin and c-Myc genes revealed significant down-regulation in colon cancer- induced group treated with low dose of RMTME (-17% and -20% respectively) and that treated with high dose (-26.6% and -26.6% respectively) as compared with untreated cancer- induced group. The effect of RMTME in amelioration of genetic alteration was dose dependent.

Table. 1: Semi-quantitative real time-PCR confirmation of β–catenin and c-Myc genes in colon tissue of the different studied groups

Groups	β-catenin gene	c-Myc gene
Control group	0.33±0.02	0.48±0.02
Cancer group	1.35±0.04[a] (309%)	1.50±0.03[a] (212.5%)
Fluorouracil group	0.38±0.03[b] (-71.85%)	0.53±0.04[b] (-64.66%)
RMTME group (1400 mg/kg)	1.11±0.04[b] (-17%)	1.20±0.04[b] (-20%)
RMTME group (2800 mg/kg)	0.99±0.03[b] (-26.6%)	1.10±0.03[b] (-26.6%)

Data are expressed as means ±standard error (SE) for 8 animals / group.
a: Significance change at P < 0.05 in comparison with control group.
b: Significance change at P < 0.05 in comparison with cancer group.
(%): percent of difference with respect to the corresponding control value.
RMTME: R. officinalis total methanolic extract extract.

Table (2): Effect of *R. officinalis* total methanolic extract (RMTME) on COX-2, Cyclin D_1 and Survivin expression in colon cancer induced in rats

Groups	COX-2 (cell count)	Cyclin D1 (cell count)	Survivin (cell count)
Control group	10.67±1.03	15.83±0.74	6.5±0.49
Cancer group	30.5±1.95[a] (185.9 %)	38.33±3.12[a] (142.1 %)	41.16±4.45[a] (533.3 %)
5- fluorouracil group	11.0±0.98[b] (-63.93 %)	15.5±1.14[b] (-59.56 %)	6.5±0.41[b] (-84.21 %)
RMTME group (1400 mg/kg)	17.67±1.48[b] (-42.07 %)	38.0±1.44 (-0.87 %)	11.16± 1.09[b] (-72.8 %)
RMTME group (2800 mg/kg)	18.17±1.02[b] (-40.43 %)	38.0±2.87 (-0.87 %)	10.33±0.58[b] (-74.9 %)

Data are expressed as means ±standard error (SE) for 8 animals / group.
a: Significance change at P < 0.05 in comparison with control group.
b: Significance change at P < 0.05 in comparison with cancer group.
(%) percent of difference with respect to the corresponding control value.
RMTME: R. officinalis total methanolic extract extract.

2.Immunohistochemical results:

Immunohistochemical examination of COX-2, cyclin D1 and survivin expressions

Table (2) and Fig. (1, 2 &3) explained the effect of *R. officinalis* total methanolic extract (RMTME) on COX-2, cyclin D1 and survivin expression in colon cancer induced in rats. The results indicated significant elevation in the number of positive cells for COX-2, cyclin D1 and survivin expression in cancer group recording 185.9%, 142.1% and 533.3% respectively as compared to control group. However, treatment of cancer induced rats with 5-fluorouracile showed a significant depletion in COX-2, cyclin D1 and survivin expression by -63.93%, -59.56% and -84.21% respectively in comparison with cancer group. Also, daily oral administration of low dose of *R. officinalis*

extract (RMTME) revealed significant decrease in the number of positive cells for COX-2 and survivin expression by -42.07% and -72.8% respectively as compared with the untreated one. Similarly, high dose of *R. officinalis* extract (RMTME) revealed a significant decrease in the number of positive cells for COX-2 and survivin expression by -40.43% and -74.9% respectively as compared with the untreated one. Treatment of cancer induced rats with either low or high doses of RMTME caused non-significant variation in the number of positive cells for cyclin D1 expression as compared with untreated one.

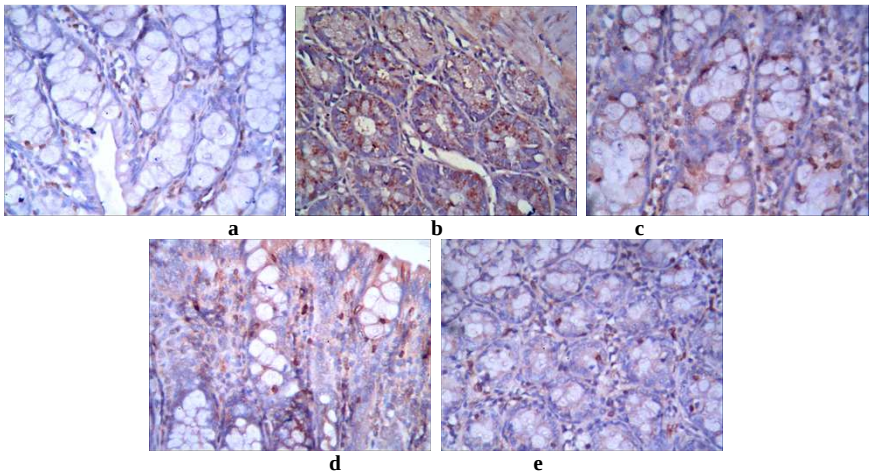

Fig.1: Photographs for immunohistochemical staining of colon tissue using antibody against COX-2 of (a) Control rat: showed mild positive reaction in interstitial stromal cells (160x), (b) Colon cancer-induced rat showed very sever positive reaction in the cytoplasm of the glandular lining epithelium (160x), (c) Colon cancer- induced rat treated with 5-fluorouracil showed moderate positive reaction in the nuclei of the glandular lining epithelium (160x), (d) Colon cancer- induced rat treated with low dose of RMTME showed sever positive reaction in the nuclei of stromal cells as well as some of the nuclei in the glandular lining epithelium (80x) and (e) Colon cancer- induced rat treated with high dose of RMTME showed moderate positive reaction in the nuclei of the glandular lining epithelium (160x).

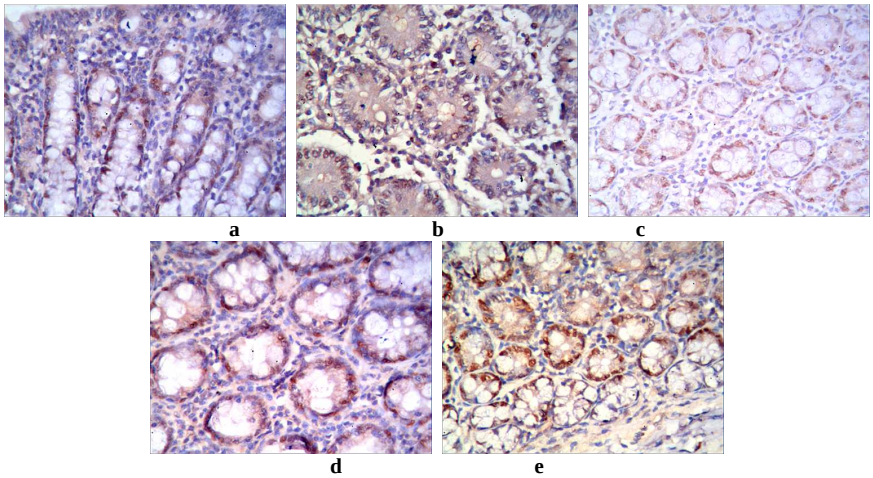

Fig. 2: Photographs for immunohistochemical staining of colon tissue using antibody against cyclin D1 of (a) Control rat showed positive reaction in the nuclei of the glandular lining epithelial cells (80x), (b) Colon cancer-induced rat showed very sever positive reaction in the nuclei of the glandular lining epithelial cells as well as the interstitial stromal cells (160x), (c) Colon cancer- induced rat treated with 5-fluorouracil showed moderate positive reaction in the nuclei of the glandular lining epithelial (80x), (d) Colon cancer- induced rat treated with low dose RMTME showed very sever positive reaction reaction in the nuclei of the glandular lining epithelial (160x) and (e) Colon cancer- induced rat treated with high dose of RMTME showed sever positive reaction in the nuclei of the glandular lining epithelial (160x).

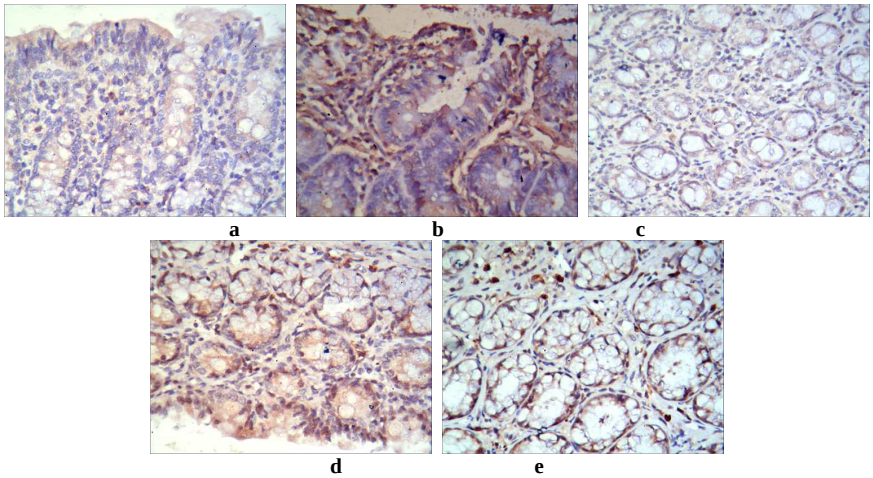

Fig. 3: Photographs for immunohistochemical staining of colon tissue using antibody against survivin of (a) Control rat showed mild negative reaction except some few interstitial stromal cell especially in their nuclei (80x), (b) Colon cancer-induced rat showed very sever positive reaction in the nuclei of the interstitial stromal cell as well as some nuclei of lining glandular epithelial cells (160x), (c) Colon cancer- induced rat treated with 5-fluorouracil showed mild positive reaction in the nuclei of the glandular lining epithelial cells as well as in the stromal interstitial cells (80x), (d) Colon cancer- induced rat treated with low dose of RMTME showed sever positive reaction in the nuclei of the glandular lining epithelial cells as well as stromal interstitial cells (160x) and (e) Colon cancer-induced rat treated with high dose of RMTME showed sever positive reaction mainly the nuclei of the glandular lining epithelial cells (160x).

3.Biochemical results

The data in Table (3) illustrate the effect of *R. officinalis* total methanolic extract (RMTME) on plasma levels of Bcl-2 and TNF-α as well as, serum levels of MMP-7, MMP-9 and EGF in colon cancer- induced rats. The results revealed a significant elevation in plasma Bcl-2 and TNF-α levels (75.37% and 21.53% respectively). Also, serum levels of MMP-7, MMP-9 and EGF (47.85%, 263% and 45.9% respectively) in cancer- induced group as compared with the control group. However, treatment of cancer- induced group with 5-fluorouracil showed a significant reduction in plasma levels of Bcl-2 and TNF-α (-40.42% and -15.32% respectively) as well as serum levels of MMP-7, MMP-9 and EGF (-33.02%, -71.52% and -28.37% respectively) as compared with untreated cancer- indued group. Treatment of cancer- induced group with either low or high dose of RMTME produced a significant depletion in Bcl-2 level (-18.6% and -20.20% respectively), TNF-α level (-4.05% and -14.9 % respectively), MMP-7 (-10.5% and -12.97% respectively), MMP-9 level (-61.3% and -63.6% respectively) and EGF level (-13.8% and -19.18% respectively) as compared to untreated cancer- induced group.

Table. 3: Effect of *R. officinalis* total methanolic extract extract (RMTME) on Bcl-2, TNF-α, MMP-7, MMP-9 and EGF circulating levels in colon cancer induced in rats.

Groups	P Bcl-2 ng/ml	P TNF-α pg/ml	S MMP-7 µ/L	S MMP-9 ng/ml	S EGF ng/L
Control group	13.40±0.51	81.25±0.47	0.180±0.015	1.19± 0.014	660 ±20
Cancer group	23.50±0.64[a] (75.37%)	98.75±0.47[a] (21.53%)	0.270±0. 024[a] (47.85 %)	4.32±0.14[a] (263%)	963.3 ±25.1[a] (45.9%)
5- fluorouracil group	14.00±0.45[b] (-40.42%)	83.62±0.89[b] (-15.32%)	0.182±0.013[b] (-33.02 %)	1.23±0.09[b] (-71.52%)	690 ±5.7[b] (-28.37%)
RMTME group (1400 mg/kg)	19.12±0.51[b] (-18.60%)	94.75±0.41[b] (-4.05%)	0.246±0.005[b] (-10.5 %)	1.67±0.08[b] (-61.3%)	830±20[b] (-13.8%)
RMTME group (2800 mg/kg)	18.75±0.47[b] (-20.20%)	93.87±0.34[b] (-14.9%)	0.240±0.011[b] (-12.97 %)	1.57±0.04[b] (-63.6%)	776.6±23.3[b] (-19.18%)

Data are expressed as means ± standard error (SE) for 8 animals / group.
a: Significance change at P < 0.05 in comparison with control group.
b: Significance change at P < 0.05 in comparison with cancer group.
(%) percent of difference with respect to the corresponding control value.
RMTME: R. officinalis total methanolic extract extract.
S: Serum
P: Plasma

4.Histopathological results:

Histological investigation of colon tissue sections of control group showed normal histological structure of the mucosa, submucosa and muscularis layers (Fig. 4a). While, colon tissue sections of colon cancer-induced group showed dysplasia and anaplasis associated with pleomorphism and hyperchromachia in the lining epithelial cells of the glandular structure (Fig. 4b). Examination of colon tissue sections of colon cancer- induced rats treated with 5-fluorouracil showed few inflammatory cells infiltration in the lamina propria of the mucosa with oedema in

muscularis (Fig.4c and 4d). Microscopic investigation of colon tissue section of cancer- induced rats treated with low dose of RMTME showed focal lymphoid cells aggregation in the mucosa (m) with massive number of inflammatory cells infiltration in submucosa (mm) and congestion in blood vessels (v) (Fig. 4e) and dysplasia in the lining epithelium of the mucosa (d) (Fig. 4f). The investigation of colon tissue section of cancer- induced rat treated with high dose of RMTME showed massive number of inflammatory cells infiltration (m) in the hyalinized lamina propria of the mucosal layer (h) (Figs 4g and 4h).

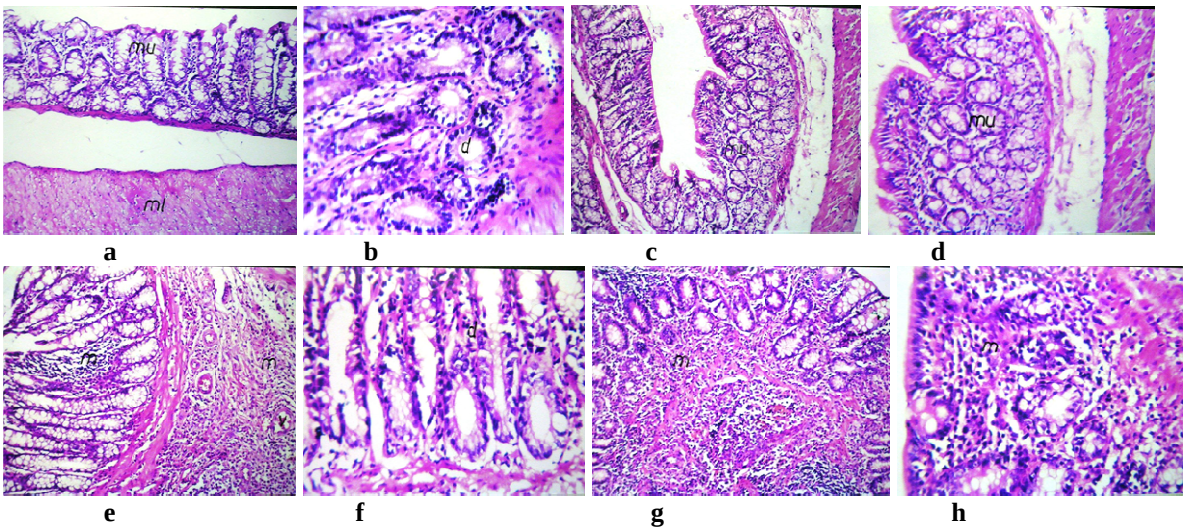

Fig. 4: (a) Micrograph of colon tissue section of control group showed normal histological structure of the mucosa (mu), submucosa (s) and muscularis (ml) layers. (H &E X40), (b) Micrograph of colon tissue section of colon cancer- induced rat showed dysplasia and anaplasis associated with pleomorphism and hyperchromachia in the lining epithelial cells of the glandular structure (d) (H&E X64), (c) Micrograph of colon tissue section of colon cancer- induced rat treated with 5-fluorouracil showed few inflammatory cells infiltration in the lamina propria of the mucosa (mu) with oedema in muscularis (ml) (H &E X40), (d) Micrograph of colon tissue section of colon cancer- induced rats treated with 5-fluorouracil showed the magnification of Fig. (4c) to identify few inflammatory cells infiltration in the lamina propria of the mucosa (mu) (H &E X64). (e) Micrograph of colon tissue section of colon cancer- induced rat treated with low dose of RMTME showed focal lymphoid cells aggregation in the mucosa (m) with massive number of inflammatory cells infiltration in submucosa (mm) and congestion in blood vessels (v) (H &E X40). (f) Micrograph of colon tissue section of colon cancer- induced rat treated with low dose of RMTME showed showed dysplasia in the lining epithelium of the mucosa (d) (H &E X64). (g) Micrograph of colon tissue section of colon cancer- induced rat treated with high dose of RMTME showed massive number of inflammatory cells infiltration (m) in the hyalinized lamina propria of the mucosal layer (h) (H &E X40) and (h) Micrograph of colon tissue section of colon cancer- induced rats treated with high dose of RMTME showed the magnification of Fig. (4h) to identify few inflammatory cells infiltration (m) in the hyalinized lamina propria of the mucosal layer (h) (H &E X80).

DISCUSSION

Colon carcinogenesis is a multistage process, involving multiple genetic and epigenetic changes that provide tumor cells with a selective advantage to expand their clones [40, 41]. The present study revealed significant increase in the expression of β- catenin and c-Myc genes in colon tissue of colon cancer group. This finding is in agreement with that in the previous studies of [42, 43 and 44].β- catenin is a subunit of cadherin protein complex and has been implicated as an integral component in the Wnt/signaling pathway. When β- catenin is mutated, β- catenin cannot be degraded but accumulates in the cytoplasm and translocates into the nucleus, where it binds to T- cell factor (TCF) and activates the Wnt target genes such as cyclin D1 and Myc that can lead to cell transformation [45-47]. Thus, the gene that codes for β- catenin can function as an oncogene. Mutations in this gene are a cause of colorectal cancer. Also, β- catenin binds to the product of adenomatous polyposis coli (APC) gene, which is mutated in adenomatous polyposis of the colon. Constitutive activation of β- catenin pathway is responsible for the initiation of the vast majority of colon cancers [48].

In the contrary, treatment of cancer group with 5-fluorouracil or *R. officinalis* extract showed significant decrease in the expression level of β-catenin and c-Myc genes. The mechanism by which 5- fluorouracil could act as effective chemotherapy against colon cancer is well understood. 5- Fluorouracil inhibits cell proliferation by: (a) forming 5-fluorodeoxyuridine monophosphate (FdUMP), which in turn blocks thymidylate synthase (TS), the enzyme that catalyzed novo synthesis of the DNA precursor thymidylate (i.e., TMP); (b) forming defective, F-RNA, which ultimately interferes with protein synthesis; and (c) forming defective, fluorinated DNA, which results in single-strand breaks and DNA fragmentation [49-51]**.**

Previous investigation from animal and cell culture studies demonstrated the anticancer potential of *R. officinalis* active constituents, including carnosol, carnosic acid, ursolic acid, and rosmarinic acid [52]. The reported anticancer properties were found to arise through the molecular changes in the multiple-stage process of cancer development, which are dose related and not tissue or species specific. This is evidenced by its ability to suppress the development of tumors in several organs including colon, breast, liver, stomach, as well as melanoma and leukemia cells [53]. From the present results it is clear that treatment with *R. officinalis* extract showed significant reduction in the expression of β-catenin gene and this result could be explained by the efficacy of *R. officinalis* to attenuate Wnt/signaling pathway resulting in reduced transcription of target genes, including cyclin D1 and COX-2. Carnosol prevents adenomatous polyposis carcinoma (APC) associated intestinal tumorigenesis; potentially *via* its ability to enhance E-cadherin- mediated adhesion and suppress β-catenin tyrosine phosphorylation. Additionally, carnosic acid attenuates transcriptional β-catenin outputs in colorectal cancer cells [54]**.**

In the same way, treatment with *R. officinalis* extract in colon cancer-induced rats resulted in a significant reduction in the level of c-Myc gene expression in the current study. This finding is in agreement with that of Makino *et al*. [55]. The proto-oncogene, c-Myc is a central regulator of cell proliferation closely linked to the cell cycle machinery [56], and is rapidly induced by mitogenic growth factor such as platelet- derived growth factor (PDGF) [57]. Rosmarinic acid has been found to exhibit anti-proliferative effects in cultured murine mesangial cells and suppress the expression of platelet- derived growth factor (PDGF) and c-Myc mRNA expression in a dose dependent manner [55]. Previous evidence revealed that reactive oxygen species act as cellular signals in PDGF- induced mitogenesis, and these include the expression of the proto-oncogenes c-Fos and c-Myc. Since rosmarinic acid has a potent antioxidative activity [58], this compound might suppress c-Myc mRNA expression by scavenging reactive oxygen species. The suppressive effects of rosmarinic acid on PDGF and c-Myc mRNA expression would contribute to its anti-proliferative activity on mesangial cells [55].

In the present study, the immunohistochemical data revealed that there was significant elevation in cyclooxagenase-2 (COX-2), cyclin D1 and survivin expression in colon tissue of colon cancer rats. This finding is in consistent with the studies of [43, 59 and 60]. COX-2 is involved in the regulation of a broad range of cellular processes including tumor onset and progression, metastases and angiogenesis [61]**.** Takahashi *et al*. [42] found that the increased expression of COX-2 in epithelial cells of colon tissue in 1, 2 Dimethylhydrazine/Azoxymethane (DMH/AOM) - induced colon adenocarcinoma, adenomas and aberrant crypt foci (ACF) with dysplasia. The mechanism of increased COX-2 expression in the current study may be related to k-ras mutation and/or protein activation which increased COX-2 expression in tumors [62]**.**

Cyclin D1 is a member of cyclins. It has been considered to be an oncogene which could regulate progression from the G1 phase of the cell cycle to the S phase [63]. Overexpression of cyclin D1 protein was also found in colon cancer [64, 65]. The study of Mao *el al*. [66] provided the first evidence that increased activation of signal transducer and activator of transcription-5 (Stat5) may contribute to the malignancy of colonic adenocarcinoma through overexpression of cyclin D1.

Survivin is an apoptosis inhibitor protein that inhibits the activation of caspases and its overexpression is implicated in the growth and progression of many types of cancers including colorectal carcinoma [67]. Our finding is in consistent with study of Chu *et al*. [68] and Jin *et al*. [69] which demonstrated that the expression levels of survivin mRNA and protein were higher in colorectal carcinoma cells than in normal cell line. On other hand, treatment of cancer group with 5-fluorouracil or *R. officinalis* extract exhibited marked decrease in COX-2, cyclin D1 and survivin expression in colon tissue accompanied with a significant reduction in the circulating levels of the all studied biomarkers.

Srimuangwong *et al*. [70] demonstrated that 5-fluorouracil at concentration of 5 μmol/L in combination with hexahydrocurcumin (HHC) at concentration of 25 μmol/L significantly down-regulate COX-2 expression in HT-29 human colon cancer cells and these results may be clarify the potential therapeutic role of 5-fluorouracil as COX-2 inhibitors. Moreover, the anti-tumorgenic effect of 5- fluorouracil against N-methylnitrosourea induced colon cancer was confirmed by Wen *et al*. [71] who found that 5-fluorouracil-triggered apoptosis of DN-HIF-transfected A549 cells was reduced by sicyclin D1 (cyclin D1-specific interference RNA) introduction. In addition, the apoptotic effect of 5- fluorouracil was greatly supported by the study of Sawai *et al*. [72] which detected the invasion inhibitory effect of 5-fluorouracil on survivin-3B gene- transfected DLD-1 cells. These authors speculated that survivin-3B expression in colon cancer is an important factor involved in the invasive capacity of cancer cells in the presence of anticancer drug.

Carnosol has been found to block protein kinase C signaling and inhibits the binding of the activator protein 1 (AP-1) to the COX-2 promoter which should be noted is fundamentally different than the synthetic COX-2 inhibitors

(e.g. celecoxib) that function as direct inhibitors of COX-2 [73]. *In vitro* study of Barni *et al*. [74] demonstrated that the treatment of Caco-2 cell line with carnosic acid leads to the down-regulation of COX-2 expression at both the mRNA and protein levels**.** In several types of human cancer including colorectal carcinoma, mutation in the β-catenin leads to cytoplasmic/nuclear accumulation of β-catenin resulting in accelerated tumor cell proliferation and tumor progression through the transcriptional activation of target genes including cyclin D1 [75]. Therefore, the inhibition of β-catenin mRNA level by *R. officinalis* extract as shown in the current study might be one of the proposed mechanisms by which its active constituents could reduce the expression of cyclin D1 in colon tissue of the treated colon cancer rats. The second proposed mechanism for the efficacy of *R. officinalis* extract to reduce cyclin D1 expression in colon tissue of the treated rats related to the PI3K/AKT pathway. PI3K/AKT has been found to promote cyclin D1 which has been found to be overexpressed in tumor with a k-ras mutation [62].

The treatment of COLO 205 human colorectal adenocarcinoma with rosmarnol for 24h displayed a strong apoptosis-inducing response *via* the involvement of caspase activation and complicated regulation of both the mitochondrial apoptotic pathway and death receptor pathway. There was an inverse correlation between caspases activation and survivin expression [67]. Thus, the activation of caspases by rosmanol might be the mechanism by which *R. officinalis* extract used in the present study could down-regulate survivin expression in colon tissue of the treated rats.

The biochemical results in the current study revealed that there was significant increase in B cell lymphoma 2 (Bcl-2), tumor necrosis factor- α (TNF-α), matrix metalloproteinase-7 (MMP-7), matrix metalloproteinase-9 (MMP-9) and epidermal growth factor (EGF) levels in colon cancer group. It well known that the key biochemical event involved in the apoptotic process was the upregulation of pro-apoptotic proteins and/or the down-regulation of anti-apoptotic protein molecules. Among these proteins, Bcl-2 family of proteins serve as critical regulators of the mitochondrial pathway involved in apoptosis, acting to either inhibit or promote cell death. The Bcl-2 proteins have been identified as anti-apoptotic proteins, which bind to the outer membrane of the mitochondria and prevent the release of cytochrome c [76]. The increment of Bcl-2 level in this study is in agreement with Sun *et al*. [77] who found high expression levels of the anti-apoptotic genes Bag-1 and Bcl-2 in colon cancer. The expression of Bcl-2 is inversely correlated with p53 expression in adenomas as well as primary and metastatic colorectal carcinomas [78, 79]. Enforced expression of β-catenin, the central mediator of Wnt signaling, induces Bcl-2 expression through its down-stream targets c-Myc [80]. Previous studies indicated that TNF-α stimulates inflammation by turning on gene transcription through signaling cascades such as the Akt/NF-κB pathway. This signaling subsequently serves as the primary mechanism to protect cancer cells against apoptotic stimuli through several transcriptional genes, such as inhibitor of apoptosis proteins (IAP), the specific inhibitor of caspases [81]**.** The anti-apoptotic signaling of TNF-α, tumor necrosis factor receptor (TNFR) is known to activate Akt/NF-κB in three ways: directly through phosphatidylinositol 3-kinase (PI3K) activation, or indirectly through crosstalk signaling to EGFR, or both together [82, 83].

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes, which degrade all components of both the basement membrane and extracellular matrix (ECM). Matrix metalloproteinases play a key role not only in normal processes of ECM degradation, but also in pathological processes such as tissue remodeling of inflammatory diseases, cancer invasion, and metastasis [84, 85]. The MMPs are frequently overexpressed in various human cancers. Moreover, enhanced expressions of MMPs have been associated with an aggressive malignant phenotype and adverse prognosis in cancer patients [86, 87]**.** It is noteworthy that only matrix metalloproteinase-7 (MMP-7) and membrane type-1 MMP (MT1-MMP) are produced by colorectal cancer cells themselves [88, 89] and because of the strong ECM-degradative activity, much evidence supports the role of MMP-7 in tumorigenesis and progression *in vitro*, and in the animal models. The levels of MMP-7 mRNA expression were correlated with the stage of colon cancer progression [90]. Also, it has been reported that colorectal tumors have increased co-expression of MMP-3 and MMP-9. Inuzaka *et al*. [91] hypothesized that urokinase plasminogen activator (uPA) co-expressed with MMP-9 in colorectal cancers is responsible for the activation of plasminogen to plasmin. Plasmin then activates proMMP-3 to MMP-3 which then activates proMMP-9, resulting in colorectal cancer progression.

Overexpression of epidermal growth factor receptor (EGFR) occurs in 65–70% of colorectal cancer (CRC) patients, and as would be suggested, it is more commonly seen in advanced stage tumors [92]. In this context, EGFR has been identified as an important therapeutic target in metastatic CRC (mCRC). Epidermal growth factor receptor, a member of the HER-erbB family of receptor tyrosine kinases, is a cell-surface receptor that binds epidermal growth factor [93]. The binding of epidermal growth factor (EGF) or other ligands to EGFR initiates a mitogenic signaling cascade through two main axes. The first axis involves the K-RAS–RAF-mitogen-activated protein kinase (MAPK) pathway, which responsible for gene transcription, cell-cycle progression and cell proliferation. While, the second axis involves membrane localization of the lipid kinase phosphatidylinositol 3- kinase (PI3K), which promotes Akt-

mammalian target of rapapycin (mTOR) activation, responsible for antiapoptosis and prosurvival signals [94, 95]**.** It may also activate phospholipase-C, signal transducer and activators of transcription protein (STAT) [96]**.** EGFR has also been implicated in the activation of eicosanoid signaling through induction of cyclooxygenase-2 (COX-2) [97]. While, *He et al*. [98] found that 5- fluorouracil induces apoptosis in colon cancer cells through the regulation of the Bcl-2/Bax protein ratio. It was demonstrated by Fang *et al*. [99] that 5-fluorouracil alone can significantly inhibit HT-29 cell proliferation and migration, block the cells in G2/M phase and induce cell apoptosis. Also, recent study of Zhou *el al*. [100] showed a reduction in TNF-α level in a canine model of severe acute pancreatitis (SAP) after treatment with 5-fluorouracil or octreotide, alone or in combination.

5-fluorouracil also can down-regulate MMP7 and estrogen receptor 2 (ERbeta) expression. Iovieno *et al*. [101] found a reduction in the expression matrix metalloproteinases (MMP)-2, MMP-9, and tissue inhibitor of metalloproteinase (TIMP)-1 in a case of conjunctival intraepithelial squamous cell carcinoma (SCC) treated with topical 5-fluorouracil chemotherapy. Guo *et al*. [102] showed that lapatinib in combination with 5-fluorouracil markedly reduced the phosphorylation of EGFR and human epidermal growth factor receptor 2 (HER2), and inhibited the activation of downstream signaling molecules, such as AKT and ERK. Also, Dörrie *et al*. [103] found that carnosol induced apoptosis with down-regulation of Bcl-2 in cell lines established from patients with acute lymphocytic leukaemia, but not in normal peripheral blood mononucleocytes. Also, Mengoni *et al*. [104] showed that inflamed skin treated with carnosic acid (CA) and carnosol (CS) exhibited a decreased expression of IL-1β and TNF-α, a selective inhibition of COX-2.

The study of Barni *et al*. [105] reported that carnosic acid induced colon cancer cell lines apoptosis after 24h of treatment and inhibited cell adhesion and migration possibly by reducing the activity of secreted protease such as urokinase plasminogen activator (uPA) and metalloproteinase. These effects may be associated through a mechanism involving the inhibition of the COX-2 pathway as carnosic acid could down-regulate the expression of COX-2 in Caco-2 cells at both the mRNA and protein levels [105]**.** Also**,** Huang *et al*. [106] found that carnosol could restrict the invasive ability of B16/F10 mouse melanoma cells by reducing MMP-9 expression and activity through suppressing extracellular signal-regulated kinase (ERK) 1/2, AKT, p38, and JNK signaling pathway and inhibition of NF-κB and activator protein-1 (AP-1) binding activity. NF-κB and AP-1 are MAPKs and AKT-responsive promoter elements. Therefore, the MAPKs and AKT signal transduction pathway may play an important role in the regulation of MMP-9 expression.

Venkatachalam *et al.* [107] studied the potent beneficial role of rosmarinic acid (RA) in DMH induced experimental rat colon carcinogenesis. Its chemopreventive effect is evidenced by the decreased incidence and distribution of tumors along the colon. This effect of RA could be associated with inhibition of cell proliferation and induction of tumor cell death [108]. Moreover, the strong anti-inflammatory compound in *R. officinalis* mainly carnosic acid and carnosol could regulate the expression of inflammation-associated genes [104]. Furthermore, carnosol has been reported to have broad anti-cancer properties in several cell line models targeting multiple deregulated pathways [109]. Additionally, carnosic acid showed powerful antioxidant effect due to its free radical scavengering property [110]. All of these properties of *R. officinalis* extract together with the present findings on the molecular levels that confirmed its anticancer activity led to suppressing of the circulating tumor biochemical markers levels as shown in the current study.

Histological examination of colon tissue section of rat received intrarectal dose of 2 mg N-methylnitrosourea for five weeks (cancer group) revealed dysplasia and anaplasia associated with pleomorphism and hyperchromachia in the lining epithelial cells of the glandular structure (adenocarcinoma). This histopathological feature is in consistent with the studies of Narisawa *et al*. [111], Narisawa and Fukaura [32] and Ousingsawat *et al*. [112] which confirmed the induction of colon carcinogenesis in rats. While, histological investigation in the present results revealed that the treatment with 5-fluorouracil showed the presence of few inflammatory cells infiltration in the lamina propria of the mucosa with oedema in the muscularis. These findings are in agreement with the study of El-Malt *et al*. [113] study. The influence of fluorouracil on colonic carcinoma mainly comes from its growth inhibitory effects on cancer cells [114]. Also, histological examination of colon tissue section of colon cancer rats treated with low dose of *R. officinais* extract (RMTME low) showed focal lymphoid cells aggregation in the mucosa with massive number of inflammatory cells infiltration in submucosa as well as dysplasia in the lining epithelium of the mucosa. In addition, the treatment with high dose of RMTME revealed massive number of inflammatory cells infiltration in the hyalinized lamina propria of the mucosal layer. These findings could be explained as that rosemary constituent mainly rosmarinic acid which possesses more or less moderate decreasing effect on the number of polyps in colon cancer reaching to 50% [107].

CONCLUSION

From the present results, it could be concluded that *R. officinalis* possess a promising therapeutic role against colon cancer induced in rats through its potential antiinflammatory property, antiproliferative capacity and apoptotic activity.

Acknowledgments
The outhors express sincere appreciation to prof. Adel Bakeer Kholoussy, professor of pathology, Faculty of Veterinary Medicine, Cairo University for his kind cooperation in conducting histopathological investigations and immunohistochemical examination in this study. The authors acknowledge the financial support of the National Research Centre, Egypt (Grant no: 9100305- 2011-2013).

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