

## ANTITUMOR EFFICACY OF *BOSWELLIA SERRATA* EXTRACT IN MANAGEMENT OF COLON CANCER INDUCED IN EXPERIMENTAL ANIMAL

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### **ABSTRACT**

**Objective:** This study aimed to investigate the efficacy of *Boswellia serrata* methylene chloride extract against colon cancer- induced in rats.

**Materials and Methods** Forty male rats were classified into 5 groups. Group (1) was control. Groups from (2) to (5) were intrarectally administered with N-methylnitrosourea 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 1666.6 mg/kg and 3333.3 mg/kg of *Boswellia serrata* methylene chloride extract respectively. Serum MMP-7, MMP-9 and EGF, plasma TGF- $\beta$  and TNF- $\alpha$  levels were assayed using ELISA procedure. Immunohistochemical technique was used for estimation of colon COX-2 and cyclin D1 expression. Colon  $\beta$ -catenin, K-ras and c- myc gene expression was detected by RT-PCR. Also, histological investigation of colon tissue was done.

**Results:** Colon cancer group showed significant elevation in the studied biochemical markers. In the contrary, the all treated groups showed significant reduction in these markers. Colon cancer group showed significant rise in COX-2 and cyclin D1 expression in colon tissue. In contrast, all treated groups exhibited marked depletion in COX-2 and cyclin D1 expression. Cancer group displayed significant up-regulation in the expression level of  $\beta$ -catenin K-ras and c-myc genes in colon tissue. While, all treated groups exhibited significant down-regulation in the expression levels of these genes. Histopathological investigation of colon tissue sections in cancer group showed dysplasia and anaplasia in the lining epithelial cells of the glandular structure. While, treatment with 5-fluorouracil or *Boswellia serrata* extract showed marked improvement in the histological feature of colon tissue.

**Conclusion:** The present study indicated that *Boswellia serrata* methylene chloride extract has a promising therapeutic role against colon cancer induced in rats through its potential antiinflammatory property, antiproliferative capacity and apoptotic activity.

**Keywords:** Colon cancer, *Boswellia serrata*, Inflammation, Proliferation, Apoptosis and Rats

### **INTRODUCTION**

In recent years, colorectal cancer (CRC) incidence has been increasing to become a major cause of morbidity and mortality worldwide from cancers, with high rates in westernized societies and increasing rates in developing countries [1]. Although its etiology is multifactorial and complex, it is generally accepted that the hereditary genetic component associated with dietary habits such as low intake of fibers, proteins, fruits and vegetables as well as diets with high red and processed meat as well as fat levels are among the greatest risk factors [2]. Also many genetic defects (mutations) in colon cancer have been found and these play important roles in carcinogenesis of colon cancer. These mutations include adenomatous polyposis coli (APC), K-ras, phosphatidylinositol 3-kinase catalytic subunit (PIK3CA), phosphatase and tensin homolog (PTEN), transforming growth factor- $\beta$  (TGF- $\beta$ ) receptor and tumor protein P53 (TP53) genes [3-5].

Development of colon cancer typically follows several consecutive steps, although initiating mutations in normal epithelial or stem cells occur at random and at low rates, cells that contain activating mutations in Wnt or  $\beta$ -catenin are the most likely to form tumors.  $\beta$ -catenin plays an important role in cell adhesion and also is an oncogenic protein. It associates with cadherin or  $\alpha$ -catenin to link the actin cytoskeleton. It is also a co-transcriptional activator of genes in the Wnt signal pathway. In the free form, it associates with the scaffolding proteins, axin and adenomatous polyposis coli (Apc), and is phosphorylated by glycogen synthase kinase-3 $\beta$ (GSK-3 $\beta$ ) then degraded by the proteasome [6]. In most colon cancer, patients have a mutation in the Apc gene and in 80% of sporadic colon cancer patients, a mutated APC cannot bind to  $\beta$ -catenin for normal function [7]. The N-terminus of  $\beta$ -catenin is also mutated in some cases, so that  $\beta$ -catenin cannot form the complex and cannot be degraded [5]. Thus, free  $\beta$ -catenin is increased and binds with the T-cell factor/lymphoid enhancer factor TCF/LEF to form a complex, which

activates gene transcription and cell proliferation [8, 9].  $\beta$ -catenin targets c-myc and cyclin D1 genes, which have important roles in proliferation, apoptosis, and cell-cycle progression and are responsible for tumour formation [10]. C-myc and cyclin D1 have been found to be overexpressed in several stages of DMH/AOM induced rat colon carcinogenesis [11, 12].

K-ras plays an important role in the carcinogenesis of colon cancer. K-ras is a small G-protein that regulates both mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase–protein kinase B/Akt (PI3K/Akt) intracellular signal pathways, which, in turn, regulate cell growth and proliferation. Both pathways play important roles in the carcinogenesis of many types of cancers including colon cancer. K-ras gene mutation and/or protein activation has been found to increase cyclooxygenase-2 (COX-2) expression in tumors [13].

Cyclooxygenase-2 is an inducible enzyme produced mainly in inflammatory cells, inflammatory sites and colorectal carcinomas [14, 15]. Research studies demonstrated the importance of COX-2 in colorectal tumorigenesis and in the development of intestinal neoplasms [16]. COX-2 overexpression enhances neovascularization thus conferring survival advantage of colon cancer cells [17].

Transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling pathway is involved in the control of several biological processes, including cell proliferation, differentiation, migration and apoptosis [18]. It is one of the most commonly altered cellular signaling pathways in human cancers [19].

Matrix Metalloproteinases (MMPs) are a family of structurally related zinc-dependent proteinases responsible for normal matrix remodeling and pathological tissue destruction by virtue of their ability to catabolize extracellular matrix ECM components [20]. MMPs have been classified according to their structures, substrate specificities and cellular localization into the following groups:

collagenases, gelatinases, stromelysins, membrane-type MMPs and others include matrilysin (MMP-7) [21]. These enzymes 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 [22, 23].

Traditional medicines, which have been used for thousands of years, are still being used by the majority of people in the world today because natural products are generally considered to be safe, inexpensive and targeted toward a number of diseases. In most cases, however, neither the active components nor their mechanisms of action are well established. Thus, identification of the active molecules used in traditional medicines and the signaling pathways that they modulate could validate their use in various diseases.

The gum resin of *Boswellia serrata*, a kind of deciduous tree grown in the dry part of China and India, has been considered throughout the ages to have a wealth of healing properties. Resins of *Boswellia serrata* have been used for the treatment of rheumatoid arthritis and other inflammatory diseases such as Crohn's disease [24, 25]. The antiinflammatory activity has been attributed to the resin's ability in regulating immune cytokine production [26] and leukocyte infiltration [27, 28]. Extracts from *Boswellia serrata* have been shown to possess anti-bacterial, anti-fungal [29], anti-carcinogenic [30], and anti-neoplastic [31] properties. Clinically, extracts from the resin have been shown to reduce the peritumoral edema in glioblastoma patients [32] and reverse multiple brain metastases in breast cancer patients [33]. Also, efficacy, safety and tolerability profile of essential oil formulation containing oil of *Boswellia serrata* has been confirmed for the treatment of acute soft tissue injuries [34].

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

## MATERIALS AND METHODS

### Materials

#### Plant material

*Boswellia serrata* was obtained from the Egyption Herbal Market, Cairo, Egypt. *Boswellia serrata* was precisely identified and differentiated in the Research Institute for Oily Crops, Cairo, Egypt.

#### Preparation of *Boswellia serrata* methylene chloride extract (BSMC)

*Boswellia serrata* methylene chloride extract (BSMC) was prepared by adding 2000 ml methylene chloride to 1 kg of *Boswellia serrata* and left for 72 hrs. The extract was filtered using filter paper and the solvent was evaporated using rotary evaporator [35].

### 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 ( $25\pm1$ ) 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 [36]. 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.

### 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 [37]. (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) [38].(4): *Boswellia serrata* methylene chloride extract-treated group (BMCE low dose) in which the rats were intrarectally administered with N-methylnitrosourea for 5 weeks and then orally treated with low dose (1666.6 mg/kg) of BMCE daily for 4 months. (5): *Boswellia serrata* methylene chloride extract-treated group (BMCE high dose) in which the rats were intrarectally administered with N-methylnitrosourea for 5 weeks and the orally treated with high dose (3333.3 mg/kg) of BMCE daily for 4 months. The selected doses of the medicinal plant extracts were 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 preserved in formalin saline (10%) for histological investigation and immunohistochemical examination and the second portion was collected in liquid nitrogen and stored at -80°C for molecular genetic analyses.

### Methods

#### Biochemical analyses

Serum matrix metalloproteinase-7 (MMP-7) activity was assayed by ELIZA technique using MMP-7 assay kit purchased from Glory Science Co., Ltd, TX, USA according to the instructions provided. Serum matrix metalloproteinase-9 (MMP-9) activity was assayed by ELIZA technique using MMP-9 assay kit purchased from Glory Science Co., Ltd, TX, USA according to the instructions provided with MMP-9 assay kit. Serum epidermal growth factor (EGF) level was assayed by ELIZA technique using EGF assay kit purchased from Glory Science Co., Ltd, TX, USA according to the instructions provided with EGF assay kit. Plasma transforming growth factor-beta (TGF- $\beta$ ) level was determined by ELIZA technique using TGF- $\beta$  assay kit purchased from Glory Science Co., Ltd, TX, USA according to the instructions provided with TGF- $\beta$  assay kit and plasma TNF- $\alpha$  activity was assayed by ELIZA technique using TNF- $\alpha$  assay kit purchased from Glory Science Co., Ltd, TX, USA the instructions provided with TNF- $\alpha$  assay kit.

#### 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  $\mu$ m by slide microtome. The obtained tissue sections were collected on glass positive slides and fixed in a 65°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 °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), and (Cyclin D1 Cat.#RB-9041-R7, Thermoscientific, CA-USA) were applied, then the slides were incubated in the humidity chamber for overnight at 4°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.

#### Molecular genetic analyses

##### Expression of $\beta$ -catenin, K-ras and c-myc genes

##### 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 for no more than 12,000 xg for 15 minutes at 4°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 °C for 10 minutes and centrifuged at not more than 12,000 xg for 10 minutes at 4 °C. The RNA was precipitated which was often invisible before centrifugation, formed 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 no more than 7,500 xg for five minutes at 4 °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).

##### 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  $\mu$ l using RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5 $\mu$ g) was used with a reaction mixture, termed as master mix (MM). The MM was consisted of 50 mM MgCl<sub>2</sub>, 5x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3; 10 mM of each dNTP, 50  $\mu$ M 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 for 5 min at 99 °C. 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).

##### 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  $\mu$ L reaction mixtures containing 12.5 $\mu$ L 1x SYBR® Premix ExTaqTM (TaKaRa, Biotech. Co. Ltd Germany), 0.5  $\mu$ L 0.2  $\mu$ M sense primers, 0.5  $\mu$ L 0.2  $\mu$ M antisense primer, 6.5  $\mu$ L distilled water, and 5  $\mu$ L of cDNA template. Each experiment included a distilled water control.

Primer sequence for  $\beta$ -catenin, 5'-CAAT GGG TCA TAT CAC AGA TTC TT-3',  $\beta$ -catenin-R, 5'-TCT CTT TTC ACC ACA ACA TTT-3' [39], for K-ras, 5'- AGT ACG ACC CTA CGA TAG AGG ACT CCT-3', K-ras-R, 5' - CAA TCT GTA CTG TCG GAT CTC TCT CAC C - 3'[40], and for c-myc (GenBank accession number Z38066) were upstream: 5'-TGA CGA GAC CTT CGT GAA GA-3' and downstream: 5'-ATT GAT GTT ATT TAC ACT TAA GGG T-3' [41]. The semi quantitative values of RT-PCR (sqRT-PCR) of the previous genes were normalized on the expression values of  $\beta$ -actin gene ( $\beta$ -actin-F: 5'- CCC CAT CGA GCA CGG TAT TG -3',  $\beta$ -actin-R ATG GCG GGG GTG TTG AAG GTC [42]. 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 [43]

$$Ef = 10^{-1}/\text{slope}$$

$$\text{Efficiency (\%)} = (Ef - 1) \times 100$$

The relative quantification of the target to the reference was determined by using the  $\Delta$ CT method if E for the target ( $\beta$ -catenin, K-ras and c-myc) and the reference primers ( $\beta$ -actin) are the same [43]

$$\text{Ratio (reference/ target gene)} = Ef^{\text{CT (reference)}} - Ct(\text{target})$$

##### 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  $\mu$ m by slide 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 [44].

##### 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 [45]. 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

### Biochemical results

The data in Table (1) illustrate the effect of treatment with 5-fluorouracil and *Boswellia serrata* methylene chloride extract (BMCE) on serum MMP-7, MMP-9, EGF, plasma TGF- $\beta$  and TNF- $\alpha$  levels in colon cancer- induced rats. The results revealed significant elevation in serum levels of MMP-7 (47.85 %) and MMP-9 (263%), EGF (45.9%), plasma levels of TGF- $\beta$  (44.97 %) and TNF- $\alpha$  (21.53%) in cancer- induced group as compared with the control group. However, treatment of cancer- induced group with 5-fluorouracil showed significant reduction in serum levels of MMP-7 (-33.02%), MMP-9 (-71.52%), EGF (-28.37%), plasma levels of TGF- $\beta$  (-27.96 %) and TNF- $\alpha$  (-15.32%) compared with untreated cancer- induced group. Treatment of cancer- induced group with either low or high

dose of BMCE produced significant depletion in serum MMP-7 (-22.3%) for BMCE low dose and (-22.54 %) for BMCE high dose, MMP-9 level (-66.89%) for BMCE low dose and (-67.12%) for BMCE high dose, serum EGF level (-22.14%) for BMCE low dose and (-20.06 %) for BMCE high dose, plasma TGF- $\beta$  level (-9.24 %) for

BMCE low dose and (-10.46 %) for BMCE high dose and TNF- $\alpha$  level (-5.51%) for BMCE low dose and (-6.53 %) for BMCE high dose, as compared to untreated cancer- induced group. Thus, the effect of BMCE in modulation of these biochemical markers was dose dependent (Table 1).

**Table 1: Effect of *Boswellia serrata* methylene chloride extract (BMCE) on MMP-7, MMP-9, EGF, TGF-  $\beta$  and TNF- $\alpha$  circulating levels in colon cancer induced in rats.**

Groups	S MMP-7 μL	S MMP-9 ng/ml	P TGF- $\beta$ pg/ml	S EGF ng/L	P TNF- $\alpha$ ng/ml
Control group	0.18±0.015	1.19± 0.014	29.33±1.68	660 ±20	81.25±0.47
Cancer group	0.27±0.024 <sup>a</sup> (47.85 %)	4.32±0.149 <sup>a</sup> (263%)	42.52±2.13 <sup>a</sup> (44.97%)	963.3 ±25.1 <sup>a</sup> (45.9%)	98.75±0.47 <sup>a</sup> (21.53%)
5- fluorouracil group	0.18±0.013 <sup>b</sup> (-33.02 %)	1.23±0.09 <sup>b</sup> (-71.52%)	30.63±2.61 <sup>b</sup> (-27.96 %)	690 ±5.7 <sup>b</sup> (-28.37%)	83.62±0.89 <sup>b</sup> (-15.32%)
BMCE group (1666.6 mg/kg)	0.21±0.0065 <sup>b</sup> (-22.3 %)	1.43±0.033 <sup>b</sup> (-66.89%)	38.59±2.21 <sup>b</sup> (-9.24 %)	750 ±37.85 <sup>b</sup> (-22.14%)	93.3±1.84 <sup>b</sup> (-5.51%)
BMCE group (3333.3mg/kg)	0.21±0.01 <sup>b</sup> (-22.54 %)	1.42±0.029 <sup>b</sup> (-67.12%)	38.07±2.59 <sup>b</sup> (-10.46 %)	770 ±23.09 <sup>b</sup> (-20.06%)	92.3±0.83 <sup>b</sup> (-6.53%)

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.

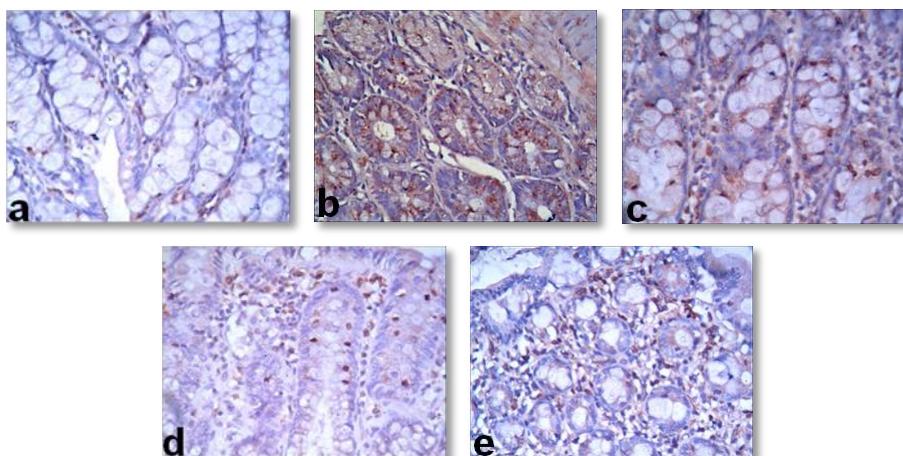
BMCE: *Boswellia serrata* methylene chloride extract.

S: Serum; P: Plasma

#### Immunohistochemical results

Photograph for immunohistochemical staining of colon tissue of control rat using antibody against COX-2 showed mild positive reaction in interstitial stromal cells (Fig. 1a). While, photograph for immunohistochemical staining of colon tissue of cancer- induced rat showed very sever positive reaction in cytoplasm of the glandular lining epithelium (Fig. 1b). Photograph for immunohistochemical

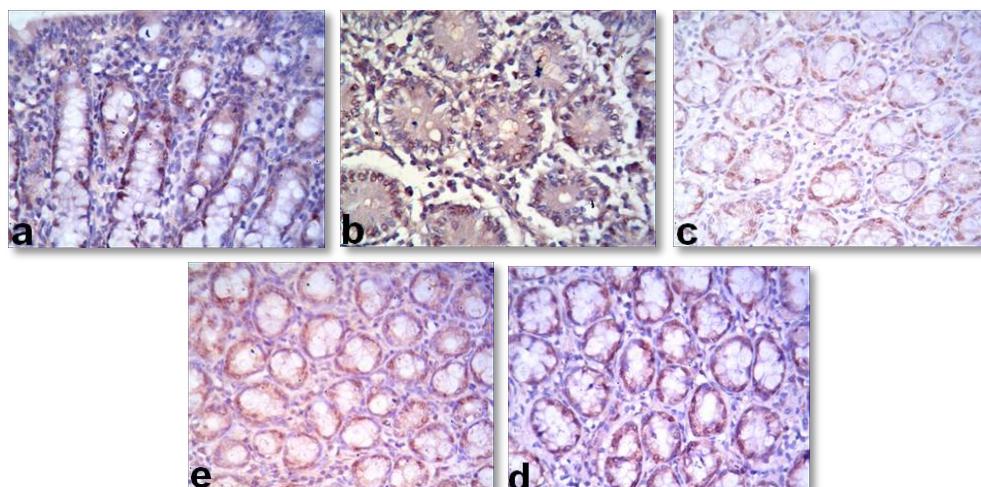
staining of colon tissue of cancer- induced rat treated with 5- fluorouracil showed moderate positive reaction in the nuclei of the glandular lining epithelium (Fig. 1c). Photograph for immunohistochemical staining of colon tissue of cancer- induced rat treated with low dose of BMCE showed mild positive reaction in the lining glandular epithelial cells (Fig. 1d) and that for cancer- induced rat treated with high dose of BMCE showed moderate positive reaction in interstitial stromal cells (Fig.1e).



**Fig 2: 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 BMCE showed mild positive reaction in the lining glandular epithelial cells (80x) and (e) Colon cancer- induced rat treated with high dose of BMCE showed moderate positive reaction in interstitial stromal cells (80x).**

Photograph for immunohistochemical staining of colon tissue of control rat using antibody against cyclin D1 showed positive reaction in the nuclei of the glandular lining epithelial cells (Fig. 2a). While, photograph for immunohistochemical staining of colon tissue of cancer- induced rat showed very sever positive reaction in the nuclei of the glandular lining epithelial cells as well as the interstitial stromal cells (Fig. 2b). Photograph for immunohistochemical

staining of colon tissue of cancer- induced rat treated with 5- fluorouracil showed moderate positive reaction in the nuclei of the glandular lining epithelial (Fig. 2c). Photograph for immunohistochemical staining of colon tissue of cancer- induced rat treated with low dose and high dose of BMCE showed sever positive reaction in the nuclei of the glandular lining epithelial as illustrated in (Figs. 2d, 2e).



**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 BMCE showed mild positive reaction in the nuclei of the glandular lining epithelial (80x) and (e) Colon cancer- induced rat treated with high dose of BMCE showed moderate positive reaction in the nuclei of the glandular lining epithelial (80x).

#### Molecular genetic results

The results of gene expression levels assessment of  $\beta$ -catenin, K-ras and c-myc in colon tissue of the different studied groups were summarized in Table (2). Our data revealed that  $\beta$ -catenin, K-ras and c-myc genes were down-regulated in the colon tissue of control group. However, the expression level of  $\beta$ -catenin, K-ras and c-myc genes were significantly up-regulated in colon cancer- induced group (309%, 17% and 212.5% respectively) as compared with the control group. In contrast, cancer- induced group treated with 5-fluorouracil exhibited significant down-regulation in the expression

levels of  $\beta$ -catenin, K-ras and c-myc genes (-71.85%, -58.95% and -64.66% respectively) compered with untreated cancer- induced group. Treatment of cancer- induced group with low or high dose of BMCE reverses the alteration of the expression levels of the two genes. Thus, the expression levels of  $\beta$ -catenin, K-ras and c-myc genes revealed significant down-regulation in colon cancer- induced group treated with low dose of BMCE (-29.62%, -30.63% and -26.66% respectively) and that treated with high dose (-37.77%, -37.57% and -34% respectively) compared with untreated cancer-induced group. The effect of BMCE in amelioration of genetic alteration was dose dependent Table (2).

**Table 2: Semi-quantitative real time -PCR confirmation of  $\beta$  -catenin, K-ras and c-myc genes in colon tissue of the different studied groups.**

Groups	$\beta$ -catenin gene	K-ras gene	c-myc gene
Control group	0.33±0.02 <sup>c</sup>	0.62±0.02 <sup>c</sup>	0.48±0.02 <sup>c</sup>
Cancer group	1.35±0.04 <sup>a</sup> (309%)	1.73±0.04 <sup>a</sup> (179%)	1.50±0.03 <sup>a</sup> (212.5%)
Fluorouracil group	0.38±0.03 <sup>c</sup> (-71.85%)	0.71±0.02 <sup>c</sup> (-58.95%)	0.53±0.04 <sup>c</sup> (-64.66%)
BMCE group (1666.6 mg/kg)	0.95±0.02 <sup>ab</sup> (-29.62%)	1.20±0.03 <sup>b</sup> (-30.63%)	1.10±0.04 <sup>ab</sup> (-26.66%)
BMCE group (3333.3 mg/kg)	0.84±0.03 <sup>ab</sup> (-37.77%)	1.08±0.02 <sup>b</sup> (-37.57%)	0.99±0.02 <sup>b</sup> (-34%)

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.

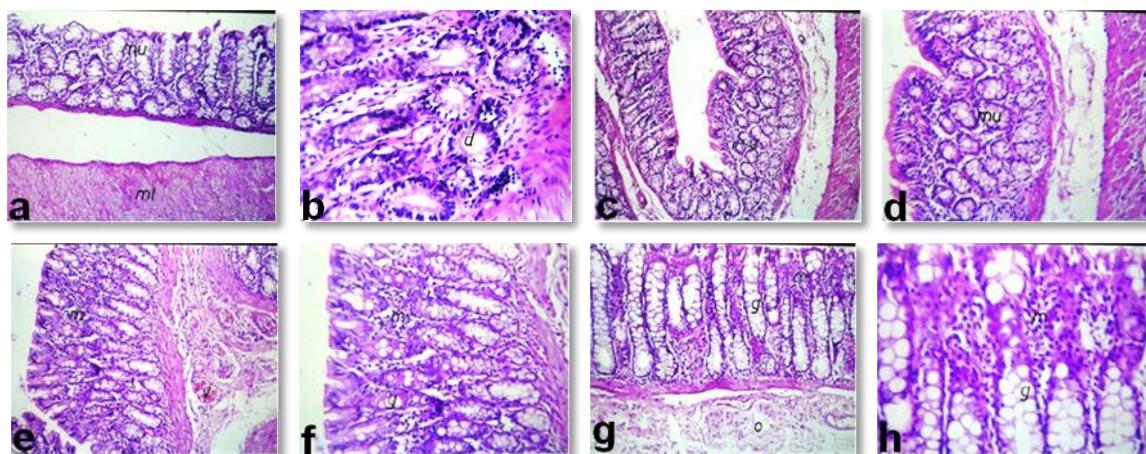
(%): percent of difference with respect to the corresponding control value.

BMCE: *Boswellia serrata* methylene chloride extract

#### Histopathological results

Histological investigation of colon tissue sections of control group showed normal histological structure of the mucosa, submucosa and muscularis layers (Fig. 3a). While colon tissue sections of colon cancer- induced group showed dysplasia and anaplasia associated with pleomorphism and hyperchromachia in the lining epithelial cells of the glandular structure (Fig. 3b). 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.3c and 3d). Microscopic investigation of colon tissue section of cancer- induced rats treated with low dose of BMCE showed few inflammatory cells infiltration in the lamina propria with dilatation and congestion in blood vessels of submucosa (Figs 3e and 3f) and the investigation of colon tissue section of cancer- induced rat treated with high dose of BMCE showed goblet cells formation in the mucosal epithelium with inflammatory cells infiltration in the lamina propria and oedema in submucosa and muscularis (Figs 3g and 3h).



**Fig. 3:** (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 anaplasia 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. (3c) 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 BMCE showed few inflammatory cells infiltration in the lamina propria (m) with dilatation and congestion in blood vessels of submucosa (v) (H & E X40). (f) Micrograph of colon tissue section of colon cancer- induced rat treated with low dose of BMCE showed the magnification of Fig. (3e) to identify the inflammatory cells infiltration in the lamina propria (H & E X64). (g) Micrograph of colon tissue section of colon cancer- induced rat treated with high dose of BMCE showed goblet cells formation in the mucosal epithelium (g) with inflammatory cells infiltration in the lamina propria (m) and oedema in submucosa and muscularis (o) (H & E X40) and (h) Micrograph of colon tissue section of colon cancer- induced rats treated with high dose of BMCE showed the magnification of Fig. (3g) to identify goblet cells formation in the mucosal epithelium (g) with inflammatory cells infiltration (H & E X80).

## DISCUSSION

The results in current study revealed that there was significant increase in matrix metalloproteinase-7 (MMP-7), matrix metalloproteinase-9 (MMP-9), transforming growth factor -beta (TGF- $\beta$ ), epidermal growth factor (EGF) and TNF-  $\alpha$  levels in cancer-induced group. Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes, which degrade all components of both the basement membrane and extracellular matrix (ECM). 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 [46]. It is noteworthy that only matrilysin (MMP-7) and membrane type-1 MMP (MT1-MMP) are produced by colorectal cancer cells themselves [47] 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 [48]. Also, it has been reported that colorectal tumors have increased co-expression of MMP-3 and MMP-9. Inuzaka et al. [49] 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.

High levels of TGF- $\beta$ 1 in the primary colorectal tumor are associated with advanced stages and a greater likelihood of recurrence and decreased survival [50]. Experimentally, prolonged exposure to high levels of TGF- $\beta$  promotes neoplastic transformation of intestinal epithelial cells [51] and TGF-  $\beta$  stimulates the proliferation and invasion of poorly differentiated and metastatic colon cancer cells [52]. TGF-  $\beta$  has been shown to increase the production of several mitogenic growth factors including transforming growth factor-  $\alpha$  (TGF-  $\alpha$ ), fibroblast growth factor (FGF) and epidermal growth factor (EGF). In addition, TGF-  $\beta$  can activate SMAD-independent pathways, such as Ras/MAPK pathway, JNK pathway and PI3 kinase/Akt pathway [53]. Thus, TGF-  $\beta$  may drive the proliferation of colorectal cancer cells in conjunction with these oncogenic pathways. During tumorigenesis, TGF-  $\beta$  frequently stimulates the

proteolytic activity of cancer cells by increasing the expression of matrix-degrading enzymes [54].

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 tumours [55]. 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, transforming growth factor  $\alpha$  (TGF- $\alpha$ ), amphiregulin, betacellulin, and epiregulin [56]. When bound, EGFR changes its conformation to activate its tyrosine activity and mediates signalling through activation of the RAS-RAF-MAPK and PI3K signalling cascades, causing cellular growth and progression, proliferation, angiogenesis, and invasion [57]. It may also activate phospholipase-C, STAT (signal transducer and activators of transcription protein), and SRC/FAK [58].

Otherwise, several cancer therapies exploiting the cytotoxic effect of TNF- $\alpha$  on solid tumors and soft-tissue sarcomas have been examined [59]. Tammali et al. [60] reported that inhibition/antisense abolition of polyol pathway enzyme, aldosereductase (AR) inhibited the TNF- $\alpha$ -induced synthesis of prostaglandin E2 and the activity of cyclooxygenase (COX) in human colon cancer cells, Caco-2. Inhibition of AR prevented TNF- $\alpha$ -induced activation of protein kinase C (PKC) and NF- $\kappa$ B which results in the abrogation of COX-2 mRNA and protein expression. The TNF- $\alpha$  stimulates inflammation by turning on gene transcription through signaling cascades such as the Akt/nuclear factor  $\kappa$ B (NF- $\kappa$ B) 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 [61]. Akt/NF- $\kappa$ B signaling also occurs downstream of EGFR and this signaling mediates cell proliferation and antiapoptotic signaling [62]. The antiapoptotic signaling of TNF- $\alpha$ , TNFR is known to activate Akt/NF- $\kappa$ B in three ways: directly through phosphatidylinositol 3-kinase activation, or indirectly through cross-talk signaling to EGFR, or both together [63].

The results of our biochemical assays also showed significant decrease in MMP7, MMP-9, TGF- $\beta$ , EGF and TNF- $\alpha$  levels due to treatment with 5-fluorouracil. Fang et al. [64] confirmed that 5-fluorouracil alone can significantly inhibit HT-29 cell proliferation and migration, block the cells in G2/M phase and induce cell apoptosis. This drug also can down-regulate MMP7 and ERbeta expression. Iovieno et al. [65] 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.

It was demonstrated by Wendling et al. [66] that 5-fluorouracil antagonizes TGF-beta driven COLA2 transcription and associated type I collagen production by dermal fibroblasts. In addition, 5-fluorouracil inhibits both SMAD3/4-specific transcription and formation of SMAD/DNA complexes induced by TGF-beta. Thus 5-fluorouracil was identified as a potent inhibitor of TGF-beta/SMAD signaling.

Guo et al. [67] 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.

Finally, recent study of Zhou et al. [68] showed a reduction in TNF- $\alpha$  level in a canine model of severe acute pancreatitis (SAP) after treatment with 5-fluorouracil or octreotide, alone or in combination.

The biochemical analysis of the present study also revealed significant suppressive effect of the treatment with *Boswellia serrata* on the levels of MMP7, MMP-9, TGF- $\beta$ , EGF and TNF- $\alpha$  in cancer-induced rats. Park et al. [69] showed that acetyl-11-keto-beta-boswellic acid (AKBA) down-regulated the expression of COX-2 and MMP in the tissue of spleen, liver and lungs. These findings suggest that boswellic acid can inhibit the growth and metastasis of colon cancer *in vivo* through down-regulation of cancer associated biomarkers. It is documented that boswellic acid have antiproliferative and apoptotic effects which was demonstrated by the increase in cytoplasmic DNA-histone complex [70]. Considering the structure of various boswellic acids, the keto group might be important for apoptotic properties of these acids and the presence of acetyl group may strongly enhance its apoptotic effects. Also, Yadav et al. [71] found that AKBA significantly suppressed NF- $\kappa$ B activation and matrix metalloproteins.

Furthermore, the role of boswellic acid in amelioration of TGF-beta expression may be the route of boswellic acid to modulate the antioxidant status. It is clear that boswellic acid is specific and potent inhibitor of TGF- $\beta$  signaling *in vivo* [72].

Signal transducers and activators of transcription (STAT) is a family of transcription factors that has been associated with inflammation, survival, proliferation, metastasis, angiogenesis, and chemoresistance of tumor cells. One of these members, namely STAT3, is constitutively expressed in multiple myeloma (MM), leukemia, lymphoma, squamous cell carcinoma, and other solid tumors, including cancers of the prostate, breast, head and neck, and nasopharynx [73]. Corvinus et al. [74] showed an increased phosphorylation of STAT3 in colorectal cancer (CRC) cells, but not in normal intestinal epithelial cells. STAT3 can be activated by certain interleukins such as IL-6 and growth factors (e.g., epidermal growth factor). Kunnumakkara et al. [75] found that AKBA inhibited constitutive STAT3 activation in human multiple myeloma (MM) cells. AKBA suppressed IL-6-induced STAT3 activation, and this finding suggests that boswellic acids can also suppress the activation of EGF.

The study of Syrovets et al. [76] supports our finding concerning the suppressive influence of *Boswellia serrata* on TNF- $\alpha$ . These authors demonstrated that acetyl-boswellic acids inhibit lipopolysaccharide-mediated TNF- $\alpha$  induction in monocytes by direct interaction with IkappaB kinases.

The present data revealed that there was significant elevation in COX-2 expression in colon tissue of colon cancer-induced rats. It is believed that overexpression of COX-2 is related with a wide variety

of diseases including colon, lung, and breast cancers. COX is a key enzyme responsible for the conversion of arachidonic acid to prostaglandins and thromboxanes. COX-2 is the inducible form of COX, which is overexpressed at inflammatory sites, and research evidence has indicated the critical role of COX-2 in tumor promotion and carcinogenesis. This finding is in consistent with that of Takahashi and Wakabayashi [77] and in agreement with the finding of Dubois et al. [78] and Takahashi et al. [79] who 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 in rats. The mechanism of increased COX-2 expression in our study may be related to K-ras mutation and/or protein activation which increased COX-2 expression in tumors [13].

Our results showed that the treatment with 5-fluorouracil in colon cancer-induced rats significantly down-regulates COX-2 expression in colon tissue. This result goes hand in hand with that of Srimuangwong et al. [80] who demonstrated that 5-fluorouracil at concentration of 5  $\mu$ mol/L in combination with hexahydrocurcumin (HHC) at concentration of 25  $\mu$ mol/L significantly down-regulate COX-2 expression in HT-29 human colon cancer cells. Moreover, Chow et al. [81] support a potential therapeutic role of 5-fluorouracil as COX-2 inhibitors in human breast cancer.

In the present study treatment with *Boswellia serrata* extract has been found to significantly reduce the expression of COX-2 in colon tissue of colon cancer-induced rats. This finding is in great agreement with that of Yadav et al. [71] who found that boswellic acid could suppress the expression of pro-inflammatory COX-2 in colorectal tumor tissue orthotopically implanted in nude mice. Also, Park et al. [69] found that boswellic acid derivatives could down-regulate the expression of COX-2 in pancreatic tumors of orthotopic nude mouse model of pancreatic cancer. These findings together with our finding suggest that *Boswellia serrata* extract with its active constituent (boswellic acid) could inhibit the growth and metastasis of colorectal carcinoma *in vivo* through down-regulation of cancer associated biomarkers including COX-2 expression.

The present data demonstrated significant increase in the expression of cyclin D1 in colon tissue of colon cancer-induced rats. This finding is in agreement with that in the *in vitro* study of Nie et al. [82] in colon cancer cell line and the study of Mao et al. [83] in resected colonic adenocarcinoma specimens. It has been found that p13/akt promotes cyclin D1, which has been found to be overexpressed in tumor with a K-ras mutation [13]. Cyclin D1, a member of cyclins has been considered to be an oncogene which could regulate progression from the G1 phase of the cell cycle to the S phase [84]. Overexpression of cyclin D1 protein was also found in colon cancer [85, 86]. The study of Mae et al. [83] 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.

It is clear from the present study that the treatment with 5-fluorouracil decreases the expression of cyclin D1 in cancer-induced rats which clarifies its anti-tumorigenic effect against N-methylnitrosourea induced colon cancer. This result was supported by Wen et al. [87] who found that 5-fluorouracil-triggered apoptosis of DN-HIF-transfected A549 cells via reduction of sicyclin D1 (cyclin D1-specific interference RNA) introduction.

Treatment of colon cancer-induced rats with *Boswellia serrata* extract caused significant down-regulation of cyclin D1 overexpression in colon tissue. Boswellia essential oil has been found to suppress cyclin D1 expression in human breast cancer cell lines [88]. Boswellic acids and their derivatives have been shown to arrest cancer cells at the G1 phase of cell cycle, suppress cyclin D1 and E, cdk2 and 4 and increase P21 expression in colon cancer cells through P21-dependent pathway [70]. Recent study of Yadav et al. [71] suggested that boswellic acid analog can inhibit the growth and metastasis of human colorectal carcinoma *in vivo* through down-regulation of the expression level of cancer-associated biomarkers in colon tumor tissue including the proliferative markers (Cyclin D1).

The present study revealed significant increase in the gene expression level of  $\beta$ -catenin in colon tissue of cancer-induced rats. This finding is in agreement with that in the previous studies of Takahashi et al. [79] and Takahashi and Wakabayashi [77].  $\beta$ -catenin is a subunit of cadherin protein complex and has been implicated as an integral component in the Wnt/signaling pathway. When  $\beta$ -catenin is mutated,  $\beta$ -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 [89]. Thus, the gene that codes for  $\beta$ -catenin can function as an oncogene and mutations in this gene are a cause of colorectal cancer. Also,  $\beta$ -catenin binds to the product of adenomatous polyposis coli (APC) gene, which is mutated in adenomatous polyposis of the colon. Constitutive activation of  $\beta$ -catenin pathway is responsible for the initiation of the vast majority of colon cancers [90]. In all dysplastic colorectal epithelial lesions,  $\beta$ -catenin expression is found to be increased in the cytosol and nucleus. Aberrant expression of  $\beta$ -catenin has been associated with mutations of APC or  $\beta$ -catenin [13]. It has been postulated that overexpression of  $\beta$ -catenin may be the result of altered expression of one of many proteins with which  $\beta$ -catenin interacts, such as axin, conductin or E-cadherin [91].

Gene expression analysis in the present study showed a significant reduction in  $\beta$ -catenin gene expression level in colon tissue of 5-fluorouracil treated group. This finding could be attributed to the chemotherapeutic effect of 5-fluorouracil and its disrupting protein-protein interactions which may reduce the levels of oncogene  $\beta$ -catenin [92]. This result needs further investigation.

Regarding the effect of *Boswellia serrata* extract on the gene expression level of  $\beta$ -catenin in colon cancer-induced rats, the present results demonstrated significant inhibition of  $\beta$ -catenin gene expression level with *Boswellia serrata* treatment. This finding could be attributed to boswellic acid and its derivatives in *Boswellia* sp. which have anti-neoplastic activity through their anti-proliferative and pro-apoptotic properties that have been shown in multiple human cancer cell lines including colon cancer cells [70]. Moreover, boswellic acid could suppress cyclin D1 expression in human colorectal tumor tissue [71]. It is well known downstream targets of APC/ $\beta$ -catenin/Tcf-4 mediated transcriptional activation in colorectal neoplasia include cyclin D1 gene. This means that the suppression of cyclin D1 by *Boswellia serrata* extract as shown in the present study might be one of the suggested mechanisms by which this extract could inhibit  $\beta$ -catenin gene expression level in colon tissue of colon cancer treated rats.

The current results revealed that there was significant elevation in gene expression level of K-ras in colon tissue of cancer-induced rats. Mutations in the K-ras gene are responsible for activation of the K-ras pathway which is implicated in colon carcinogenesis in humans and rats [79, 93]. Mutations of proto-oncogenes ras are most commonly found in colorectal carcinoma, appearing early in the process of carcinogenesis, already in the phase of hyperproliferating epithelium, in anaplastic crypt foci (ACF), in adenocarcinomas and cancers [94]. Functional studies in cell culture [95] and mouse models [96] support a critical role for K-ras mutation in colorectal cancer progression and maintenance. In human tumors, as well as in tumors of chemically induced colon cancer in rats, increased expression of Akt, a downstream target of the K-ras pathway, has been found [97].

Our data revealed that the K-ras gene expression level was down-regulated in 5-fluorouracil treated group. The chemosensitizing effect of 5-fluorouracil could be due to its ability to suppress AKT/NF-KappaB signaling in colon cancer carcinoma cells (MCS) *in vitro* [98].

Treatment with *Boswellia serrata* extract in colon cancer-induced rats resulted in significant reduction in K-ras gene expression level in colon tissue. This finding could be attributed to the activity of the active constituents of *Boswellia serrata* (boswellic acid and its derivatives). This explanation stems from the ability of *Boswellia* sp.

essential oil to suppress Akt activation in human breast cancer cell lines [88]. It is well known that Akt expression is increased in human colon tumors and chemically-induced colon tumor in rats and Akt is a downstream target of the K-ras pathway [97]. Therefore, the suppression of Akt expression might be the proposed mechanism by which *Boswellia serrata* extract could suppress K-ras gene expression level in colon cancer-induced rats.

The data in the present study showed there was significant increase in gene expression level of c-myc in colon tissue of colon cancer-induced rats. C-myc has been found to be overexpressed in several stages of chemically-induced rat colon carcinogenesis [11, 12]. Also, the c-myc gene is frequently deregulated and overexpressed in human colon malignancy, and strategies designed to inhibit c-myc expression in cancer cells may have considerable therapeutic value [99].

The present study showed a significant decrease in c-myc expression in colon cancer tissue due to treatment with 5-fluorouracil which was detected and confirmed by RT-PCR. This result is in agreement with that of Zhao et al. [100] who found decreased expression of c-myc mRNA and phosphorylated c-myc in human colon cancer KM12C cells treated with 5-fluorouracil.

Treatment of colon cancer-induced rats with *Boswellia serrata* extract produced significant down-regulation in c-myc gene expression level in colon tissue as shown in our data. Park et al. [69] reported that boswellic acid derivatives, could down-regulate the expression level of COX-2 in pancreatic cancer tissue. The inhibitory effect of boswellic acid derivatives on COX-2 might be the suggested mechanism by which *Boswellia serrata* could inhibit the expression level of c-myc in colon tissue of the treated rats. This proposed mechanism depends on the study of Kishimoto et al. [101] who demonstrated that COX-2 inhibitors could decrease the c-myc expression level in rat colon carcinogenesis induced by azoxymethane.

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 that in the studies of Narisawa et al. [37], Narisawa and Fukaura [102] and Ousingsawat et al. [103] which confirmed the induction of colon carcinogenesis in rats.

Histological investigation of colon tissue section of colon cancer-induced rats treated 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 those of El-Malt et al. [104] study. The influence of 5-fluorouracil on colonic carcinoma mainly results from its growth inhibitory effects [105].

Histological examination of colon tissue section of colon cancer-induced rats treated with low dose of *Boswellia serrata* methylene chloride extract (BMCE) showed few inflammatory cells infiltration in the lamina propria and the treatment with high dose of BMCE revealed goblet cells formation in the mucosal epithelium with inflammatory cells infiltration in the lamina propria associated with oedema in the submucosa and muscularis. These findings could be attributed to active constituents of *Boswellia serrata* which are called boswellic acids which are pentacyclic triterpenes appear in the resin exudates of the plant and make up to 25-35% of the resin. Boswellic acid and its derivatives have been implicated in apoptosis of cancer cells particularly that of brain tumors and colon cancer [106]. Moreover, Liu et al. [70] reported that boswellic acids have anti-inflammatory properties, antiproliferative and apoptotic effects.

In conclusion, based on our results, we could conclude that *Boswellia serrata* has a promising therapeutic role against colon cancer induced by N-methylnitrosourea as indicated by the observed improvement in the biochemical, immunohistochemical and molecular markers which was confirmed by histological examination. These effects were achieved through the powerful anti-inflammatory properties, antiproliferative and apoptotic effects of

the active constituents of this plant. These findings represented good therapeutic approaches for intervention against progressive of colon cancer with special reference to the inflammation, proliferation and apoptosis.

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