



***Boswellia Serrata* Suppresses Colorectal Carcinogenesis: *In-vitro* and *In-vivo* Studies**

Hanaa H. Ahmed¹, Amal H. Hamza^{*2,3}, Sayed A. El-Toumy⁴, Amal Z. Hassan⁵

¹Hormones Department, National Research Centre, Dokki, Cairo, Egypt.

²Biochemistry Department, King Abdulaziz University, Jeddah, KSA.

³Biochemistry and Nutrition Department, Faculty of Women, Ain Shams University.

⁴Chemistry of Tannins Department, National Research Centre, Dokki, Cairo, Egypt.

⁵Chemistry of Natural product Department, National Research Centre, Dokki, Cairo, Egypt.

*Corresponding author's E-mail: amal_hamza@hotmail.com

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ABSTRACT

A growing evidence of literature supports that natural dietary compounds generally have multiple molecular targets within cancer cells, and are ordinarily considered quite safe. *Boswellia serrata* has been shown to inhibit the growth of a wide variety of tumor cells including glioma, hepatoma, and prostate cancer. The possible biochemical and molecular mechanisms of *Boswellia serrata* as antitumorogenic agent were investigated in this study. The cytotoxic effect of methanolic and methylene chloride extracts of *Boswellia serrata* on Caco-2 cell lines was estimated using MTT assay *in-vitro*. *In-vivo* study, several biochemical and immuno histochemical assays were performed to explore the biochemical and molecular pathways concerning the anti carcinogenic activity of *Boswellia serrata* against colorectal cancer induced in rats. The results revealed significant elevation in the circulating levels of CEA, CCSA-4, TGF- β , and MMP7 in colorectal cancer group, while these biomarkers decreased significantly in *Boswellia serrata* extract treated groups. The circulating levels of the apoptotic markers (Cyto-c and PDCDP-4) were significantly declined in colorectal cancer group. However, these markers were significantly increased by the treatment with *Boswellia serrata* extract. The immunohistochemical results showed up-regulation of COX-2, survivin and cyclin D expression in colon tissue of colorectal cancer group while these indicators displayed down regulation in *Boswellia serrata* treated groups. The histopathological examination supports the biochemical and immunohistochemical results. The present study clarifies that *Boswellia serrata* methanolic extract has a promising anti tumorogenic effect against colorectal cancer through its cytotoxic effect, apoptotic activity and anti-inflammatory property.

Keywords: Anti-inflammatory, Apoptosis, *Boswellia serrata*, Colorectal cancer, Cytotoxicity.

INTRODUCTION

Gum resins obtained from trees of the Burseraceae family (*Boswellia sp.*) are important ingredients in incense and perfumes. *Boswellia sp.* includes *Boswellia sacra* from Oman and Yemen, *Boswellia carteri* from Somalia, and *Boswellia serrata* from India and China. Aroma from the resins of *Boswellia sp.* is valued for its superior qualities for religious rituals since the time of ancient Egyptians.¹ *Boswellia sp.* resins have also been considered throughout the ages to have a wealth of healing properties. As these resins have been used for the treatment of rheumatoid arthritis and other inflammatory diseases such as Crohn's disease. *Boswellia sp.* extracts have been shown to possess anti-bacterial, anti-fungal, anti-carcinogenic, and anti-neoplastic properties.² clinically, extracts from the resin have been shown to reduce the peritumoral edema in glioblastoma patients and reverse multiple brain metastases in a breast cancer patient. These data suggest that resins from *Boswellia sp.* contain active ingredients that modulate important biological and health supporting activities.³

Colorectal carcinogenesis is defined by a complex multistep process that includes genetic, epigenetic, and environmental factors, and the dietary habits are among the greatest risk factors.⁴ Despite the identification of

several important genes and the two -fold increase in risk of colorectal cancer in first-degree relatives of colorectal cancer patients, the genetic basis of the majority of sporadic colorectal cancers is unknown.⁵

Currently, fluorouracil (5-FU) remains the single most effective chemotherapeutic agent for the treatment of colorectal cancer.⁶ The cytotoxic effects of 5-FU have been well characterized, and include the inhibition of the target enzyme thymidylate synthase by the fluorouracil metabolite 5-fluoro-2'-deoxyuridine-5'-monophosphate.⁷

The present study was designed to explore the *in-vitro* and *in-vivo* mechanisms underlying the antitumorogenic effect of *Boswellia serrata* extract against colorectal cancer.

MATERIALS AND METHODS

Preparation of *Boswellia serrata* extracts

The methanolic (BME) and methylene chloride extracts (BMCE) of *Boswellia serrata* were prepared by adding 2000 ml of methanol or methylene chloride (70%) to 1 kg of *Boswellia serrata* and left for 72 hrs. The extracts were filtered using filter paper and the solvents were evaporated under vacuum at 40°C using rotary evaporator.⁸

In-vitro study**Cell propagation and maintenance**

Human cancer colon cell line (Caco-2) was supplied by Naval American Research Unit, Egypt (NAMRU). Cells were propagated and maintained in RPMI-1640 medium with L-glutamine and supplemented with 10% fetal calf serum for growth and 2% of the maintenance medium and 1% antibiotic mixture in 75 cm² tissue culture flasks.

Cell Proliferation and cytotoxicity assay

The proliferation effect of *Boswellia serrata* (methanolic and methylene chloride extracts) were investigated using MTT assay.⁹ The human cancer colon (Caco-2) at approximately 80% confluence were selected for trypsinization, and stained with trypan blue and their numbers were recorded. The percentage of cells that resisted staining ought to be above 97%. The cells were seeded in 96-well microplates, after the cell concentrations were adjusted to (3¹⁰³ cells / well) in 100 µl RPMI-1640 culture medium and incubated at 37 °C and 5 % CO₂ overnight. The cells were treated with each one of the tested extracts that was dissolved individually in DMSO in seven concentrations (10, 9, 7, 5, 2, 1, and 0.5 µg/ml) and re-incubated for 24h. Cells were washed with sterile phosphate buffer solution (PBS) and then 100 µl of the tetrazolium dye (MTT) (0.5mg/ml) solution was added to each well, and the cells were incubated for an additional 4h. The medium was discarded; 100µl of DMSO was added to dissolve the purple formazan crystals formed. The optical density (OD) of solubilized formazan was measured at 570 nm (reference filter 690 nm) using an automatic microplate reader (Wako, Japan). The results were expressed as percent of cell growth inhibition compared with the control.

To evaluate the cytotoxic and apoptotic effect of the tested extracts, different concentrations of both extracts were used (50, 100, 150, 200, 250, and 300µg/ml). Using Sulphorhodamine B (SRB) method according to Patel et al.,¹⁰ The percentage of growth inhibition was calculated using the following formula and values were plotted by using GraphPad PRISM program (GraphPad, UK). Generated graphs were used to calculate the inhibitory concentration 50 (IC 50).

$$\% \text{ Growth inhibition} = 100 - \left\{ \frac{(At - Ab)}{(Ac - Ab)} \right\} \times 100$$

Where At = mean optical density (OD) of individual test group

Ab = mean OD of blank

Ac = mean OD of control group (untreated cells)

In vivo study**Experimental design**

Sixty adult male *Sprague Dawley* rats weighting 150-160 g were supplied from the Animal House Colony of the National Research Centre, Cairo, Egypt. The acclimatization period takes 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 of water and 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.¹¹ 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.

Rats were classified into 5 groups (12 rats /group). Group (1) assigned as healthy control group received 1 ml of vehicle (Dimethyl sulfoxide DMSO 5% in saline). Groups (2 -5) were intrarectally injected with N-methylnitrosourea in a dose of 2 mg dissolved in 0.5 ml water/rat three times weekly for 5 weeks¹² to induce colorectal cancer . Then, group (3: FU), treated with fluorouracil in which the rats were 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 4 weeks over the duration of the study period (4 months).¹³ Group (4), assigned as *Boswellia serrata* methanolic extract treated group (BME low) in which the rats were orally treated with low dose (137.5 mg/kg) of BME daily for 4 months after colorectal cancer induction. Group (5), assigned as *Boswellia serrata* methanolic extract-treated group (BME high) in which rats were orally treated with high dose (270 mg/kg) of BME daily for 4 months after colorectal cancer induction. The dose of BME were selected according to the chronic toxicity study for this extract (data not shown).

At the end of the experimental period, the rats were fasted overnight and subjected to diethyl ether anesthesia. The blood samples were immediately withdrawn from the retro-orbital venous plexus for biochemical analyses. Each blood sample was collected in two tubes, the first one free from anticoagulant to separate serum and the second tube contained anticoagulant to separate plasma. Then, the rats were sacrificed by cervical dislocation and the colon was dissected carefully cleaned and dried on filter paper, then, it was preserved in formalin saline (10%) for histopathological and immunohistochemical examinations.

Biochemical analyses

Serum cytochrome C (CYT-c) and programmed cell death protein-4 (PDCDP-4) were measured according to the manufacture instructions of Glory Science Co., assay kits, TX, USA. The kits use a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to determine the level of cytochrome C (CYT-c) and PDCDP-4 in serum samples. Plasma transforming growth factor-beta (TGF-β) level was estimated by ELIZA technique using TGF-β assay kit purchased from WKEA MED SUPPLIES Co., New York, USA according to the instructions provided with TGF-β assay kit. Serum



carcinoembryonic antigen (CEA) level was detected by ELIZA technique using CEA assay kit according to the method of Schwartz.¹⁴ Serum matrix metalloproteinase-7 (MMP-7) activity was assayed using MMP-7 assay kit according to the method of Nagase *et al.*¹⁵ Serum colon cancer specific antigen-4 (CCSA-4) level was estimated by enzyme linked immunosorbent assay (ELIZA) technique using CCSA-4 assay kit purchased from Glory Science Co. TX, USA according to the manufacture instructions.

Immunohistochemical examination (IHC)

After fixation of colon tissues in formalin saline (10%) for 24 hours. Sections of fixed colon cancer tissue of rats in the different studied groups were washed in tap water then; serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns by slide microtome. Sections were fixed in 65°C oven for 1 hr. Slides were placed in a coplin jar filled with 200 ml of triology working solution and 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 TBS to adjust the pH, this is repeated between each step of the IHC 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. 2-3 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), and applied, then slides were incubated in the humidity chamber overnight at 4°C. Henceforward, poly HRP enzyme conjugate was applied to each slide for 20 mins. DAB chromogen was prepared and 2-3 drops were applied on each slide for 2 min. DAB was rinsed, after which counterstaining with mayer hematoxylin and cover slipping were performed as the final steps before slides were examined under the light microscope.¹⁶ Image J software (NIH, version v1.45e, USA) was calibrated and the image is opened on the computer screen for image analysis.

Histopathological investigation

Colon tissues were prepared as described above in immunohistochemical analysis. Then, the obtained tissue sections (4 microns) were collected on glass slides, deparaffinized and stained by hematoxylin and eosin stain and examined through the electric light microscope.¹⁷

Statistical Analysis

In the present study, our 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. Difference was considered significant when P value was < 0.05.

RESULTS

In-vitro study

Cytotoxic effect and apoptotic activity of *Boswellia serrata* extracts against human Caco-2 cell line

In this study, the cytotoxicity BME and BMCE against human Caco-2 cell line was carried out using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Results in Table 1 and Figures 1-2 showed the capability of the tested extracts at different concentrations to inhibit human Caco-2 cell line proliferation. Significant (P < 0.05) inhibition of proliferation was recorded on treatment of human Caco-2 cell line with BME at different concentrations after 24h incubation as compared to the untreated control cells. Insignificant inhibition of proliferation was recorded on treatment of human Caco-2 cell line with BMCE at concentration 10, 9, 7, and 5 µg/ml after 24 h incubation as compared to the untreated control cells. However, treatment of human Caco-2 cell line with BMCE at concentration 2, 1 and 0.5 g/ml showed significant inhibition of cells proliferation after 24 h incubation when compared with the untreated control cells.

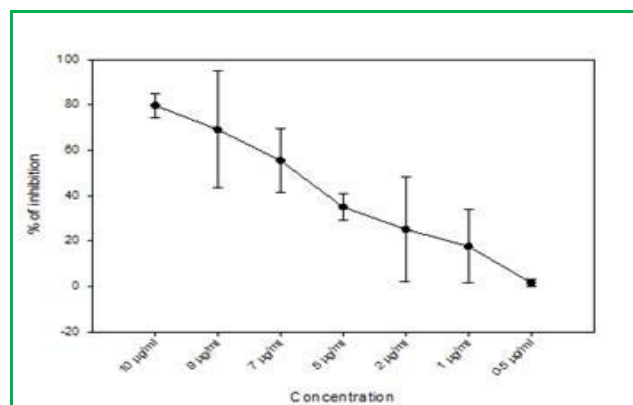


Figure 1: Effect of BME at different concentrations on human Caco-2 cell line proliferation

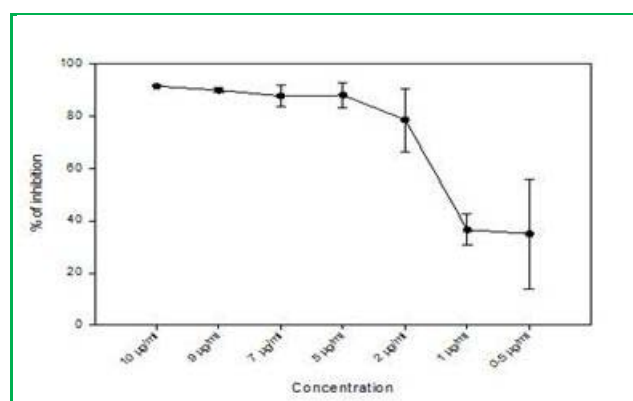


Figure 2: Effect of BMCE at different concentrations on human Caco-2 cell line proliferation

Table 1: Inhibitory effect of *Boswellia serrata* extract at different concentrations on human Caco-2 cell line proliferation

| Ext. Conc. | % of inhibition | | | | | | |
|------------|-------------------------|-------------------------|-------------------------|-------------------------|------------|-------------|-------------|
| | 10 µg/ml | 9 µg/ml | 7 µg/ml | 5 µg/ml | 2 µg/ml | 1 µg/ml | 0.5 µg/ml |
| Control | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| BME | 79.7±5.2* | 69.1±25.79* | 55.4±13.87* | 34.9±5.68* | 25.1±22.9* | 17.7±16.03* | 1.62±1.8* |
| BMCE | 91.2±0.26 ^{NS} | 89.7±0.95 ^{NS} | 87.5±4.08 ^{NS} | 87.8±4.67 ^{NS} | 78.4±12.1* | 36.5±6.02* | 34.9±20.99* |

Data expressed as percent of inhibition ± S.E; *: Significant change at P < 0.05 compared to control group; NS: Insignificant change at P > 0.05 compared to control group.

Table 2: Effect of *Boswellia Serrata* methanolic extract on tumor markers (TGF-β, CEA, CCSA-4, and MMP7) levels in colorectal cancer model.

| Groups | TGF-β pg/ml | CEA ng/ml | CCSA-4 ng/ml | MMP-7 ng/ml |
|--------------------|-----------------------------------|------------------------------------|-------------------------------------|-------------------------------------|
| Control group | 29.33±1.68 | 2.59±0.065 | 69.46±3.43 | 0.18 ±0.015 |
| Cancer group | 42.52±2.13 ^a (45.42 %) | 4.32±0.14 ^a (66.67 %) | 98.98±2.24 ^a (42.49 %) | 0.27 ±0.024 ^a (47.85 %) |
| Fluorouracil group | 30.63±3.61 ^b (-28.2 %) | 2.89±0.12 ^b (-32.93 %) | 70.83±1.51 ^b (-28.43 %) | 0.18±0.013 ^b (-33.02 %) |
| BME (low dose) | 37.74±3.79 (-11.53 %) | 3.09±0.19 ^{bc} (-28.44 %) | 78.67±2.48 ^{bc} (-20.52 %) | 0.198±0.014 ^b (-27.85 %) |
| BME (high dose) | 33.82±3.04 (-20.71 %) | 3.07±0.2 ^{bc} (-28.88 %) | 78.56±1.34 ^{bc} (-20.63 %) | 0.19±0.039 ^b (-28.1 %) |

a: Significance change at P < 0.05 in comparison with -ve control group; b: Significance change at P < 0.05 in comparison with cancer group; c: Significance change at P < 0.05 in comparison with fluorouracil group; (%): percent of difference with respect to the corresponding control value.

Immunohistochemical Results

Figures (3-7) illustrated the immunohistochemical results of COX2 in colon tissue of the different studied groups

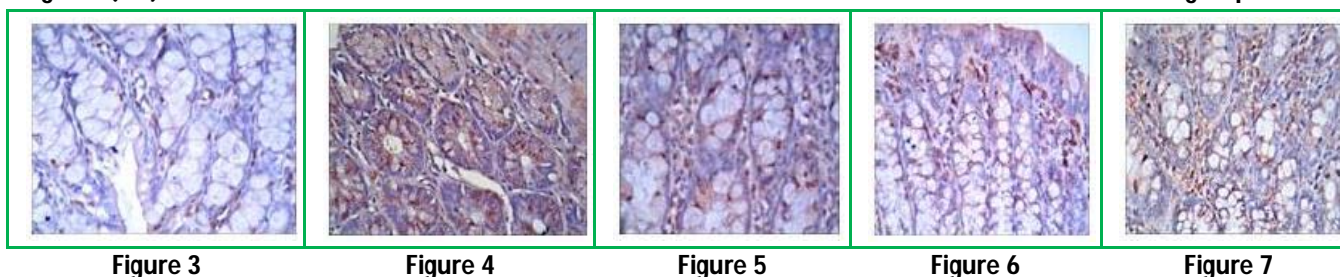


Figure 3: Photomicrograph for immunohistochemical staining of colon tissue of control group using antibody against COX-2 showed mild positive reaction in interstitial stromal cells (160x); **Figure 4:** Photomicrograph for immunohistochemical staining of colon tissue of cancer group using antibody against COX-2 showed very severe positive reaction in cytoplasm of the glandular lining epithelium (160x); **Figure 5:** Photomicrograph for immunohistochemical staining of colon tissue of fluorouracil treated group using antibody against COX-2 showed moderate positive reaction in the nuclei of the glandular lining epithelium (160x); **Figure 6:** Photomicrograph for immunohistochemical staining of colon tissue of BME low dose treated group using antibody against COX-2 showed moderate positive reaction in the lining mucosal epithelium and glandular structure (160x); **Figure 7:** Photomicrograph for immunohistochemical staining of colon tissue of BME high dose treated group using antibody against using antibody against COX-2 showed moderate positive reaction in interstitial stromal cells (160x).

Figures (8-12) illustrated the immunohistochemical results of Cyclin-D in colon tissues of the different studied groups

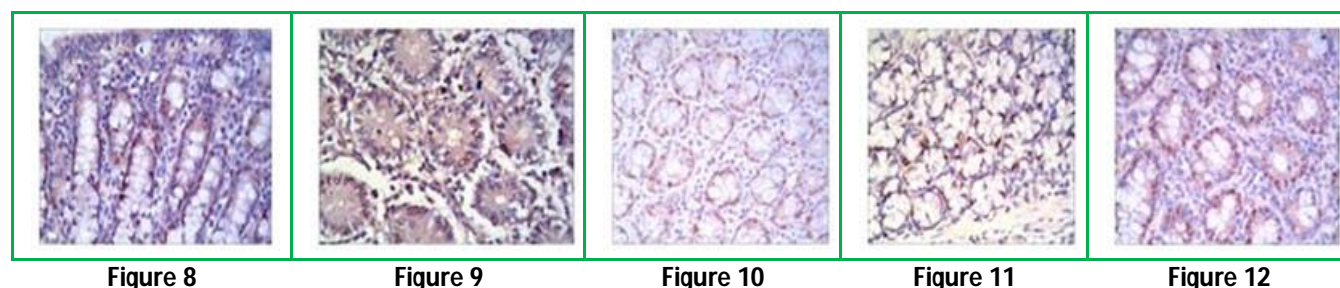


Figure 8: Photomicrograph for immunohistochemical staining of colon tissue of control group using antibody against cyclin D1 showed moderate positive reaction in the nuclei of the glandular lining epithelial cells; **Figure 9:** Photomicrograph for immunohistochemical staining of colon tissue of cancer group using antibody against cyclin D1 showed very severe positive reaction in the nuclei of the glandular lining epithelial cells as well as the interstitial stromal cells (160x); **Figure 10:** Photomicrograph for immunohistochemical staining fluorouracil treated group using antibody against cyclin D1 showed mild positive reaction in the nuclei of the glandular lining epithelial (80x); **Figure 11:** Photomicrograph for immunohistochemical staining of colon tissue of BME low dose treated group using antibody against cyclin D1 showed severe positive reaction in the nuclei of the glandular lining epithelial (160x); **Figure 12:** Photomicrograph for immunohistochemical staining of colon tissue of BME high dose treated group using antibody against cyclin D1 showed severe positive reaction in the nuclei of the glandular lining epithelial (80x).

Figures (13-17) illustrated the immunohistochemical results for Survivin in colon tissues of the different studied groups

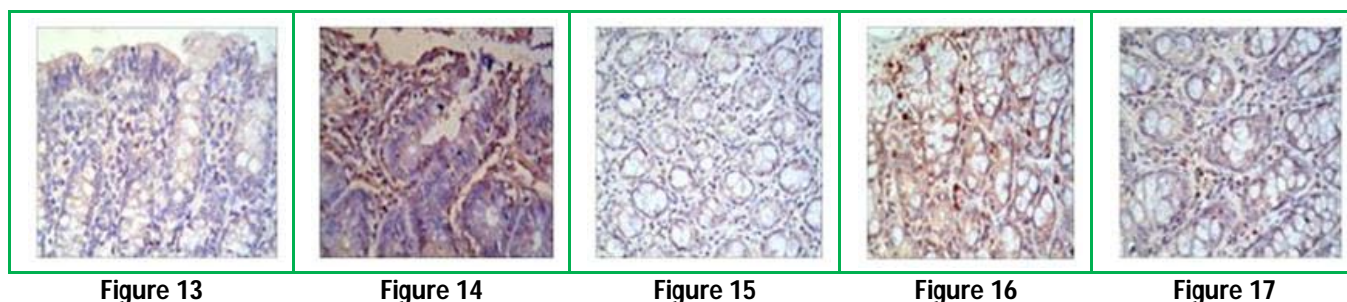


Figure 13: Photomicrograph for immunohistochemical staining of colon tissue of negative control group using antibody against survivin showed mild positive reaction except some few interstitial stromal cell especially in their nuclei (80x); **Figure 14:** Photomicrograph for immunohistochemical staining of colon tissue of cancer group using antibody against survivin showed very severe positive reaction in the nuclei of interstitial stromal cell as well as some nuclei of lining glandular epithelial cells (160x); **Figure 15:** Photomicrograph for immunohistochemical staining of colon tissue- fluorouracil treated group using antibody against survivin showed mild positive reaction in the nuclei of the glandular lining epithelial cells as well as stromal interstitial cells (80x); **Figure 16:** Photomicrograph for immunohistochemical staining of colon tissue of BME low dose group using antibody against survivin showed moderate positive reaction in both interstitial stromal cells and glandular epithelial cells (160x); **Figure 17:** Photomicrograph for immunohistochemical staining of colon tissue of BME high dose group using antibody against survivin showed mild positive reaction in the cells of glandular structure (160x).

Figures (18-22) illustrated the histopathological results of colon tissues in the different studied groups

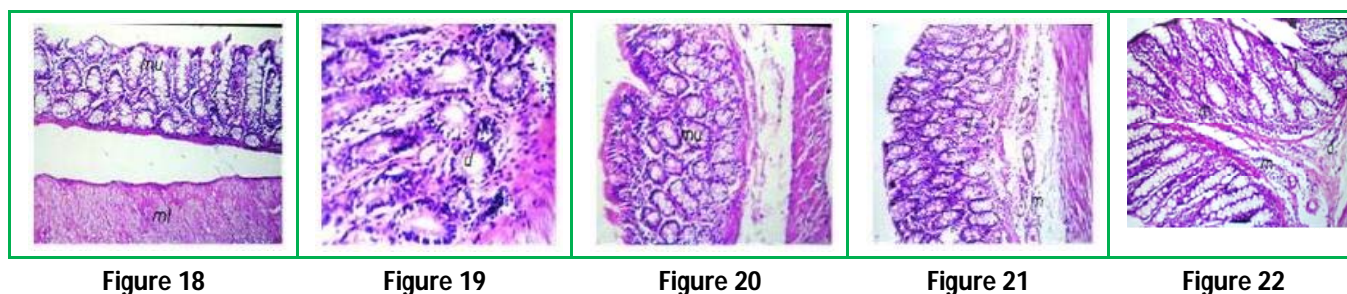


Figure 18: Photomicrograph of colon tissue section of control group showed normal histological structure of the mucosa (mu), submucosa (s) and muscularis (ml) layers. (H &E X40); **Figure 19:** Photomicrograph of colon tissue section of cancer group showed dysplasia and anaplasia associated with pleomorphism and hyperchromachia in the lining epithelial cells of the glandular structure (H &E X64); **Figure 20:** Photomicrograph of colon tissue section of fluorouracil treated group showed few inflammatory cells infiltration in the lamina propria of the mucosa (mu) (H &E X64); **Figure 21:** Photomicrograph of colon tissue section of group treated with low dose of BME showed few inflammatory cells infiltration in the lamina propria (m) and submucosa associated with congestion in blood vessels of submucosa (v) (H &E X40); **Figure 22:** A photomicrograph of colon section of colon cancer- induced rats treated with high dose of BME showed diffuse inflammatory cells infiltration in the lamina propria (m) and sub mucosa with oedema (o) (H &E X40).

The present results revealed that when applying BME or BMCE on Caco-2 cell line with concentration 50 g/ml the IC_{50} was 70 μ g/ml. The treatment of Caco-2 cell line with 50 μ g/ml of BME displayed significant increase in G_0 - G_1 phase (77.84 % vs control 61.88 %) accompanied with a decrease in total S phase (18.64% vs control 31.80%). Thus, *Boswellia serrata* methanolic extract (BME) possesses moderate apoptosis *in vitro*. On the other hand, *Boswellia serrata* methylene chloride extract (BMCE) showed no change in cell cycle.

Biochemical Results

The data presented in table (2) illustrated the effect of *Boswellia serrata* methanolic extract (BME) on the circulating levels of tumor markers in colorectal cancer model. The results revealed significant elevation ($P < 0.05$) in plasma transforming factor –beta (TGF- β) 45.42% and serum carcinoembryonic antigen (CEA) 66.67%, serum colon cancer specific antigen-4 (CCSA-4) 42.49%, and serum metalloproteinase-7 (MMP7) 47.85% levels in cancer group as compared to the control group. FU group showed significant decrease in these parameters as compared to cancer group. The percent of change for

TGF- β was -28.2%, CEA was -32.93%, CCSA-4 was -28.43% and MMP-7 was -33.02% from cancer group (Table 2). Treatment of cancer group with BME low or high dose caused significant decrease ($P < 0.05$) in the above mentioned parameters except for TGF- β which showed insignificant reduction ($P < 0.05$) on treatment with BME low or high dose as compared with cancer group. The percent of change of CEA was (-28.44%) for low dose and (-28.88%) in high dose, the percent of change of CCSA-4 was (-20.52%) for low dose and (-20.63%) for high dose and the percent of change of MMP-7 was (-27.85% and 28.1%) for both low and high dose of BME from cancer group respectively (Table 2).

Table 3 illustrated the effect of the *Boswellia serrata* methanolic extract on serum levels of CYT-C, PDCDP-4, in colorectal cancer model. It is clear from the present data that cancer group showed significant depletion ($P < 0.05$) in CYT-c (-59.3%) and PDCDP-4 (-69.26 %) levels as compared to the control group. While, FU and BME treated groups showed significant elevation ($P < 0.05$) in these indicators compared with cancer group.

Table 3: Effect of *Boswellia serrata* methanolic extract on apoptotic biomarkers (Cyto-c & PDCDP-4) serum levels in colorectal cancer model

| Groups | CYT – C ng/ml | PDCDP-4 ng/ml |
|--------------------|--|--|
| Control group | 4.871 ± 0.069 | 4.493 ± 0.112 |
| Cancer group | 1.981 ± 0.029 ^a (-59.3%) | 1.38 ± 0.125 ^a (-69.26%) |
| Fluorouracil group | 3.454 ± 0.0755 ^b (49.1%) | 3.0191 ± 0.072 ^b (118.76%) |
| BME (low dose) | 3.037 ± 0.193 ^b (53.3%) | 2.583 ± 0.114 ^{bc} (87.17%) |
| BME (high dose) | 3.269 ± 0.35 ^b (75.11%) | 2.232 ± 0.0651 ^{bc} (61.73%) |

a: Significance change at $P < 0.05$ in comparison with -ve control group.

b: Significance change at $P < 0.05$ in comparison with cancer group.

c: Significance change at $P < 0.05$ in comparison with fluorouracil group.

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

Histopathological Results

Histological investigation of colon tissue sections of control group showed normal histological structure of the mucosa, submucosa and muscularis layers (Figure 18). While sections in colon tissue of cancer group showed dysplasia and anaplasia associated with pleomorphism and hyperchromachia in the lining epithelial cells of the glandular structure (Figure 19). Histological investigation of colon tissue sections of fluorouracil treated group showed few inflammatory cells infiltration in the lamina propria of the mucosa with oedema in muscularis (Figure 20). Microscopic examination of colon tissue section of group treated with low dose of BME showed few inflammatory cells infiltration in the lamina propria and submucosa associated with congestion in blood vessels of submucosa (Figure 21). Histological examination of colon tissue section of group treated with high dose of BME showed diffuse inflammatory cells infiltration in the lamina propria and submucosa with oedema (Figure 22).

DISCUSSION

The detected cytotoxic effect of *Boswellia serrata* extracts on Caco-2 cell line is in agreement with that of liu *et al.*¹⁸ and Suhail *et al.*³ This finding could be explained by the growth inhibitory effect and antiproliferation potential of the main active ingredient in *Boswellia serrata* (boswellic acid and its derivatives). The mechanism by which this boswellic acid could be cytotoxic on colon cancer cell line is related to the ability of this acid to inhibit both topoisomerase I and II, increase the intracellular calcium and activate mitogen activated protein (MAP) kinase.¹⁹ Also, the induction of apoptosis and the activation of apoptotic cascade which is associated with the loss of mitochondrial membrane potential and release of cytochrome c in colon cancer cell line have been reported.²⁰ Moreover, The apoptotic effect of boswellic acid may be initiated by a pathway involving the activation of caspase-8, leading to the activation of

caspase-3 and the cleavage of PARP.²¹ Considering the structure of various boswellic acids, the keto group might be important for apoptotic properties of the acids and the presence of acetyl group may strongly enhance its apoptotic effects.²²

In view of our data in *in-vivo* study, biochemical results revealed that there was significant increase in plasma transforming growth factor -beta (TGF- β), serum carcinoembryonic antigen (CEA), colon cancer specific antigen-4 (CCSA-4) and matrix metalloproteinase-7 (MMPs) levels in cancer group. Experimentally, prolonged exposure to high levels of TGF- β promotes neoplastic transformation of intestinal epithelial cells²³ and TGF- β stimulates the proliferation and invasion of poorly differentiated and metastatic colon cancer cells.²⁴

Currently, CEA is used as a tumor marker for the clinical management of colorectal cancer.²⁵ There is increasing evidence that CEA is involved in multiple biological aspects of neoplasia such as cell adhesion, metastasis, suppression of cellular immune mechanisms, and inhibition of apoptosis.²⁶ Aberrant upregulation of CEA and alteration of TGF- β signaling are common features of colorectal cancers. Because both CEA and TGF- β signaling are involved in the development and progression of colorectal tumors, it is known that TGF- β induces CEA secretion in a dose-dependent manner.²⁷

It was suggested by Brunagel *et al.*²⁸ that both CCSA-3 and CCSA-4 are expressed before the onset of cancer and thus may be used as markers of early detection.

Leman *et al.*²⁹ showed that both CCSA-3 and CCSA-4 can be used as highly specific and sensitive serum-based markers for detecting individuals with colon cancer and separating them from those with other benign diseases and cancer types as well as normal individuals.²⁹

Extracellular 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.³⁰ The MMPs are frequently overexpressed in various human cancers. Moreover, expressions of MMPs have been accompanied by an aggressive malignant phenotype and adverse prognosis in cancer patients.³¹ It is noteworthy that only MMP-7 and membrane type-1 MMP (MT1-MMP) are produced by colorectal cancer cells themselves.³² Much evidence supports the role of MMP-7 in tumorigenesis and progression *in vitro*, and in the animal models.

Our biochemical studies showed that fluorouracil significantly decreased plasma TGF- β , and serum CEA, CCSA-4 and MMP-7 levels. It was demonstrated by Wendling *et al.*³³ that fluorouracil antagonizes TGF- β driven COLA2 transcription and associated type I collagen production by dermal fibroblasts. Also, the results are in accordance with Ghiringhelli *et al.*³⁴ In addition, fluorouracil inhibits both SMAD3/4-specific transcription and formation of SMAD/DNA complexes induced by TGF-

β . Fluorouracil was identified as potent inhibitor of TGF- β /SMAD signaling. This explains the significant reduction in TGF- β plasma level in fluorouracil treated group in the present study. Fang *et al.*³⁵ proved that fluorouracil alone, can significantly inhibit HT29 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.

The biochemical analysis of the present study revealed a significant suppression of serum CEA, MMP7, CCSA4 levels and insignificant decrease in plasma TGF- β levels as a result of treatment with *Boswellia serrata* methanolic extract. This finding was supported by the results of Ali and Mansour³⁶ and Saraswati *et al.*³⁷ It is clear that boswellic acid is specific and potent inhibitor of TGF- β signaling *in vivo*. Suppression of leukotriene synthesis *via* inhibiting 5-lipoxygenase is considered the main mechanism underlying boswellic acid as anti-inflammatory agent.³⁶ Also, the study of Yadav *et al.*³⁸ supported our finding as they found that acetyl-keto- β -boswellic acid (AKBA) significantly suppresses NF- κ B activation and matrix metalloproteins. Furthermore, Park *et al.*³⁹ showed that AKBA down regulated the expression of COX-2 and MMP in the tissue of spleen, liver and lungs. These findings suggest that boswellic acid could inhibit the growth and metastasis of colon cancer *in vivo* through down regulations of cancer associated biomarkers.

The current results revealed significant decrease in serum level of CYT-C in cancer group. This finding is greatly supported by the study of Cheng *et al.*⁴⁰ The pivotal role of cytochrome c in apoptosis was confirmed through the identification of its downstream binding partner, Apaf-1 and also through Bcl-2 which inhibits cell death by preventing cytochrome c release from mitochondria.⁴¹ Through activation of a variety of apoptotic stimuli cause cytochrome c release from mitochondria, which in turn induces a series of biochemical reactions that result in caspase activation and subsequent cell death.⁴¹

Programmed cell death protein-4 (PDCDP-4) was first identified as being differently upregulated during apoptosis. Research evidence suggests that PDCDP-4 established as a novel tumor suppressor gene and might represent a promising target for future antineoplastic therapy.⁴² The present study revealed significant decrease in PDCDP-4 serum level in cancer group which is in agreement with Lim and Hong,⁴³ They showed that PDCDP-4 expression is often decreased in progressed carcinomas of the lung, ovary and colon. During the tumorigenesis of colorectal adenocarcinoma, loss of nuclear PDCDP-4 expression occurs and during tumor progression, aberrant cytoplasmic expression is present suggesting a higher clinical stage.⁴³

Treatment of cancer group with fluorouracil resulted in significant increase in serum level of CYT-c and PDCDP-4. Fluorouracil, as well as the nucleoside analog 5-fluoro-2'-deoxyuridine, are part of a class of cytotoxic drugs known as anti-metabolites. The anti-metabolite fluorouracil is

employed clinically to manage solid tumors including colorectal and breast cancer.⁴⁴ Fluorouracil has demonstrated activity against colorectal cancer, leading to apoptosis of neoplastic cells.⁴⁵ Also, fluorouracil exhibited anti-proliferative effects against human colorectal cancer cells.⁴⁶ These result agrees with that of Stevenson *et al.*⁴⁷ who reported that fluorouracil induced loss of mitochondrial membrane potential in colorectal cancer cell lines leading to cytochrome c and smac release from the mitochondria. Thus, fluorouracil could induce apoptosis in colorectal cancer cell lines through the intrinsic mitochondrial pathway.⁴⁷ These properties of fluorouracil might be responsible for its influence in increasing PDCDP-4 serum level in the treated group as shown in the present study.

The apoptotic effect of *Boswellia serrata* extract was clear in increasing serum level of CYT-c and PDCDP-4 level as compared to cancer group. This result may be attributed to the effect of boswellic acid which is in agreement with Liu *et al.*²² who found that boswellic acid has anti proliferative and apoptotic effect on colon cancer cells. Apoptosis occurs *via* a major different activation pathways. One pathway involves changes in mitochondrial transmembrane potential, leading to the release of cytochrome c. Cytochrome c then binds the apoptosis activating factor 1 and procaspase-9, resulting in the activation of caspase-9 by proteolytic cleavage. The other pathway starts with death receptor ligation or Fas/FasL interaction followed by activation of caspase-8.⁴⁸ Also, the released cytochrome c could activate caspase-9 and lead to the activation of caspase-3 leading to apoptotic effect.⁴⁹ Raja *et al.*⁵⁰ demonstrated that the anticancer activity of acetyl-keto- β -boswellic acid (AKBA) is attributed to the inhibition of cell proliferation and induction of apoptosis. AKBA also arrests cancer cells at the G1 phase of cell cycle, suppresses levels of cyclin D1 and E, cdk 2 and 4, and Rb phosphorylation, as well as increases expression of p21 through a p53-independent pathway.¹⁸ AKBA activates death receptor-5 through elevated expression of CATT/enhancer binding protein homologous protein in human prostate cancer LNCaP and PC-3 cells. Moreover, boswellic acids including AKBA strongly induce apoptosis through activation of caspase-3, -8, and -9 and cleavages of PARP in colon cancer HT29 cells and hepatoma HepG2 cells.⁵¹

Our data revealed that there was significant elevation in the expression of COX-2, cyclin D1 and survivin in colon tissues of cancer group. This finding is in consistent with that of Takahashi and Wakabayashi⁵² and in agreement with the finding of Dubois *et al.*⁵³ and Takahashi *et al.*⁵⁴ Also, this finding is in agreement with that in the *in vitro* study of Nie *et al.*⁵⁵ in colon cancer cell line and the study of Mao *et al.*⁵⁶ 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.^{57,58} It has been found that p13/akt promotes cyclin D1, which has been found to be overexpressed in tumor with a k-ras mutation.⁵⁷ Cyclin D1



has been considered to be an oncogene which could regulate progression from the G1 phase of the cell cycle to the S phase.⁵⁹ Overexpression of cyclin D1 protein was also found in colon cancer.^{60,61} The study of Mao *et al.*⁵⁶ 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.⁶² Our finding is in consistent with that of Jin *et al.*⁶³ and Chu *et al.*⁶⁴ who demonstrated that the expression levels of survivin mRNA and protein were higher in colorectal carcinoma cells than in normal cell line.

Treatment with fluorouracil significantly down regulated COX-2, cyclin D1 and survivin expression in colon tissue of cancer group. This results go hand in hand with Srimuangwong *et al.*⁶⁵, Moreover Chow *et al.*⁶⁶ support a potential therapeutic role of fluorouracil as COX-2 inhibitors in human breast cancer.

The decreased expression of cyclin D1 clarified its antitumorigenic activity. This results was previously supported by Wen *et al.*⁶⁷ who found that fluorouracil-triggered apoptosis of DN-HIF-transfected A549 cells and reduced sicyclin D1 (cyclin D1-specific interference RNA) introduction.

The apoptotic effect fluorouracil against colon cancer was clear from survivin downregulation in this study. This result is in agreement with Wei *et al.*⁶⁸ Furthermore Sawai *et al.*⁶⁹ confirmed the invasion-inhibitory effect of fluorouracil on survivin-3B gene-transfected DLD-1 cells. They speculate 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.

In the present study, treatment with *Boswellia serrata* extract resulted in significant reduction in the expression of COX-2, cyclin D1, survivin in colon tissue of cancer group. This finding is in great agreement with that of Yadav *et al.*³⁸ 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.*³⁹ found that boswellic acid derivatives could downregulate 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 could inhibit the growth and metastasis of colorectal carcinoma *in vivo* through downregulation of cancer associated inflammatory markers including COX-2 expression.

Boswellia essential oil has been found to suppress cyclin D1 expression in human breast cancer cell lines.³ 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.²² Recent study of Yadav *et al.*,³⁸ suggest that the boswellic acid analog can inhibit the growth and metastasis of human colorectal carcinoma *in vivo* through downregulation of the expression level of proliferative indicators in colon tumor tissue including (Cyclin D1). Furthermore, our finding is in agreement with that of Yadav *et al.*³⁸ who demonstrate that boswellic acid can suppress the expression of the apoptosis inhibitors (survivin) in colon tissue *in vivo* and by this way boswellic acid could inhibit the growth and metastasis of colorectal carcinoma. It is documented that boswellic acid have antiproliferative and apoptotic effects which was demonstrated by the increase in cytoplasmic DNA-histone complex.²² Considering the structure of various boswellic acids, the keto group might be important for apoptotic properties of the acids and the presence of acetyl group may strongly enhance its apoptotic effects.

The suggested mechanism for the downregulation of survivin expression in colon tissue of the treated rats in our study may be correlated to the activation of caspases as it has been demonstrated that boswellia essential oil could activate caspases cascade in breast cancer cell line.³ By this way boswellic acids and their derivatives trigger apoptosis of cancer cells particularly colon cancer.²²

The histopathological features in cancer group are in consistent with that in the studies of Narisawa *et al.*⁷⁰ Narisawa and Fukaura¹² and Ousingawat *et al.*⁷¹ which confirmed the induction of colon carcinogenesis in rats.

Histological investigation of colon tissue section of cancer group treated with 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 in El-Malt *et al.*⁷² study. The influence of fluorouracil on colonic carcinoma may be attributed to its growth inhibitory effects on cancer cells.⁷³

Treatment of colon cancer-induced rats with either low or high dose of methanol extract of *Baswellia serrata* (BME) showed inflammatory cells infiltration in the lamina propria and submucosa. These findings could be attributed to active constituents of *Baswellia serrata* (boswellic acids). Boswellic acid and its derivatives have been implicated in apoptosis of cancer cells particularly that of brain tumors and colon cancer.⁵⁰ Moreover, Liu *et al.*,²² reported that boswellic acids have anti-inflammatory properties, anti proliferative and apoptotic effects.

In conclusion, the present study highlighted the promising therapeutic role of *Boswellia serrata* methanolic extract against colorectal cancer *in vitro* as well as in experimental model. This was evidenced by the growth inhibitory effect and cytotoxicity activity of the selected extracts on Caco-2 cell line which was documented by the *in vivo* study as indicated by the marked improvement in the measured biochemical and immunohistochemical

markers as well as histological features of colon tissue. These effects were achieved through the powerful anti-inflammatory properties, antiproliferative and apoptotic effects of *Boswellia serrata* extract. These results gave a hope that *Boswellia serrata* eventually could be used for cancer chemoprevention and treatment in the future.

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