

***Curcuma longa* and Colon Cancer: Evidence and Mechanisms**

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Abstract: The present study aims to evaluate the role of *Curcuma longa* (turmeric) methanolic extract against colon cancer- induced in rats. Forty male rats were classified into 5 groups as follows: Group (1) control. Groups from (2) to (5) intrarectally injected with N-methylnitrosourea in a dose of 2 mg dissolved in 0.5 ml water/rat three times weekly for five weeks for induction of colon cancer and group (2) left untreated (cancer group); group (3) intraperitoneally injected with 5-fluorouracil and groups (4 and 5) treated orally with 437.5 mg/kg b.wt and 875 mg/kg b.wt of turmeric extract respectively daily for four months. Histological investigation of colon tissue was done. Colon β -catenin and K-ras genes expression was detected by RT-PCR. Immunohistochemical technique was applied used for estimation of colon COX-2 and survivin expression. Plasma TGF- α , Bcl-2 and serum CEA and CCSA-4 levels were assayed using ELISA procedure. Histopathological investigation of colon tissue sections in cancer group showed dysplasia and anaplasia in the lining epithelial cells of the glandular structure. Additionally, cancer group declared significant increase in the expression levels of β -catenin and K-ras genes in colon tissue. Furthermore, colon cancer induced-rats demonstrated significant increase in colon COX-2 and survivin expression in colon tissue associated with significant elevation in the circulating levels of all studied biochemical markers. In the contrary, treatment with 5-fluorouracil or turmeric extract showed marked improvement in the histological structure of colon tissue. As well as, significant decrease in the expression levels of β -catenin and K-ras genes was detected. Also, all treated groups exhibited marked decrease in COX-2 and survivin expression in colon tissue accompanied with significant reduction in the circulating levels of the all studied biomarkers. Thus, it could be concluded that *Curcuma longa* methanolic extract has a promising therapeutic role against colon cancer induced in rats, as it has anti-inflammatory, anti-proliferation and apoptotic effects.

Key words: Colon cancer • *Curcuma longa* • Anticancer • N- methylnitrosourea • Rat

INTRODUCTION

Colon cancer is a malignant tumor with high morbidity and mortality. It is the fourth most common form of cancer and the third leading cause of cancer-related death worldwide [1]. The occurrence of colon cancer is mainly associated with the incidence of aberrant crypt foci (ACF), an earliest neoplastic lesion, which is a cluster of mucosal cells with an enlarged and thicker layer of epithelia than the surrounding normal crypts that progress into polyps followed by adenomas and adenocarcinomas [2]. Also, these carcinomas are often

accompanied by histological features of inflammation [3]. These sequences of events are considered to be a consequence of the accumulation of multiple genetic alterations in colonic epithelium along the Wnt signaling pathway. Some of the mutations are inherited and others are acquired. The most commonly mutated gene in colon cancer is the tumor suppressor gene, adenomatous polyposis coli (APC), which produces the APC protein. This protein is the brake on the β -catenin protein. Without APC, β -catenin moves into the nucleus, binds to DNA and activates more proteins [4]. The other most proto-oncogenes in colon carcinogenesis are a member of

the RAS family of genes K-ras [5]. Oncogenic mutations of K-ras disrupt the GTPase activity of K-ras and allow it to remain in an activated state [6]. The activated K-ras then interacts with downstream signaling molecules to propagate cell proliferation. K-ras gene mutation and/or protein activation increased COX-2 expression in tumors [7]. Cyclooxygenase-2 (COX-2) is an inducible enzyme produced mainly in inflammatory cells, inflammatory sites and colorectal carcinomas [8]. It was demonstrated the importance of COX-2 in colorectal tumorigenesis and in the development of intestinal neoplasms [9]. COX-2 overexpression enhances neovascularization thus, conferring survival advantage of colon cancer cells [10]. Apoptosis and associated cellular events have profound effect on the progression of benign to malignant phenotype [11]. Survivin is highly expressed in numerous solid tumor types including colon, breast, lung and liver and its expression is associated with aberrant cell survival and tumor progression [12]. Transforming growth factor- β (TGF- β), signaling pathway is involved in the control of several biological processes, including cell proliferation, differentiation, migration and apoptosis [13]. It is one of the most commonly altered cellular signaling pathways in human cancers [14]. Experimentally, prolonged exposure to high levels of TGF- β promotes neoplastic transformation of intestinal epithelial cells [15] and TGF- β stimulates the proliferation and invasion of poorly differentiated and metastatic colon cancer cells [16]. As well as TGF- β signaling promotes angiogenesis and immunosuppression [17]. A growing body of evidence indicates that, natural products can modulate various molecular pathways involved in cancer initiation and progression. It is expected that, studies with natural products will define various molecular mechanisms and targets for tumor growth inhibition, apoptosis and especially angioprevention. To date, chemoprevention clinical trials with natural products conducted in cancer are very limited. Extensive clinical research is warranted to evaluate further safety and chemopreventive efficacy of natural products either alone or in combination with chemotherapeutic agents against cancer [18]. *Curcuma longa* (Turmeric) is a traditional herbal remedy belongs to *Zingiberaceae* family. Curcumin, an orange- yellow major component of turmeric, is a natural polyphenol product isolated from the *Curcuma longa* plant rhizome. Curcumin is used intraditional medicinal preparation and as a food-coloring agent [19]. Curcumin is used in India and China for many diseases and health problems, including biliary disorders, hepatic disorders, diabetic

wounds, respiratory conditions, anorexia, rheumatism, sinusitis, liver disease and cancer [20-22]. Previous studies have reported that, curcumin exerts antiviral [23], antiarthritic [24], anti-amyloid [25], antioxidant [26], anti-obesity [27] and anti-inflammatory properties [28]. Moreover, extensive research has shown that curcumin possesses anti-proliferative [29] and anti-carcinogenic properties in a wide variety of cell lines [30].

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

MATERIALS AND METHODS

Materials

Plant Material: *Curcuma longa* (turmeric) was obtained from the Egyptian Herbal Market, Cairo, Egypt. *Curcuma longa* was precisely identified and differentiated by Oil Crops Department, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt.

Preparation of Turmeric Total Methanolic Extract (TTME):

The methanolic extract of turmeric was prepared by adding 300 ml of methanol (70 %) to 50g of turmeric and left for 10-12 hrs. The extract was filtered using filter paper and the solvent was evaporated using rotary evaporator. The resultant extract was dehydrated in an oven at 50°C for 24 hours [31].

Animals: Forty adult male Sprague-Dawley rats weighing 150-170 g were obtained from the Animal House of National Research Centre, Cairo, Egypt and acclimatized for one week in a specific pathogen free (SPF) barrier area where the temperature (25 \pm 1) and humidity (55%). Rats were controlled constantly with a 12 h light/dark cycle at National Research Centre Animal Facility Breeding Colony. Rats were housed with *ad libitum* access standard laboratory diet consisting of casein 10%, salts mixture 4 %, vitamins mixture 1%, corn oil 10 % and cellulose 5% completed to 100 g with corn starch [32]. 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: Rats were classified into five groups (8 rats/group): (1): Normal healthy animals served as negative control group in which rats received 1 ml of dimethyl sulfoxide (DMSO) 5% in saline. (2): Colon cancer induced group in which rats were intrarectally injected with N-methylnitrosourea in a dose of 2 mg dissolved in 0.5 ml water/rat three times weekly for five weeks [33]. (3): 5-fluorouracil-treated group in which rats were intrarectally injected with N-methylnitrosourea three times weekly for five weeks as in group 2 then intraperitoneally treated with 5-fluorouracil in a dose of 12.5 mg/kg b.wt on days 1, 3 and 5 with the cycle being repeated every four weeks over the duration of the four months [34]. (4): Turmeric total methanolic extract (TTME) treated group in which rats were intrarectally injected with N-methylnitrosourea three times weekly for five weeks as in group 2 & 3, then orally treated with low dose (437.5 mg/kg b.wt) of TTME daily for four months (TTME low dose). (5): Turmeric total methanolic extract treated group in which the rats were intrarectally injected with N-methylnitrosourea for five weeks and then orally treated with high dose (875 mg/kg b.wt) of TTME daily for four months (TTME high dose). The doses of TTME treated groups were selected according to the chronic toxicity study (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 contained anticoagulant agent for separation of plasma samples and the second tube was free from any anticoagulant agent for separation of serum samples for biochemical analysis. 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 analysis and the second portion was collected in liquid nitrogen and stored at - 80°C for molecular genetic analysis.

Methods

Preparation of Colon Tissue Sections for Histological Examination:

After fixation of colon tissue in formalin saline (10%) for 24 hours, one portion of 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 4µm 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 [35].

Molecular Genetics Methods

Expression of β -catenin and K-ras 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 x g 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 µl using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5µg) was used with a reaction mixture, termed as master mix (MM). The MM was consisted of 50 mM MgCl₂, 5x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3; 10 mM of each dNTP, 50 µM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M- MuLV reverse transcriptase. The mixture of each sample was centrifuged for 30 sec at 1000 xg and transferred to the thermocycler (Biometra GmbH, Göttingen, Germany). The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C and the reaction was stopped by heating 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 rats cDNA copy number. PCR reactions were set up in 25 µL reaction mixtures containing 12.5µL 1× SYBR® Premix ExTaq™ (TaKaRa, Biotech. Co. Ltd, Germany), 0.5 µL 0.2 µM sense primers, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water and 5 µL of cDNA template. Each experiment included a distilled water control. Primer sequence for β-catenin, 5'-CAAT GGG TCA TAT CAC AGA TTC TT-3', β-catenin-R, 5'-TCT CTT TTC TTC ACC ACA ACA TTT-3' [36] and for K-ras, 5'- AGT ACG ACC CTA CGA TAG AGG ACT CCT-3' (92 to 118), K-ras-R, 5'- CAA TCT GTA CTG TCG GAT CTC TCT CAC C- 3', (477 to 504) [37]. The semi quantitative values of RT-PCR (sqRT-PCR) of the previous genes were normalized on the expression values of β-actin gene (β-actin-F: 5'- CCC CAT CGA GCA CGG TAT TG -3', β-actin-RATG GCG GGG GTG TTG AAG GTC [38]. 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 [39]:

$$\text{Ef} = 10^{-1/\text{slope}}$$
$$\text{Efficiency (\%)} = (\text{Ef} - 1) \times 100$$

The relative quantification of the target to the reference was determined by using the ΔCT method if E for the target (K-ras and β-catenin) and the reference primers (β-Actin) are the same [39]:

$$\text{Ratio (reference/ target gene)} = \frac{\text{EfCT (reference)}}{\text{CT (target)}}$$

Immunohistochemical Method for Estimation of COX-2 and Survivin: The second portion of fixed colon tissue of rats in the different studied groups was washed in tap water then, subjected to serial dilutions of alcohol (methyl, ethyl and absolute ethyl) for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for 24 hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 µm by 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. 2-3 drops of the rabbit polyclonal primary antibody (COX-2 Cat#RB-9072-R7, ThermoScientific, CA-USA) and (survivin Cat#RB-9245-R7, ThermoScientific, CA-USA) were applied, then the slides were incubated in the humidity chamber for overnight at 4°C. Henceforward, poly horse reddish peroxidase (HRP) enzyme conjugate was applied to each slide for 20 min. 3, 3'- Diaminobenzidine (DAB) chromogen was prepared and 2-3 drops were applied on each slide for 2 min. DAB was rinsed, after which counterstaining 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.

Biochemical Analyses: Plasma transforming growth factor-beta (TGF-β) level was determined by ELIZA technique using TGF-β assay kit purchased from Glory Science Co., Ltd, TX, 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 purchased from Glory Science Co., Ltd, TX, USA according to the instructions provided with CEA assay kit. Serum colon cancer specific antigen-4 (CCSA-4) level was estimated by ELIZA technique using CCSA-4 assay kit purchased from Glory Science Co., Ltd, TX, USA according to the instructions provided with CCSA-4 assay kit. Plasma B-cell lymphoma 2 (Bcl-2) levels was assayed by ELIZA technique using Bcl-2 assay kit purchased from Glory Science Co., Ltd, TX, USA according to the instructions provided with Bcl-2 assay kit.

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 followed by least significant difference (LSD) to compare significance between groups

[40]. Percentage difference representing the percent of variation with respect to the corresponding control group was also calculated using the following formula.

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

Results

Histopathological Investigation: Histological investigation of colon tissue sections of control group showed normal histological structure of the mucosa, submucosa and muscularis layers (Fig. 1A). 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. 1B). 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.1C). Microscopic investigation of colon tissue section of cancer- induced rats treated with low dose of TTME showed normal histological structure of the mucosa with mild oedema in muscularis (Fig. 1D) and

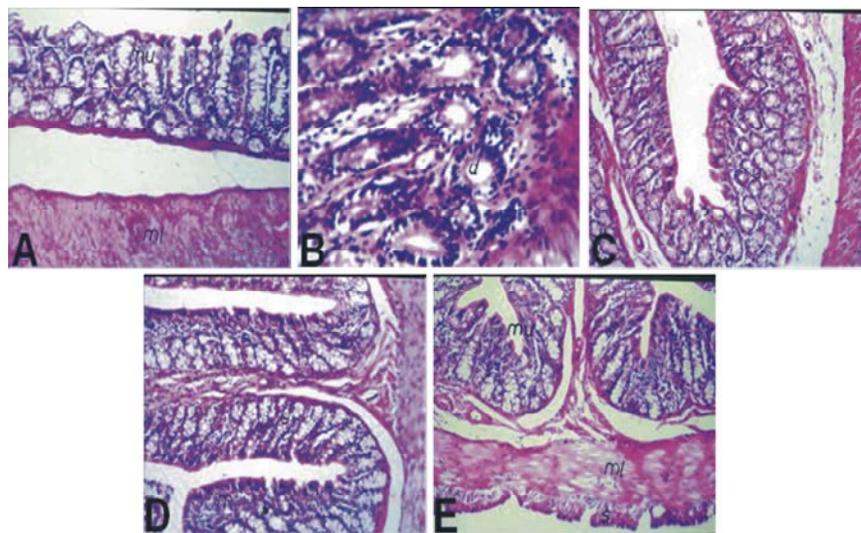


Fig. 1(A): Micrograph of colon tissue section of control group showed normal histological structure of the mucosa (mu), submucosa (s) and muscularis (ml) layers. (H &E X40), (B)Micrograph of colon tissue section of colon cancer- induced group showed dysplasia and anaplasia in the lining epithelial cells of the glandular structure (d) (H&E X64), (C) Micrograph of colon tissue section of colon cancer- induced rats 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 low dose of TTME showed normal histological structure of the mucosa (m) with mild oedema in muscularis (o) (H &E X40) and (E)Micrograph of colon tissue section of colon cancer- induced rats treated with high dose of TTME showed mild oedema in muscularis (ml) and serosa (s) (H &E X40).

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

Groups	β -catenin gene	K-ras gene
Control group	0.33±0.02	0.62±0.02
Cancer group	1.35±0.04 ^a (309%)	1.73±0.04 ^a (179%)
5-fluorouracil group	0.38±0.03 ^b (-71.85%)	0.71±0.02 ^b (-58.95%)
TTME group (437.5 mg/kg b.wt)	0.67±0.03 ^b (-43.70%)	0.88±0.03 ^b (-49.13%)
TTME group (875 mg/kg b.wt)	0.61±0.02 ^b (-54.81%)	0.77±0.02 ^b (-55.49%)

Data are expressed as means \pm 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.

TTME: Turmeric total methanolic extract.

the investigation of colon tissue section of cancer-induced rat treated with high dose of TTME showed mild oedema in muscularis and serosa (Fig. 1E).

Molecular Genetic Analysis: The results of gene expression levels assessment of β -catenin and K-ras in colon tissue of the different studied groups were summarized in (Table 1). Data revealed that β -catenin and K-ras genes were down-regulated in the colon tissue of control group. However, the expression level of β -catenin and K-ras genes were significantly up-regulated in colon cancer- induced group (309 % and 179% respectively), as compared to the control group. In contrast, cancer-induced group treated with 5- fluorouracil exhibited significant down-regulation in the expression levels of β -catenin and K-ras genes (-71.85% and - 58.95%, respectively) as compared to untreated cancer- induced group. Treatment of cancer-induced group with low and/or high dose of TTME significantly normalized the levels of two genes expression. Thus, the expression of β -catenin and K-ras genes revealed significant down-regulation in colon cancer- induced group treated with TTME (-43.70% and -49.13% respectively for low dose and - 54.81% and -55.49%, respectively for high dose), as compared to untreated cancer-induced group. The effect of TTME in amelioration of genetic alteration was dose dependent (Table 1).

Immunohistochemical Results: Photograph for immunohistochemical staining of colon tissue of the control rat using antibody against COX-2 showed mild positive reaction in interstitial stromal cells (Fig. 2A). While, photograph for immunohistochemical staining of colon tissue of cancer- induced rat demonstrated very sever positive reaction in the cytoplasm of the glandular lining epithelium (Fig.2B). Photograph for immunohistochemical staining of colon tissue of cancer- induced rat treated with 5-fluorouracil exhibited moderate positive reaction in the nuclei of the glandular

lining epithelium (Fig. 2C). Moreover, photograph for immunohistochemical staining of colon tissue of cancer- induced rat treated with low dose of TTME declared moderate positive reaction in the nuclei of the glandular lining epithelium (Fig. 2D) and that for cancer- induced rats treated with high dose of TTME, mild positive reaction in the nuclei of the lining epithelium was observed (Fig. 2E). Photograph for immunohistochemical staining of colon tissue of the control rat using antibody against survivin showed mild negative reaction except some few interstitial stromal cell especially in their nuclei (Fig. 3A). While, photograph for immunohistochemical staining of colon tissue of cancer- induced rat showed very sever positive reaction in the nuclei of interstitial stromal cell as well as in some nuclei of the lining glandular epithelial cells (Fig. 3B). Photograph for immunohistochemical staining of colon tissue of cancer- induced rat treated with 5- fluorouracil showed mild positive reaction in the nuclei of the glandular lining epithelial cells as well as in the stromal interstitial cells (Fig. 3C). Photograph for immunohistochemical staining of colon tissue of cancer- induced rat treated with low dose of TTME showed moderate positive reaction in the cells of the glandular structure (Fig. 3D) and that for cancer- induced rat treated with high dose of TTME showed mild positive reaction in the interstitial stromal cells (Fig. 3D).

Biochemical Analyses: Data presented in Table 2 illustrate the effect of treatment with 5-fluorouracil and TTME on plasma TGF- β , serum CEA and serum CCSA-4 levels in colon cancer- induced rats. The results revealed significant elevation in plasma TGF- β (60.99 %), serum CEA (66.79%) and serum CCSA-4 (93.5%) levels in cancer- induced group as compared to the control group. However, treatment of cancer- induced group with 5-fluorouracil showed significant reduction in plasma TGF- β (-35.13 %), serum CEA (-33.10%) and serum CCSA-4 (-47.42%) levels as compared to untreated

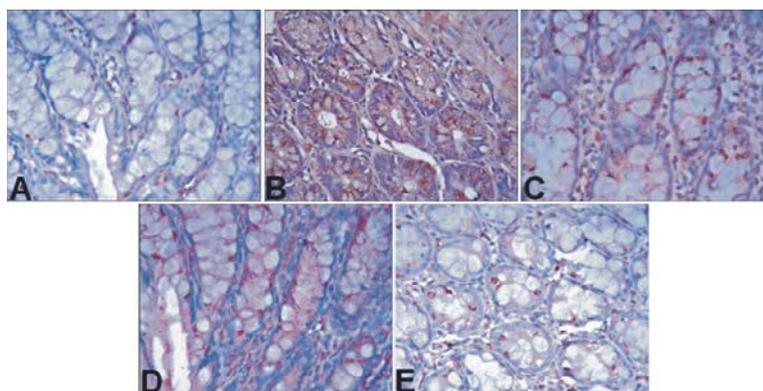


Fig. 2: Photographs for immunohistochemical staining of colon tissue using antibody against COX-2 of (A) Control rat: showed mild positive reaction in interstitial stromal cells (160x), (B) Colon cancer-induced rat showed very severe positive reaction in the cytoplasm of the glandular lining epithelium (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 TTME showed moderate positive reaction in the nuclei of the glandular lining epithelium (160x) and (E) Colon cancer- induced rat treated with high dose of TTME showed mild positive reaction in the nuclei of the lining epithelium (160x).

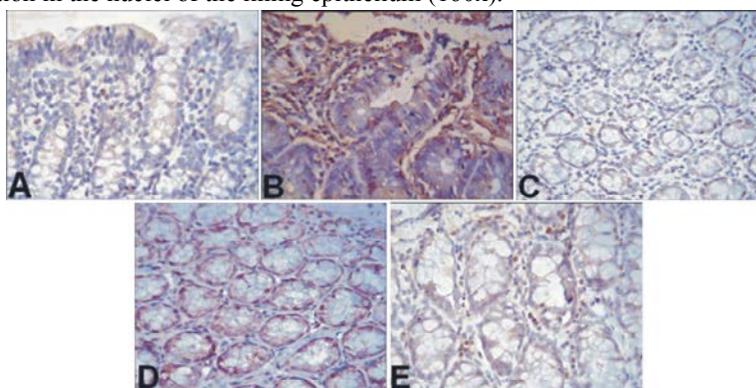


Fig. 3: Photographs for immunohistochemical staining of colon tissue using antibody against survivin of (A) Control rat showed mild negative reaction except some few interstitial stromal cell especially in their nuclei (80x), (B) Colon cancer-induced rat showed very severe positive reaction in the nuclei of the interstitial stromal cell as well as some nuclei of lining glandular epithelial cells (160x), (C) Colon cancer- induced rat treated with 5-fluorouracil showed mild positive reaction in the nuclei of the glandular lining epithelial cells as well as in the stromal interstitial cells (80x), (D) Colon cancer- induced rat treated with low dose of TTME: showed moderate positive reaction in the cells of the glandular structure (80x) and (E) Colon cancer- induced rat treated with high dose of TTME showed mild positive reaction in the interstitial stromal cells (160x).

Table 2: Effect of treatment with 5-fluorouracil and turmeric total methanolic extract (TTME) on plasma TGF- β , serum CEA and CCSA-4 levels in colon cancer-induced group

Groups	TGF- β pg/ml	CEA ng/ml	CCSA-4 g/ml
Control group	29.33 \pm 0.37	2.59 \pm 0.065	1.70 \pm 0.08
Cancer group	47.22 \pm 0.59 ^a (60.99%)	4.32 \pm 0.14 ^a 66.79%	3.29 \pm 0.08 ^a (93.5%)
5- Fluorouracil group	30.63 \pm 0.58 ^b (-35.13 %)	2.89 \pm 0.12 ^b (-33.10%)	1.72 \pm 0.03 ^b (-47.72%)
TTME group (437.5 mg/kg b.wt)	32.27 \pm 0.57 ^b (-31.66%)	2.93 \pm 0.16 ^b (-32.17%)	1.88 \pm 0.13 ^b (-42.85%)
TTME group (875 mg/kg b.wt)	31.44 \pm 0.34 ^b (-33.41 %)	2.91 \pm 0.11 ^b (-32.63%)	1.74 \pm 0.03 ^b (-47.11%)

Data are expressed as means \pm 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.

TTME: Turmeric total methanolic extract.

Table 3: Effect of treatment with 5-fluorouracil and turmeric total methanolic extract (TTME) on plasma Bcl-2 level in colon cancer-induced group

Groups	Bcl-2 ng/ml
Control group	13.4±0.5
Cancer group	23.5±0.6 ^a (75.37%)
5- Fluorouracil group	14±0.4 ^b (-40.42%)
TTME group (437.5 mg/kg b.wt)	15.8±1 ^b (-32.76%)
TTME group (875 mg/kg b.wt)	15±0.8 ^b (-36.61%)

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.

TTME: Turmeric total methanolic extract.

cancer- induced group. Treatment of cancer- induced group with either low or high dose of TTME produced significant depletion in plasma TGF- β level (-31.66%) for TTME low dose and (-33.41%) for TTME high dose, serum CEA level (-32.17%) for TTME low dose and (-32.63 %) for TTME high dose and serum CCSA-4 level (-42.85 %) for TTME low dose and (-47.11 %) for TTME high dose as compared to untreated cancer- induced group. Thus, dose-dependent relationship was noticed for the effect of TTME in modulation of these biomarkers. Data in Table 3 also illustrate the effect of treatment with 5- fluorouracil and TTME on plasma Bcl-2 level in colon cancer- induced group. The results revealed significant elevation in plasma Bcl-2 level in cancer- induced group (75.37%) as compared to the control group. However, treatment of cancer-induced group with 5-fluorouracil showed significant reduction in plasma Bcl-2 level (-40.42%), as compared to cancer-induced group. Also, treatment of cancer- induced group with either low or high dose of TTME showed significant decrease in plasma Bcl-2 level (-32.76 %) for TTME low dose and (-36.61%) for TTME high dose as compared to untreated cancer- induced group.

DISCUSSION

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.* [41], Narisawa and Fukaura [42] and Ousingsawat *et al.* [43] as they 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 obtained by El-Malt *et al.* [44]. The influence of fluorouracil on colonic carcinoma mainly due to its growth inhibitory effects on cancer cells [45]. Histopathological investigation of colon tissue section of colon cancer-induced rats treated with low or high dose of turmeric total methanolic extract (TTME) showed normal histologic structure of the mucosa with mild oedema in the muscularis. The observed improvement in the histological feature of colon tissue due to administration of turmeric could be attributed to its active constituent called curcumin. Curcumin has been shown to suppress multiple signaling cascades of cell proliferation, invasion, metastasis and angiogenesis [46]. Moreover, curcumin can induce apoptosis and cell cycle arrest in colon cells. Doses up to 8000 mg per day of curcumin were well tolerated [47].

The present data revealed significant increase in the gene expression level of β - catenin in colon tissue of cancer- induced rats. This finding runs in parallel with the studies of Takahashi *et al.* [48] and Takahashi and Wakabayashi [49]. β -catenin is a subunit of cadherin protein complex has been implicated as an integral component in the Wnt/signaling pathway. When β - catenin is mutated, β - catenin cannot be degraded but accumulates in the cytoplasm and translocates into the nucleus, where it binds to T- cell factor (TCF) and activates the Wnt target genes [50]. Thus, the gene that codes for β - catenin can function as an oncogen. Mutations in this gene are a cause of colorectal cancer. Also, β - catenin binds to the product of adenomatous polyposis coli (APC) gene, which is mutated in adenomatous polyposis of the colon. Constitutive activation of β - catenin pathway is responsible for the initiation of the vast majority of colon cancers [51]. In all dysplastic colorectal epithelial lesions, β - catenin expression is found to be increased in the cytosol and nucleus. Aberrant expression of β - catenin has been associated with mutations of APC or β - catenin [7]. It has been postulated that, overexpression of β - catenin may be the result of altered expression of one or many proteins with which β - catenin interacts, such as axin, conductin or E- cadherin [52].

Gene expression analysis in the present study showed a significant reduction in β - catenin gene expression level 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 β -catenin [53]. This result needs further investigation.

Treatment of colon cancer-induced rats with turmeric extract led to significant reduction in β -catenin gene expression level in colon tissue as shown in the current data. This result could be attributed to curcumin compound (the most active ingredient of turmeric). Curcumin treatment impairs both Wnt/signaling and cell-cell adhesion pathways, resulting in the cell cycle arrest at G2/M phase and induction of apoptosis in HCT-116 colon cancer cells. Curcumin induced the activation of caspase-3 that, in turn, mediated the cleavage of β -catenin, decreased the transactivation of β -catenin/Tcf-Lef, decreased promoter DNA-binding activity of the β -catenin/Tcf-Lef complex [54]. Mahmoud *et al.* [55], investigated the efficacy of curcumin in tumor prevention in C57BL/6JMin/+ (Min/+) mice and found that curcumin decreases the expression of the oncoprotein β -catenin in the enterocytes of the Min/+ mouse, which reflects its antitumor effect. These animals bear a germ-line mutation in the APC gene and spontaneously develop numerous intestinal adenomas by 15 weeks of age. Curcumin decreased tumor formation in Min/+ mice by over 60%. Tumor prevention by curcumin was associated with increased enterocyte apoptosis [55].

With respect to K-ras gene expression, the current results declared significant elevation in its level in colon 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 [49, 56]. 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, anaplastic crypt foci (ACF), adenocarcinomas and cancers [57]. Functional studies in cell culture [58] and mouse models [59] supported the 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. Akt plays an important role in the PI3K/Akt pathway, which is implicated in a variety of cellular processes, such as glucose metabolism, cell proliferation, apoptosis, transcription and cell migration [60]. 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 of AKT/NF-KappaB signaling in MCS colon cancer

carcinoma cells *in vitro* [61]. *Curcuma longa* (turmeric) extract could down-regulate the gene expression level of K-ras in colon tissue of colon cancer- induced rats in our study. Curcumin appears to interact with all of the key pathways associated with the adenoma-adenocarcinoma sequence including APC, TP53, K-ras and c-myc [62]. *In vivo* study suggested that, curcumin can prevent disease propagation by modulating cellular mechanisms involved in proliferation, angiogenesis and metastasis [63]. The speculated mechanism for the inhibition of k-ras gene expression level post treatment of colon cancer rats with turmeric is the ability of curcumin to suppress the activation of Akt [64, 65].

The present data demonstrated 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 to 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. Research evidence has indicated the critical role of COX-2 in tumor promotion and carcinogenesis. Our finding is in consistent with the finding of Takahashi *et al.* [48], Takahashi and Wakabayashi [49] and Dubois *et al.* [66], they 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 colon tumors [7]. Our results showed the treatment with 5- fluorouracilin colon cancer- induced rats significantly down regulates COX-2 expression in colon tissue. This result go hand in hand with that of Srimuangwong *et al.* [67], who demonstrated that 5-fluorouracil at concentration of 5 μ mol/L in combination with hexahydrocurcumin (HHC) at concentration of 25 μ mol/L significantly down-regulate COX-2 expression in HT-29 human colon cancer cells. Moreover Chow *et al.* [68] support a potential therapeutic role of 5-fluorouracil as COX-2 inhibitors in human breast cancer.

Treatment with *Curcuma longa* (Turmeric) extract in colon cancer-induced rats resulted in significant depletion of COX-2 expression in colon tissue. Curcumin has the ability to inhibit COX-2, but not COX-1 gene expression in colon carcinogenesis [69]. This effect could be due to the inhibition of I (kappa) B kinase (IKK) signaling complex

that is responsible for the phosphorylation of I (κ)B (I κ B) and the hindering of the nuclear translocation of the functionally active subunit of NF- κ B, thereby blocking improper activation of NF- κ B [70]. In human colon epithelial cells, curcumin has shown to inhibit COX-2 initiation by the colon tumor promoters TNF through the inhibition of NF- κ B [71]. Furthermore, a growing body of evidence indicated that curcumin suppressed the chenodeoxycholate-(CDC) or phorbol ester 12-O-tetradecanoylphorbol-13-acetate-(TPA) mediated induction of COX-2 protein and inhibited the synthesis of prostaglandin E2 in a number of gastrointestinal cell lines [65]. Dietary agents such as curcumin can interfere with the non-receptor tyrosine kinases such as Src and FAK, thereby inhibiting the downstream PI-3 kinase signaling responsible for the induction of angiogenic target genes as COX-2 [72] and in turn its expression in the target tissue.

Our results indicated that, survivin expression is significantly increased in colon tissue of colon cancer-induced rats. 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 [73]. Our finding is in agreement with that of Jin *et al.* [74] and Chu *et al.* [75], who demonstrated that the expression levels of survivin mRNA and protein were higher in colorectal carcinoma cells than in normal cell line. Immunohistochemical results of the present study showed a decrease in the expression level of survivin in colon tissue of colon cancer-induced rats treated with 5-fluorouracil which may indicate the apoptotic effect of 5-fluorouracil against colon cancer. This result is in accordance with Wei *et al.* [76]. Furthermore, Sawai *et al.* [77] confirmed the invasion inhibitory effect of 5-fluorouracil on survivin-3B gene-transfected DLD-1 cells that showed significant lower survivin expression level than their empty vector gene-transfected counterparts. These authors speculated that, survivin-3B expression in colon cancer is an important factor involved in the invasive capacity of cancer cells in the presence of anticancer drug. *Curcuma longa* (Turmeric) extract has been shown to suppress the expression of survivin in colon tissue of the treated colon cancer-induced rats. The ability of curcumin to induce apoptosis selectively in cancerous and transformed cells contributes to its anticancer potential. Curcumin has been reported to efficiently induce apoptosis in various cell lines, including HL-60, K562, MCF-7 and HeLa [78]. One of the major signaling pathways involved in apoptotic cell death

includes the intracellular caspases, a family of structurally related cysteine proteases [79]. Caspase activity is responsible, either directly or indirectly, for the proteolytic cleavage of certain cellular proteins, which is characteristic of apoptotic cell death. Curcumin was shown to activate caspases 9, 3 and 8 in the colon cancer cell lines SW480 and SW620 [80]. The mechanisms responsible for apoptosis induction by curcumin include the release of cytochrome-c and modulation of cell survival and death signaling pathways involving Akt, NF- κ B, AP-1, or JNK and down-regulation of the expression of survival genes and inhibitor of apoptosis (IAP) [81].

The results in current study revealed that there was significant increase in plasma transforming growth factor- β (TGF- β), serum carcinoembryonic antigen (CEA) and serum colon cancer specific antigen-4 (CCSA-4) levels in cancer-induced group. High levels of TGF- β 1 in the primary colorectal tumor are associated with advanced stages and a greater likelihood of recurrence and decreased survival [82]. Experimentally, prolonged exposure to high levels of TGF- β promotes neoplastic transformation of intestinal epithelial cells [15] and TGF- β stimulates the proliferation and invasion of poorly differentiated and metastatic colon cancer cells [16]. Carcinoembryonic antigen (CEA) is a membrane-bound glycoprotein expressed by cancerous and to some extent by normal epithelial cells of gastrointestinal tract. Currently, it is used as a tumor marker for the clinical management of colorectal cancer [83]. Elevated blood levels of CEA indicate metastasis and poor prognosis [84]. 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 [85]. CEA increases the ability of weakly metastatic colorectal cancer to colonize in the liver and develop spontaneous hematogenous liver and lung metastasis [86]. Four mechanisms have been proposed for the release of membrane associated glycoproteins *in vivo*, including cell lysis, shedding of plasma membrane vesicles, cleavage by proteases and release due to the action of phospholipases [87, 88]. Aberrant up-regulation of CEA and alteration of TGF- β signaling are common features of colorectal cancers. Because of 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 [89]. Also, CEA and CEACAM6 are identified as target genes for Smad3-mediated TGF- β signaling [90].

Colon cancer-specific antigen CCSA-4 is a novel colon cancer markers identified by focused proteomic analysis of nuclear structural proteins. Analyses on colon polyps and cancer tissues revealed that CCSA-3 and CCSA-4 were present in 83% to 100% of advanced adenomas and in all colon cancer specimens [91]. These results suggested that both CCSA-3 and CCSA-4 are expressed before the onset of cancer and thus may useful as markers of early detection. Leman *et al.* [92] 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. The mechanisms by which CCSA-3 and CCSA-4 are found in the serum are under study.

Our results showed that 5-fluorouracil significantly decreased plasma TGF- β , serum CEA and serum CCSA-4 levels in colon cancer- induced rats. Wendling *et al.* [93] demonstrated that 5-fluorouracil antagonizes TGF- β driven COLA2 transcription and associated type I collagen production by dermal fibroblasts via inhibition of SMAD3/4-specific transcription and formation of SMAD/DNA complexes induced by TGF- β . Thus, 5-fluorouracil was identified as a potent inhibitor of TGF- β /SMAD signaling. Concerning the inhibitory effect of 5-fluorouracil on serum CEA level, our finding is in accordance with Ghiringhelli *et al.* [94]. However, Aldulaymi *et al.* [95] showed stable plasma CEA level during adjuvant chemotherapy. With respect to the reducing action of 5-fluorouracil on serum CCSA-4 level, our results could be explained as 5-fluorouracil has been reported to be the single most effective chemotherapeutic agent for the treatment CRC [96]. The mechanism by which 5- fluorouracil could act as effective chemotherapy against colon cancer is well understood. 5- Fluorouracil inhibits cell proliferation by: (a) forming FdUMP, which in turn blocks TS, the enzyme that catalyzed novo synthesis of the DNA precursor thymidylate (i.e., TMP); (b) forming defective, F-RNA, which ultimately interferes with protein synthesis; and (c) forming defective, fluorinated DNA, which results in single-strand breaks and DNA fragmentation [97,98,99]. Treatment with *Curcuma longa* (Turmeric) extract in colon cancer-induced rats significantly represses the plasma level of TGF- β , serum CEA and serum CCSA-4 levels as shown in our results. Regarding to, the inhibitory effect of turmeric extract on plasma TGF- β in the treated rats, the present finding is in agreement with the study of Awad and Sharif [100] and Song *et al.* [101]. Multiple evidences have indicated that curcumin might be an effective blocker of TGF- β signaling

in a dose dependent manner. Curcumin could also block the proliferatic actions of TGF- β through down-regulation of TGF- β RII and the inhibition of c-jun activity [101]. Concerning the inhibitory effect of turmeric extract on colon cancer biomarkers (CEA and CCSA- 4) levels, our results were strongly supported by the present molecular, immunohistochemical and histopathological data which revealed the strong anti-inflammatory and anti-tumorigenic effect of turmeric extract against colon cancer- induced in rats.

The present results indicated that plasma Bcl-2 level is significantly increased in colon cancer- induced group. The key of biochemical events involved in the apoptotic process was the upregulation of pro-apoptotic proteins and/or the down-regulation of anti-apoptotic protein molecules. Among these proteins, Bcl-2 family of proteins, including Bcl-2, Bcl-xL, Bim and Bax proteins, serve as critical regulators of the mitochondrial pathway involved in apoptosis, acting to either inhibit or promote the cell death. The Bcl-2 proteins have been identified as anti-apoptotic proteins, which bind to the outer membrane of the mitochondria and prevent the release of cytochrome c. Bax and Bim are thought to be pro-apoptotic effect or proteins and are responsible for permeabilizing the membrane due to damaging cellular stress. While, Bad and Bik can bind and regulate the anti-apoptotic Bcl-2 proteins to promote apoptosis [102]. Our finding agrees with that of Sun *et al.* [103] who found high expression levels of the anti-apoptotic genes Bag-1 and Bcl-2 in colon cancer. It is clear from the present results that 5-fluorouracil could decrease Bcl-2 plasma level clarifying its apoptotic effect against N-methylnitrosourea- induced colon cancer. This result was greatly supported by He *et al.* [104] who found that 5- fluorouracil induces apoptosis in colon cancer cells through the regulation of the Bcl-2/Bax protein ratio.

In present study we observed that *Curcuma longa* (Turmeric) extract down-regulates the plasma level of Bcl-2 in the treated colon cancer-induced rats. Bcl-2 is a key anti-apoptotic molecule and it has been suggested that curcumin-induced tumor cell apoptosis through a Bcl-2-associated mechanism. For example, curcumin-induced down-regulation of Bcl-2 expression in the tumor cells of the head, neck, lung and prostate [105-107]. Furthermore overexpression of Bcl-2 has been demonstrated to be completely blocked by curcumin which induce apoptotic pathway involving cytochrome c release and activation of caspases 8 and 3 in human myeloid leukemic cell line, HL-60 [108]. Also, Yu and Shah

[109] observed that curcumin down-regulates the basal level of Bcl-2 expression in a time- and dose- dependent manner in HEC-1 cells supporting a Bcl-2-associated mechanism as the anti-tumor activity of curcumin.

CONCLUSION

In conclusion, based on our results, we could be concluded that *curcuma longa* has a promising therapeutic role against colon cancer induced by N-methylnitrosourea as indicated by the observed improvement in the measured histological, molecular, immunohistochemical and biochemical markers. These effects were achieved through the powerful anti-inflammatory properties, antiproliferative and apoptotic effects of the active ingredient (curcumin) of this plant. These results represented good therapeutic approaches for intervention against progressive of colon cancer with special reference to the inflammation, proliferation and apoptosis.

ACKNOWLEDGMENTS

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

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