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Research Article

PROMISING THERAPEUTIC ROLE OF *ROSMARINUS OFFICINALIS* SUCCESSIVE METHANOLIC FRACTION AGAINST COLORECTAL CANCER

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ABSTRACT

Objective: Colorectal cancer is one of the leading causes of death in the world. Plant-derived products have proven to be valuable sources for discovery and development of unique anticancer drugs.

Methods: In this study, the possible therapeutic effect of rosemary (*Rosmarinus officinalis L.*) successive methanolic fraction on colorectal cancer induced in experimental animals was studied through several biochemical and molecular genetics analyses as well as histological investigation of colon tissue.

Results: The biochemical results revealed significant elevation in serum colon cancer specific antigen-4 (CCSA-4) and carcinoembryonic antigen (CEA) in colorectal cancer induced group while programmed cell death protein-4 (PDCDP-4) and cytochrome C (CYT-C) showed significant depletion as compared to the control group. Molecular genetics results indicated that colorectal cancer induced group exhibited significant up regulation in the expression level of β-catenin, K-ras and c-myc genes in colon tissue. On the other hand, treatment with rosemary successive methanolic fraction (RSMF) could ameliorate the biochemical markers and down regulate β-catenin, K-ras and c-myc gene expression levels in colon tissue of the treated groups compared with untreated cancer induced group. These findings were greatly supported by histopathological results.

Conclusion: Thus, it could be concluded that rosemary (*Rosmarinus officinalis L.*) has a promising therapeutic effect against colorectal cancer induced in experimental animals through its apoptotic, anti-inflammatory, and antiproliferative effects.

Keywords: Rosemary, Colorectal cancer, Apoptosis, Inflammation, Proliferation, Rats.

INTRODUCTION

Colorectal cancer (CRC) is the second most fatal and the third most diagnosed type of cancer worldwide. Despite having multifactorial causes, most CRC cases are mainly determined by dietary factors [1]. It is well known that ingestion of a diet rich in calories and lipids , red meats, N-nitroso compounds, and aromatic polycyclic hydrocarbons, administration of high amounts of ethanol and certain diseases could raise the incidence risk of CRC [2].The etiology of CRC may be hereditary or sporadic. Chronic inflammatory bowel disease is considered as an etiologic factor in the development of CRC because high oxidative stress burden present in the inflamed mucosa alters the important cellular functions [3]. 5-fluorouracil (fluorouracil) has been the major chemotherapeutic agent for the treatment of colorectal carcinoma for the past four decades. This regimen is noncurative, and its impact on survival is unclear [4].

The relationship between the more than 8000 polyphenols present in the diet and the prevention of diseases in humans has been an intense field of research during the last years. One of the reasons for the growing interest in studying these compounds resides in their protective role against colorectal cancer [5].

Rosemary (*Rosmarinus officinalis L.*) is a common household plant grown in many parts of the world. Rosemary leaves are often used as spices and flavoring agents. Because of the desirable flavor and antioxidant activity of the dried leaves of the plant, they are widely used in food processing, including the preparations of meats, sausages, soups, salads or potato chips. There is also an increasing interest in the pharmaceutical properties of rosemary, being used in traditional medicine to improve memory and relieve pain, or for its antimicrobial, hepatoprotective , and anti-inflammatory, antitumorigenic or chemopreventive activity [6]. However, the literature concerning the mechanisms of the rosemary constituents in managing cancer is still limited. Carnosic acid (CA) and carnosol (CS) are the major phenolic constituents in fresh, and dried leaves from the plant, and they are believed to be mainly responsible for their antioxidant, anti-inflammatory and cytotoxic properties [7]. Carnosic acid is the major phenolic diterpene derived from rosemary and has been reported to have antioxidant antimicrobial , anti obesity anti platelet and antitumor activities as well as inhibitory effects on the anticancer drug efflux transporter P-glycoprotein [8]. Carnosic acid can be converted to carnosol (CAL) and rosmanol by oxidative dehydrogenation processes. It has been shown that rosmanol can inhibit the oxidation of low density lipoprotein (LDL) *in vitro* [9]. Lai *et al,* 2009 [10]. has found that rosmanol can significantly inhibit lipopolysaccharide induced iNOS and Cox-2 expression through down regulating MAPK, NF-κB, STAT3, and C/EBP signaling pathways and thereby exhibits anti-inflammatory effect [10]. Thus, the principal goal of this study was to investigate the possible biochemical and molecular mechanisms by which rosemary might display antitumor effect against colorectal carcinoma.

MATERIALS AND METHODS

Preparation of *Romarinus officinalis* (Rosemary) Successive Methanolic Fraction (RMSMF)

The successive methanol fraction of rosemary was prepared by adding 300 ml of methanol (70 %) to 50 g of rosemary after applying methylene chloride (300 ml for 50 g powdered rosemary for 6 hours) and left for 10-12 hrs. The fraction was filtered using filter paper and the solvent was evaporated using rotary evaporator. The resultant extract was dehydrated in an oven at 50 ^{o}C for 24 hours [11].

Experimental Deign

Sixty adult male Sprague-Dawley rats weighting 150-160 g were obtained from the Animal House Colony of the National Research Centre, Cairo, Egypt and acclimated for one week in a specific pathogen free (SPF) barrier area where the temperature (25±1) and humidity (55%). Rats were controlled constantly with a 12 h light/dark cycle at National Research Centre Animal Facility Breeding Colony. Rats were housed with *ad libitum* access standard laboratory diet consisting of casein 10%, salts mixture 4 %,vitamins mixture 1%,corn oil 10 % and cellulose 5% completed to 100 g with corn starch [12]. 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.

After the acclimatization period, the rats in the current study 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 weeks [13] 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) [14]. Group (4), assigned as rosemary successive methanolic fraction-treated group (RMSMF low) in which the rats were orally treated with low dose (1666.6 mg/kg/wt) of RMSMF daily for 4 months after colorectal cancer induction. Group (5), assigned as rosemary successive methanolic fraction-treated group (RMSMF high) in which rats were orally treated with high dose (3333.3 mg/kg/wt) of RMSMF daily for 4 months after colorectal cancer induction.

At the end of the experimental period, the rats were fasted overnight and subjected to diethyl ether anesthesia. The blood samples were immediately collected from the retro-orbital venous plexus in tube free from any anticoagulant agent for separation of serum samples for biochemical analysis. Then the rats were sacrificed by cervical dislocation and the colon was dissected and divided into two portions , the first portion was preserved in formalin saline (10%) for histological investigation and the second portion was collected in liquid nitrogen and stored at -80° C for molecular genetic analysis.

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 immuno asorbent assay (ELISA) to assay the level of cytochrome C (CYT-C) and PCDP-4 in serum samples. Serum carcinoembryonic antigen (CEA) level was detected by ELIZA technique using CEA assay kit purchased from Bio Check, Inc Co., Foster city, Canada according to the method of Schwartz, (1987) [15]. Serum colon cancer specific antigen-4 (CCSA-4) level was estimated by enzyme linked immuno absorbent assay (ELIZA) technique using CCSA-4 assay kit purchased from Glory Science Co., Ltd, TX, and USA according to the manufacture instructions.

4-Molecular genetic analysis

Expression of β-catenin, K-ras and c-myc genes

Isolation of total RNA

Total RNA was isolated from colon tissue of male rats by the standard TRIzol® reagent extraction method (cat#15596-026, Invitrogen, Germany). Total RNA was treated with 1 U of RQ1 RNAase-free DNAase (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 male rats colon tissue was reverse transcribed into cDNA in a total volume of 20 µl using RevertAidTM first strand cDNA synthesis kit (MBI Fermentas, Germany). An amount of total RNA (5µg) was used with a reaction mixture, termed as master mix (MM). The MM was consisted of 50 mM MgCl2, 5x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3; 10 mM of each dNTP, 50 µM oligo-dT primer, 20 U ribo nuclease inhibitor (50 kDa recombinant enzyme to inhibit RNAase activity) and 50 U M- MuLV reverse transcriptase. The mixture of each sample was centrifuged for 30 sec at 1000 g and transferred to the thermo cycler (Biometra GmbH, Göttingen, Germany). The RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and the reaction was stopped by heating for 5 min at 99 °C. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through semi-quantitative real time-polymerase chain reaction (sqRT-PCR).

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

An iQ5-BIO-RAD Cycler (Cepheid. USA) was used to determine the rat's cDNA copy number. PCR reactions were set up in 25 µL reaction mixtures containing 12.5µL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µL 0.2 µM sense primer, 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 sequences for β-catenin: (5'-CAAT GGG TCA TAT CAC AGA TTC TT-3') and (5'-TCT CTT TTC TTC ACC ACA ACA TTT-3') [16], for K-ras: (5′- AGT ACG ACC CTA CGA TAG AGG ACT CCT-3′) and (5′ - CAA TCT GTA CTG TCG GAT CTC TCT CAC C - 3′) [17] , and for c-myc (GenBank accession number Z38066) were upstream: (5′-TGA CGA GAC CTT CGT GAA GA-3′) and downstream: (5′-ATT GAT GTT ATT TAC ACT TAA GGG T-3′) [18]. The semi quantitative values of RT-PCR (sqRT-PCR) of the previous genes were normalized on the expression values of ß-actin gene (ß-actin-F: 5′- CCC CAT CGA GCA CGG TAT TG -3′, ß-actin-R ATG GCG GGG GTG TTG AAG GTC) [19]. 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.

Histopathological examination of colon tissue

After fixation of colon tissues in formalin saline (10%) for 24 hours, the colon tissues 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 sledge 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 [20].

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

The biochemical results of the present study revealed that serum levels of CYT-C and PDCDP-4 showed significant decrease in colorectal cancer group as compared to the control group. While fluorouracil treated group showed significant increase in both CYT-C and PDCDP-4 levels as compared to colorectal cancer group. Treatment with low or high dose of RMSMF produced significant increase in both parameters as compared to cancer induced group. There was no significant difference between the two doses of RMSMF (Table 1).

Also, the results of the current study showed significant elevation in the level of CEA and CCSA-4, in colorectal cancer-induced group as compared to the control group ($p < 0.05$). Fluorouracil significantly decreased these parameters in the treated groups as compared to colorectal cancer group. RMSMF treated group With low or high dose showed significant decrease in CEA and CCSA-4 levels ($P \leq 0.05$) as compared with colorectal cancer –induced group. Herein, the effect of rosemary was dose dependent (Table 1).

Furthermore, the results of the current study showed that the expression level of β-catenin, K-ras and c-myc genes were significantly unregulated in colon tissue of rats administered with N-methylnitrosourea induced colorectal cancer compared to the control group. On the other hand, β-catenin, K-ras and c-myc genes were down regulated in colon tissue of rats treated with fluorouracil compared with colorectal cancer group. Treatment with RMSMF was effective in down regulation of the expression levels of β-catenin, K-ras and c-myc genes significantly compared with colorectal cancer-induced group. Both doses were similarly effective (Fig. 1).

Table 1: Effect of rosemary successive methanolic fraction on biochemical parameters (CYT-C, PDCDP-4, CEA, and CCSA-4) serum levels in the different studied groups

Groups	CYT - Cng/ml	PDCDP-4ng/ml	CEAng/ml	CCSA-4 ng/ml
Control group	4.887±.000552[a]	4.493 ± 0.134[a]	2.59±0.065	69.46±3.43
Cancer group	2.025 ± 0.00062	1.38 ± 0.12	4.32±0.14[a]	98.98±2.24[a]
Fluorouracil group	3.849±.00696[bc]	3.05± 0.256[bc]	2.89±0.12[b]	70.83±1.51[b]
RMSMF group (low dose)	2.997±.0007[b]	2.441± 0.141[b]	3.36±0.25[b]	83.34±3.21[bc]
RMSMF group (high dose)	3.161±.128[b]	2.709 ± 0.1119[b]	3.34±0.25[b]	82.51±0.65[bc]

Data are expressed as means ± standard error (SE)

a: Significance change at P < 0.05 in comparison with the 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.

CYT-C: Cytochrome C; PDCDP-4: Programmed cell death protein-4; CEA: Carcinoembryonic antigen; CCSA-4: Colon cancer specific antigen-4

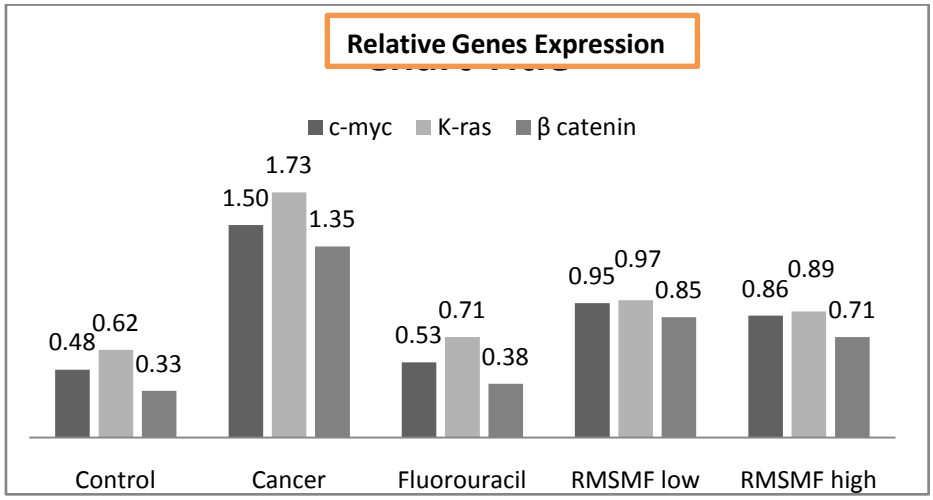

Fig. 1: Effect of rosemary successive methanolic fraction on the expression level of β-catenin, K-ras and c-myc genes in colon tissue of the different studied groups.

Histological investigation of colon tissue sections of control group showed normal histological structure of the mucosa, sub mucosa and muscularis layers (Fig. 2). While sections in colon tissue of colorectal cancer- induced group showed dysplasia and anaplasis associated with pleomorphism and hyperchromachia in the lining epithelial cells of the glandular structure (Fig. 3). Colorectal cancer- induced group treated with fluorouracil showed few inflammatory cells infiltration in the lamina propria of the mucosa with edema in muscularis (Figs 4). Microscopic examination of colon tissue section of colorectal cancer-induced group treated with low dose of RMSMF showed lymphoid cells aggregation in sub mucosa (Fig. 5). While, colon tissue section of colorectal cancer–induced group treated with high dose of RMSMF showed lymphoid cells aggregation (Fig. 6).

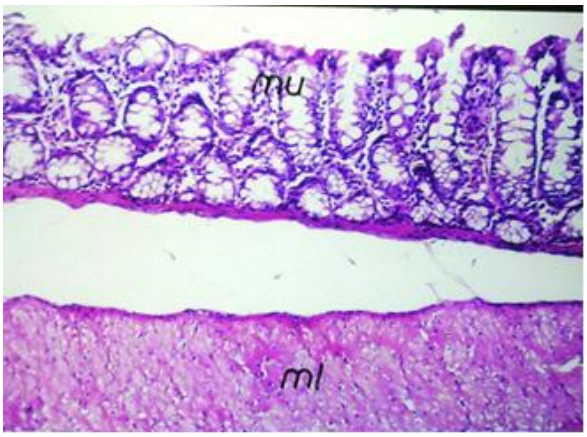

Fig. 2 :A photomicrograph of colon tissue section of control rat showed normal histological structure of the mucosa (mu), submucosa (s) and muscularis (ml) layers. (H &E X40).

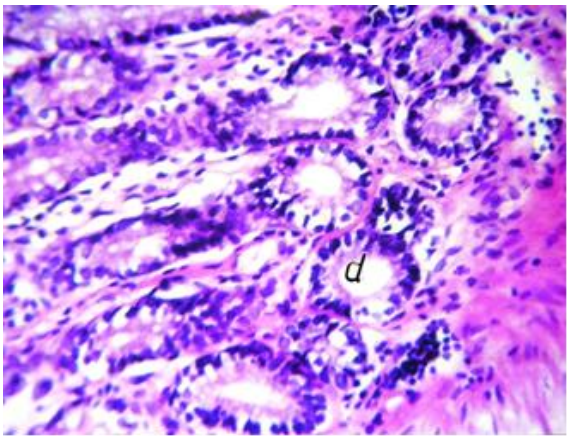

Fig. 3:A photomicrograph of colon tissue section of colorectal cancer- induced in rat showed dysplasia and anaplasis associated with pleomorphism and hyperchromachia in the lining epithelial cells of the glandular structure (d) (H&E X40).

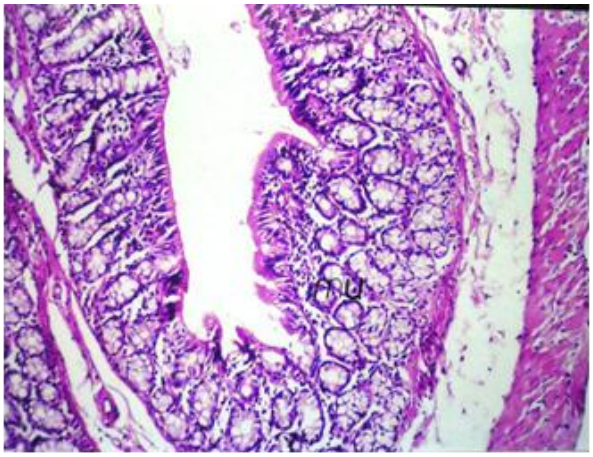

Fig. 4: A photomicrograph of colon tissue section of colorectal cancer- induced in rat treated with fluorouracil showed few inflammatory cells infiltration in the lamina propria of the mucosa (mu) with oedema in muscularis (ml) (H &E X40).

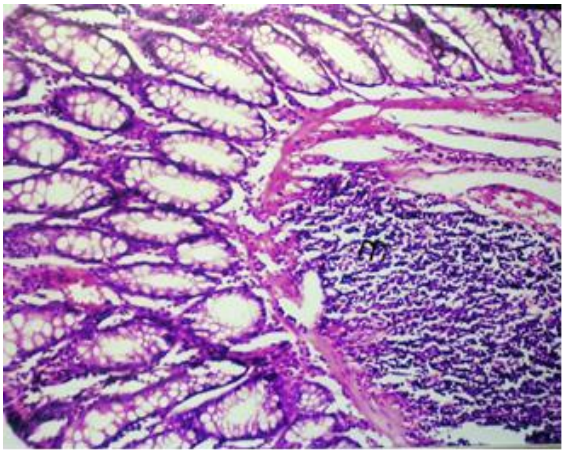

Fig. 5:A photomicrograph of colon tissue section of colorectal cancer –induced in rat treated with low dose of RMSMF showed lymphoid cells aggregation (m) in submucosa (H &E X40).

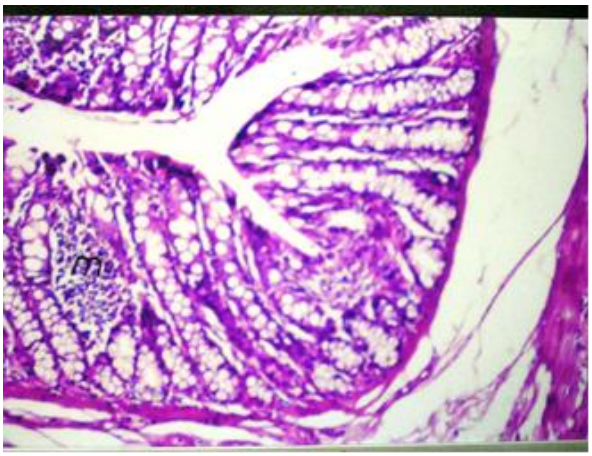

Fig. 6: A photomicrograph of colon tissue section of colorectal cancer –induced in rat treated with high dose of RMSMF showed lymphoid cells aggregation (m) (H &E X40).

DISCUSSION

The current results revealed significant decrease in serum level of CYT-C in colorectal cancer induced group. This finding is greatly supported by the study of Cheng *et al*. (2011), [21]. In mammalian cells, a major caspase activation pathway is the cytochrome c-initiated pathway. In this pathway, 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 [22]? 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 [22]. Treatment of colorectal cancer-induced group with fluorouracil resulted in significant increase in serum level of CYT-C. This result agrees with that of Stevenson *et al*. (2011) [23] were 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 [23]. Rosemary fraction exhibited antitumor effect through increasing the serum level of CYT-C in colorectal cancer- induced animals. The efficacy of rosemary to suppress tumor may be attributed to its major components including carnosic acid, carnosol, rosmarinic acid, rosmanol and ursolic acid [21]**.** Rosmanol has been shown to increase the expression of Fas and FasL leading to cleavage and activation of pro-caspase-8 and tBid and mobilization of Bax from cytosol into mitochondria. The mutual activation between tBid and Bad decreased the mitochondrial membrane potential and released cytochrome c and apoptosis- inducing factor (AIF) to cytosol. In turn, cytochrome c induced the processing of pro-caspase-9 and pro-caspase-3, followed by the cleavage of poly-ADP-ribose polymerase (PARP) and DNA fragmentation factor (DFF-45). This mechanism demonstrates that rosmanol-induced apoptosis is mediated by the involvement of caspase activation and complicated regulation of both mitochondrial apoptotic pathway and death receptor pathway. Moreover, Kim *et al*. (2011), [24] found that ursolic acid could inhibit tumorigenesis, tumor promotion, and angiogenesis. Ursolic acid decreases cell proliferation rate and induces apoptosis in human breast cancer cell line, MDA-MB-231. Also, ursolic acid induces various apoptotic molecules related to either extrinsic or intrinsic apoptosis signal pathway in MDA-MB-231 cells. Also, these authors demonstrated that ursolic acid induces the appearance of Fas receptor and cleavage of caspase-8, -3 and PARP. Furthermore, ursolic acid induces Bax up-regulation and Bcl-2 down-regulation and release of cytochrome C to the cytosol from mitochondria. Moreover, ursolic acid cleaves caspase-9 and decreases mitochondrial membrane potential. These data indicate that ursolic acid induces apoptosis through both mitochondrial death pathway and extrinsic death receptor dependent pathway [24]**.**

Programmed cell death protein-4 (PDCDP-4) was first identified as being differently up regulated 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 [25]. PDCDP-4 has been identified as a suppressor of transformation, tumorigesesis, progression, invasion, matrix-metalloproteinase activation and tumor growth. The present study revealed significant decrease in PDCDP-4 serum level in colorectal cancer-induced group which was in agreement with Lim and Hong (2011), [26]. 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 suggestion a higher clinical stage [26]. Fluorouracil treatment in colorectal cancer-induced group resulted in significant increase in serum level of PDCDP-4. Fluorouracil, as well as the nucleoside analog 5-fluoro-2′-deoxyuridine, is part of a class of cytotoxic drugs known as anti-metabolites. The anti-metabolite 5-fluorouracil is employed clinically to manage solid tumors including colorectal and breast cancer [27]. 5-Fluorouracil has demonstrated activity against colorectal cancer, leading to apoptosis of neoplastic cells [28]. 5-Fluorouracil exhibited anti-proliferative effects against human colorectal cancer cells [29]. 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 anticarcinogenic effect of RMSMF was clearly demonstrated in the present study as it could increase serum level of PDCDP-4 in colorectal cancer treated groups. The effect of RMSMF may due to the presence of carnosol which acts as anticancer and anti-inflammatory agent. Carnosol targets multiple deregulated pathways associated with inflammation and cancer including nuclear factor kappa B (NFκ-B), apoptotic related protein, phosphatidyl inositol-3-kinase, androgen and estrogen receptors as well as molecular targets [30]. In addition, carnosol appears to be well tolerated as it has a selective toxicity towards cancer cells versus non-tumorigenic cell and is well tolerated when administered to animals [30].

Our biochemical findings in the current study revealed that there was significant increase in CEA, and CCSA-4 levels in colorectal cancer-induced group as compared to the control group. Currently, CEA is used as a tumor marker for the clinical-induced management of colorectal cancer. Elevated blood levels of CEA indicate metastasis and poor prognosis. 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. Also, aberrant up regulation of CEA is common feature of colorectal cancers [31].

Brunagel et al. (2004), [32] 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*. (2007),[33] 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 [33].

The current biochemical data showed that fluorouracil administration in colorectal cancer-induced group significantly decreased CEA and CCSA-4 serum levels level as compared to the control group. These findings are in accordance with Ghiringhelli *et al.* (2009), [34]. However, Aldulaymi *et al.* (2010), [35] showed stable plasma CEA level during adjuvant chemotherapy. In the present study, treatment of colorectal cancer-induced group with rosemary fraction resulted in significant decrease in serum CEA, and CCSA-4 levels. These results could be attributed to rosmarinic acid constituent of rosemary. This explanation was supported by Venkatachalam *et al.* (2012), [36]. The chemopreventive effect of rosmarinic acid on colon carcinogenesis is evidenced by the decreased incidence and distribution of tumors along the colon. This effect of rosmarinic acid could be associated with inhibition of cell proliferation and induction of tumor cell death. Moreover, the strong anti-inflammatory compounds in rosemary mainly carnosic acid and carnosol could regulate the expression of inflammation-associated genes. Furthermore, carnosol has been reported to have broad anticancer properties in several cell line models targeting multiple deregulated pathways. Additionally, carnosic acid showed powerful antioxidant effect, free radical scavenging property [37]. All of these properties of rosemary containing compounds confirmed the anticancer activity of this plant and in turn its ability to suppress circulating tumor biochemical markers (CEA and CCSA-4) levels as shown in the current study.

The present study revealed significant increase in the gene expression level of β- catenin, K-ras and c-myc in colon tissue of colorectal cancer-induced group. This finding is in agreement with that in the study of Takahashi and Wakabayashi (2004), [38]. 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 Thus, and the gene that codes for β- catenin can function as an oncogen. Mutations in this gene are a cause of colorectal cancer. It has been found that over expression of β- catenin may be the result of altered expression of one of the many proteins with β- catenin interacts, such as axin, conduction or E- cadherin [39].

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 [40]. Mutations of proto-oncogenes ras are most commonly found in colorectal carcinoma, appearing early in the process of carcinogenesis. Functional studies in cell culture and mouse models support a critical role for K-ras mutation in colorectal cancer progression and maintenance [41].

C-myc has been found to be over expressed in several stages of chemically-induced rat colon carcinogenesis [42]. Myc protein is a transcription factor that activates expression of a great number of genes; it can also act as a transcriptional repressor. The c-myc gene is frequently deregulated and over expressed in colon malignancy, and strategies designed to inhibit c-myc expression in cancer cells may have considerable therapeutic value [43].

The molecular gene expression analysis in the present study showed significant inhibition of β-catenin, K-ras and c-myc gene expression in colon tissue of fluorouracil treated group as compared to colorectal cancer- induced group. The suggested explanation of this result could be attributed to the chemotherapeutic effect of fluorouracil which disrupts protein-protein interactions and in turn reduces the levels of oncogenic beta-catenin. This result needs further investigations since it is a pioneer study [44]. The chemo sensitizing effect of fluorouracil may be attributed to its suppressive effect on AKT/NF-KappaB signaling in colon cancer cell line (MCS) [45]. Also the present results were supported by the results of Zhao *et al*. (2008), [46] who found decreased expression of c-myc mRNA and phosphorylated c-myc in human colon cancer KM12C cells treated with fluorouracil.

Treatment of colorectal cancer-induced group with rosemary led to significant reduction in the gene expression level of β-catenin as shown in the current study. Literature evidence from animal and cell culture studies demonstrated the anticancer potential of rosemary active constituents; carnosol, carnosic acid, ursolic acid, and rosmarinic acid. The reported anticancer properties were found to arise through the molecular changes in the multiple-stage process of cancer development, which are dose related and not tissue or species specific. This is evidenced by the ability of rosemary to suppress the development of tumors in several organs including colon, breast, liver, stomach, as well as melanoma and leukemia cells [47]. This result could be explained by the efficacy of rosemary to attenuate Wnt/signaling pathway resulting in reduced transcription of target genes, including cyclin D1 and Cox-2. Carnosol, the active component of rosemary prevents adenomatous polyposis carcinoma (APC) associated intestinal tumorigenesis, potentially *via* its ability to enhance E-cadherin- mediated adhesion and suppress β-catenin tyrosine phosphorylation. Additionally, carnosic acid from rosemary attenuates transcriptional β-catenin outputs in colorectal cancer cells [44].

Oleanolic acid and ursolic acid are naturally occurring triterpenoids that have been used in traditional medicine for centuries as antibacterial, antifungal, anti-inflammatory and anticancer agents *[48-49].* It has been found that a synthetic

triterpenoid compound (CDDO-Me) could inhibit the growth of both K-ras mutated and wild-type K-ras pancreatic cancer cells at very low concentrations *[50].* Thus, we could suggest that ursolic acid in rosemary may have anti-tumor efficacy through inhibition of K-ras gene in colon tissue of the treated colorectal cancer-induced group as shown in present study.

Rosmarinic acid has been found to exhibit antiproliferative effects in cultured murine mesangial cells and suppress the expression of platelet- derived growth factor (PDGF) and c-myc mRNA expression in a dose dependent manner [51]. A growing body of evidence revealed that reactive oxygen species act as cellular signals in PDGF-induced mutagenesis, and these include the expression of the proto-oncogene's c-foes and c-myc. Since rosmarinic acid has a potent antioxidative activity [52], this compound might suppress c-myc mRNA expression by scavenging reactive oxygen species. The suppressive effects of rosmarinic acid on PDGF and c-myc mRNA expression would contribute to its antiproliferative activity on mesangial cells [51].

Histological examination of colon tissue section of colorectal cancer induced rats revealed dysplasia and anaplasia associated with pleomorphism and hyperchromachia (adenocarcinoma). This histopathological feature is in consistent with that in the studies of [13-53-54] which confirmed the induction of colon carcinogenesis in rats by N-methylnitrosourea.

Furthermore, histological investigation of colon tissue section of colon cancer-induced rats treated with 5-fluorouracil (fluorouracil group) 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*. (2003), [55] study. The influence of fluorouracil on colonic carcinoma showed conflicting results and its influence mainly comes from its growth inhibitory effects on cancer cells [56].

Low and high dose of rosemary successive methanolic fraction (RMSMF) showed lymphoid cells aggregation in the submucosa. These findings could be explained as that rosemary constituent mainly rosmarinic acid which possesses more or less moderate decreasing effect on the number of polyps in colon cancer reaching to 50% [56].

CONCLUSION

The current data suggest that *Rosmarinus officinalis* (rosemary) has a promising therapeutic role against colorectal cancer induced by N-methylnitrosourea as indicated by the observed improvement in the biochemical, molecular and histological findings. These effects could be achieved through the powerful apoptotic, anti-inflammatory properties, and antiproliferative effect of this plant. The present study represented good therapeutic approach for intervention against progressive colorectal cancer with special reference to apoptosis, inflammation and proliferation.

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