

Astaxanthin inhibits tumor invasion by decreasing extracellular matrix production and induces apoptosis in experimental rat colon carcinogenesis by modulating the expressions of ERK-2, NFκB and COX-2

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Summary Colon cancer is the third most malignant neoplasm in the world and it remains an important cause of mortality in Asian and Western countries. Astaxanthin (AST), a major component of carotenoids possesses attractive remedial features. The purpose of this study is to investigate the possible mechanism of action of astaxanthin against 1, 2 dimethyl hydrazine (DMH)-induced rat colon carcinogenesis. Wistar male rats were randomized into five groups, group 1 were control rats, group 2 were rats that received AST (15 mg/kg body wt p.o. everyday), rats in group 3 were induced with DMH (40 mg/kg body wt, s.c.), DMH-induced rats in groups 4 and 5 were either pre or post initiated with AST, respectively as in group 2. DMH-induced rats exhibited elevated expressions of Nuclear factor kappa B-p65 (NF-κB-p65), Cyclooxygenase-2 (COX-2), Matrix metallo proteinases (MMP) 2/9, Proliferating cell nuclear antigen (PCNA), and Extracellular signal-regulated kinase-2 (ERK-2) as confirmed by immunofluorescence. Further, Western blot analysis of MMPs-2/9, ERK-2 and Protein kinase B (Akt) revealed increased expressions of these proteins in DMH-induced groups of rats. AST-treatment decreased the expressions of all these vital proteins, involved in colon carcinogenesis. The ability of AST to induce apoptosis in the colon of DMH-induced rats was confirmed by Annexin-V/PI staining in a confocal microscopy, DNA fragmentation analysis and expression of caspase-3 by Western blotting. In conclusion, astaxanthin exhibits anti-inflammatory and anti-cancer effects

by inducing apoptosis in DMH-induced rat colon carcinogenesis by modulating the expressions of NFκB, COX-2, MMPs-2/9, Akt and ERK-2.

Keywords Astaxanthin · Colon carcinogenesis · COX-2 · NF-κB · ERK-2, Matrix metalloproteinase

Abbreviations

AST	Astaxanthin
DMH	Dimethyl hydrazine
COX-2	Cyclooxygenase-2
NF-κB	Nuclear factor- κB
MMP	Matrix Metalloproteinases
ERK-2	Extracellular signal-regulated kinase-2

Introduction

Colorectal cancer affects both the genders in Asia and developed countries and threatens to be one of the leading causes of morbidity and mortality [1]. Hereditary genetic component, alcohol consumption and high fat diets are among the greatest risk factors that can lead to colon cancer [2]. DMH is a powerful colon carcinogen which induces colorectal tumors in experimental animals [3]. DMH gets converted to an active carbonium ion through several processes, and is excreted in the bile, where it mediates its carcinogenic activities on the colonic mucosa while passing through the digestive tract [4].

Chemoprevention has the potential to be a major component in the control of colorectal cancer [5]. Recently, considerable attention has been drawn in identifying phytochemicals, particularly those included in our diet, which has the ability to interfere with carcinogenic or mutagenic

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processes [6, 7]. AST is a non-provitamin A carotenoid found in the red pigment of shrimp, crab, salmon, and asteroidean [8, 9]. AST has been reported to possess antioxidant, anti-inflammatory and anti-cancer properties [10–12]. Cyclooxygenases also known as prostaglandin H2 synthases are the rate-limiting enzymes involved in the conversion of arachidonic acid into prostaglandins (PGs) [13]. Two isoforms of COX exist, with diverse tissue distributions. COX-1 is constitutively expressed in many tissues and cell types, whereas COX-2 is an inducible isoform that participates in pro-inflammatory responses in response to certain stimuli such as mitogens, cytokines and growth factors [14, 15]. Upregulation of COX-2 has also been implicated in cancer development and growth. Recent reports on COX-2 expression in cancers show that this enzyme stimulates angiogenesis and is associated with tumor growth, invasion, and metastasis [14, 16]. NF- κ B, a transcriptional factor, is critically involved in tumor progression due to its transcriptional regulation of invasion-related factors such as matrix metallo proteases [17]. Moreover, NF- κ B signaling pathway has been shown to block apoptosis and promote proliferation of cancer cells [18].

One of the early events of cancer invasion involves proteolytic degradation of extracellular matrix (ECM) components which are necessary for cell migration during tissue remodeling and tumor invasion [19]. The regulation of tissue remodeling is accomplished by complex control of the activities of matrix metalloproteinases (MMPs). MMPs are a family of zinc metallo-endopeptidases that are secreted as inactive zymogens and are activated extracellularly. MMPs are increasingly involved in the breakdown of ECM and suggested to play an important role in the process of tumor invasion and metastasis [20, 21]. Over expressions of MMP (2 and 9) have been demonstrated in human colorectal cancers [22, 23]. The extracellular signal-regulated kinase (ERK) signaling pathway plays a major role in the control of diverse cellular processes such as proliferation, survival, differentiation and motility. This pathway is often up-regulated in various tumors and represents an attractive target for the development of anticancer drugs [24]. The role of ERK signalling has been documented in colon cancer [25]. Apoptosis refers to morphological changes that include chromatin condensation, compartmentalization of nuclear and cytoplasmic material into structurally-preserved, membrane-bound fragments with the participation of a family of proteases, called as caspases [26, 27]. A defective apoptotic signalling pathway is another common event in colorectal cancer. Therefore, strategies to induce apoptosis in colorectal cancer through activation of signalling mechanisms by novel drugs are a pioneering area in cancer research. Recently, we have demonstrated that AST possesses antioxidant and antiproliferative effect in DMH-induced colon carcinogenesis [28]. However, the mechanism by which AST-mediate its

action remains elusive. Therefore, in this study, we show evidence that AST induces apoptosis in DMH-induced rat colon through modulated expressions of proteins that are known to be involved in the progression of colorectal cancer.

Materials and methods

Animals

Male adult Wistar rats of body weight 180–210 g were obtained from the Central Animal House, Tamilnadu Veterinary and Animal Sciences University (TANUVAS), Madhavaram, Chennai, India and were acclimatized to the control diet for 1 week. Animals were maintained as per the principles and guidelines of the Ethics Committee (IAEC No.01/020/08). Six animals were housed per cage and were maintained in a controlled environmental condition of temperature and humidity on alternatively 12 h dark/light cycles. The animals were fed with commercial pellet diet (Hindustan Lever Ltd., Bangalore, India).

Chemicals

DMH and AnnexinV/PI apoptosis detection kit were purchased from Sigma Chemical Company, St Louis, USA. AST was purchased from Cayman chemicals, USA. The antibodies used in this study were procured from Santacruz Biotech, USA. All other chemicals used were of analytical grade.

Induction of DMH

For induction of colonic cancer, male Wistar rats received a single dose of 40 mg/kg (based on body weight) DMH dissolved in 0.9% NaCl solution by subcutaneous injection, twice a week for 2 weeks. Control rats were given a single subcutaneous dose of saline alone.

Experimental protocol

The experimental animals were divided into five groups, each groups comprising of six animals.

- Group I Control rats.
- Group II AST-treated rats (15 mg/kg body weight) once daily throughout the experimental period [16 weeks] as reported earlier in our laboratory [28].
- Group III DMH induced rats.
- Group IV Rats received AST as in Group II, starting one week before the DMH injections and continued till the end of the experiment [DMH + AST (Pre-initiation)].

Group V Rats received AST as in Group II starting one week after the cessation of DMH injections and continued till the end of the experiment [DMH + AST (post initiation)].

The experiments were terminated 16 weeks after the end of the experimental period, and all the animals were killed by cervical dislocation after an overnight fast.

Mast cell staining

Histochemical analysis of mast cells were carried out as reported earlier [29]. Briefly, 5 μ m thickness tissue sections were dewaxed in xylene and rehydrated through decreasing concentrations of ethanol to distilled water. The sections were stained with toluidine blue for 2 min and washed with distilled water followed by staining with light green SF for 30 s and washed using distilled water and dehydrated in increasing concentrations through alcohol series, xylene and mounted using DPX. High power objective field (40 \times)

was chosen for counting total number of mast cells at ten different fields per slide.

Immunohistochemical analysis of NF- κ B and COX-2

Immunohistochemical analysis was performed as standardized earlier in our laboratory [30]. Paraffin embedded tissue sections of 3 μ m thickness were rehydrated first in xylene and then in graded ethanol solutions. The slides were then blocked with 5% BSA in TBS (Tris buffered saline) for 2 h. The sections were then immunostained with respective primary antibodies of Rabbit polyclonal NF- κ B p65 (1:3000 dilution), and rabbit polyclonal COX-2 (1: 2000 dilution); incubated overnight at 4 $^{\circ}$ C. After washing the slides thrice with TBS, the sections were then incubated with anti-rabbit secondary antibody (Bangalore Genei, India), diluted 1:2000 with 5% BSA in TBS and incubated for 2 h at room temperature. Sections were then washed with TBS and incubated for 5–10 min in a solution of 0.02% diaminobenzidine containing 0.01% hydrogen peroxide. Counter staining was performed

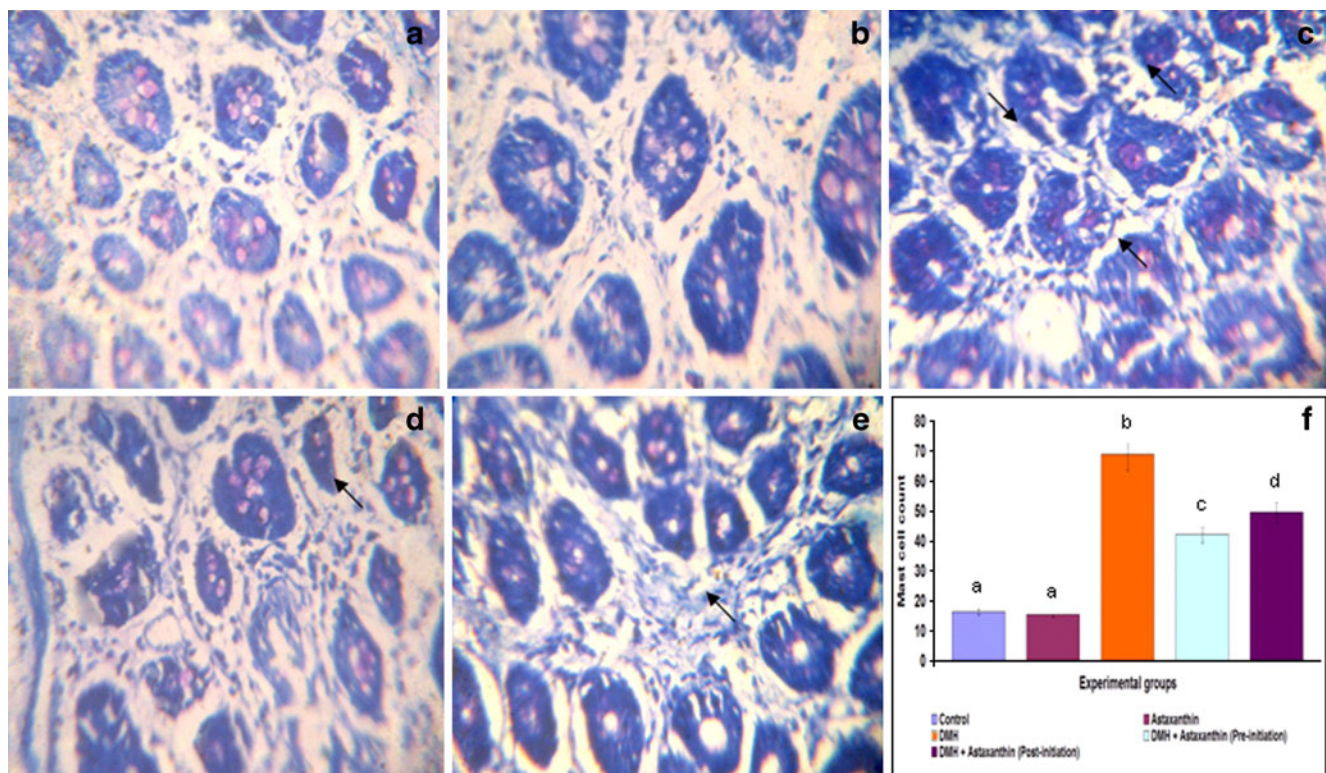


Fig. 1 Histochemical analysis of mast cells in the colon of control and experimental groups of rats. Control and experimental groups of rat tissue sections were stained with toluidine blue as mentioned in materials and methods. Arrow shows mast cells **A.** Control. **B.** AST alone treated group. **C.** DMH-induced group. **D.** DMH + AST (Pre-initiation) group. **E.** DMH + AST (Post-initiation) group. **F.**

Quantitative analysis of total mast cell count. The number of stained (positive) cells per 20x field was averaged across 20 fields for each rat. Hypothesis testing method included one-way analysis of variance (ANOVA) followed by least significant difference (LSD). Values are given statistically significant at $P < 0.05$; Values not sharing a common superscript letter (a–d) differ significantly

using hematoxylin, and the slides were visualized under a light microscope (Nikon XDS-1B).

Immunofluorescence analysis of MMPs-2/9, PCNA and ERK-2

Paraffin embedded tissue sections of three-micrometer thickness were rehydrated first in xylene and then in graded ethanol solutions. The slides were then blocked with 3% BSA in TBS for 2 h. The non inflated sections were then immunostained with primary antibody goat polyclonal IgG to rat MMPs-2/9 (1: 3000 dilutions each), PCNA (rabbit polyclonal, 1: 5000 dilution) and antirabbit ERK-2 (phycoerythrin (PE) conjugated secondary antibody, 1: 5000 dilution), incubated overnight at 4°C. After washing the sections thrice with TBS, the slides were then incubated with their corresponding fluorescence isothio cyanate (FITC) conjugate secondary antibody (Bangalore Genei, India), diluted 1:40 with TBS and incubated in dark for 2 h at room temperature. Sections were then washed with TBS and incubated with the nucleus specific counter stain 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI, Vector Laborato-

ries, UK) and propidium iodide (PI, Sigma, St. Louis, USA) respectively to highlight cell nuclei. Slides were visualized under a fluorescent microscope (Nikon TE 300) using Excitation wavelength/Emission wavelength of 345 nm/456 nm for DAPI; 529 nm/620 nm for PI ; 494 nm/525 nm for FITC and 488 nm/578 nm for PE. For quantification of MMPs-2 and 9, cell population examinations of 25 grid field (each field measuring 0.245 mm²) were counted and the mean score per colon was calculated. FITC positive cells were analyzed from six locations per rat colonic tissue section, and analyzed for statistical significance.

Confocal microscopic analysis of apoptotic cells

De-paraffinized colonic tissue sections of control and experimental groups of rats after re-hydration were permeabilized using Triton-X-100 and washing buffer. Specific binding of annexin V was performed by incubating the sections for 15 min at room temperature in a binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂ and pH 7.4) containing a saturating concentration of 1 µg ml⁻¹ annexin V-FITC and PI (2 µg ml⁻¹). Finally, the sections

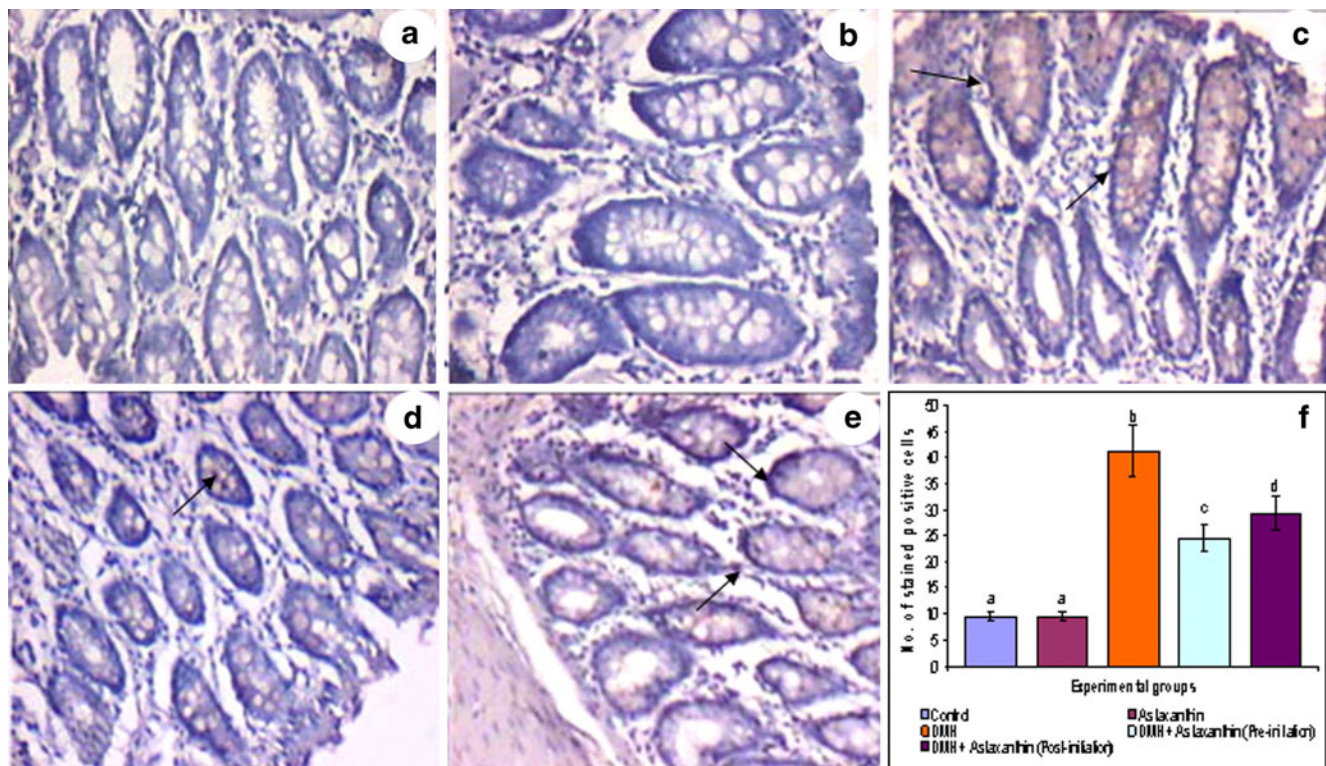


Fig. 2 Immunohistochemical analysis of NF-κB in the colon of control and experimental groups of rats. **A.** Control. **B.** AST alone treated group. **C.** DMH-induced group. **D.** DMH+AST (Pre-initiation) group. **E.** DMH + AST (Post-initiation) group. Arrows indicate NF-κB positive cells. **F.** Quantitative analysis of NF-κB expression. The

number of stained (positive) cells per 20x field was averaged across 20 fields for each rat. Hypothesis testing method included one-way analysis of variance (ANOVA) followed by least significant difference (LSD). Values are given statistically significant at $P < 0.05$; Values not sharing a common superscript letter (a–d) differ significantly

were washed and analyzed under a confocal scanning laser microscope to view the phosphatidyl serine expression (green fluorescence) against PI (red fluorescence). For analysis of green fluorescence, cells were illuminated with 488 and 550 nm band pass filter and the objective used was 20x. The co-localized images were captured in a confocal scanning laser microscopy (Leica TCS-SP2 XL, Germany).

DNA fragmentation analysis

DNA fragmentation, a hallmark of apoptosis was performed according to the method of Wu et al. [31] with some modifications. Briefly, 100 mg of tissue from control and experimental groups of rats were weighed and homogenized with 1 ml saline-EDTA reagent to get 10% tissue homogenate. 300 μ l of the homogenate from all the groups were mixed with 300 μ l of Tris saturated phenol and 300 μ l of chloroform-isoamyl alcohol mixture. To this content, 25 μ l of SDS was added. The contents were mixed thoroughly and centrifuged at 11,000 rpm for 15 min. The resultant aqueous phase was collected; 9 μ l of NaCl and 2 volumes of 100% ethanol (twice the volume of aqueous phase) were added. The contents were mixed and were

centrifuged at 12,000 rpm for 5 min. The pellet fraction containing DNA was dissolved in TE buffer. The DNA was detected on a 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized by UV light.

Protein extraction and Western blotting

Colonic tissues of control and experimental groups of rats were homogenized in 135 mM NaCl, 20mMTris, 2 mM EDTA and 1 mM PMSF (pH 7.4). The homogenates were centrifuged (15 min, 10000 rpm at 4°C) and the protein content of the supernatant was determined. Aliquots of supernatant (30 μ g total protein) were boiled for 5 min in sample buffer (0.2 M Tris-HCl buffer, 10% glycerol, 2% SDS, 0.02% β -mercaptoethanol). Equal amounts of protein from each of the samples were resolved by sodium dodecyl sulfate—Polyacrylamide gel electrophoresis (SDS—PAGE) on 12% gel and transferred electrophoretically to a nitrocellulose membrane (Amersham Biosciences, NJ). The membrane was blocked with 5% BSA in Tris-Tween buffered saline at room temperature for 1 h. The membrane was then incubated with respective primary antibodies [rabbit polyclonal NF-kB

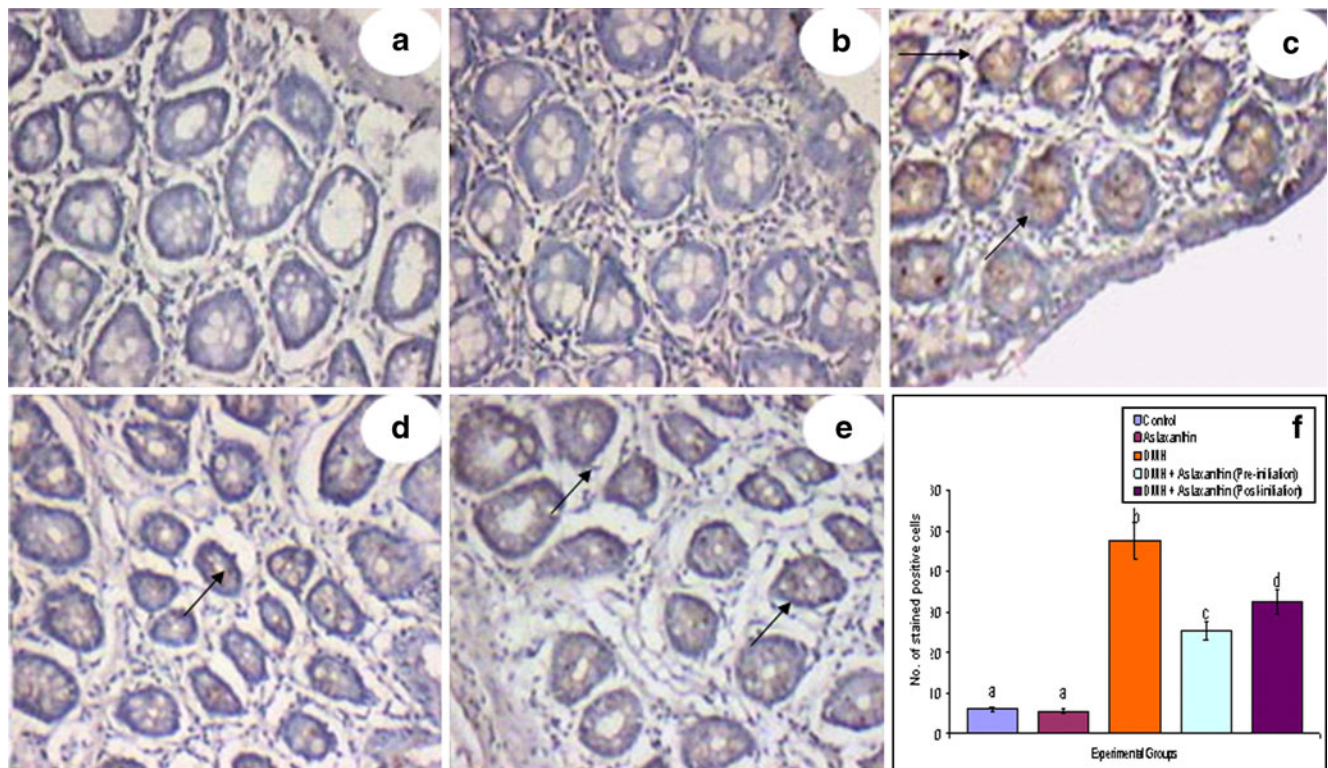
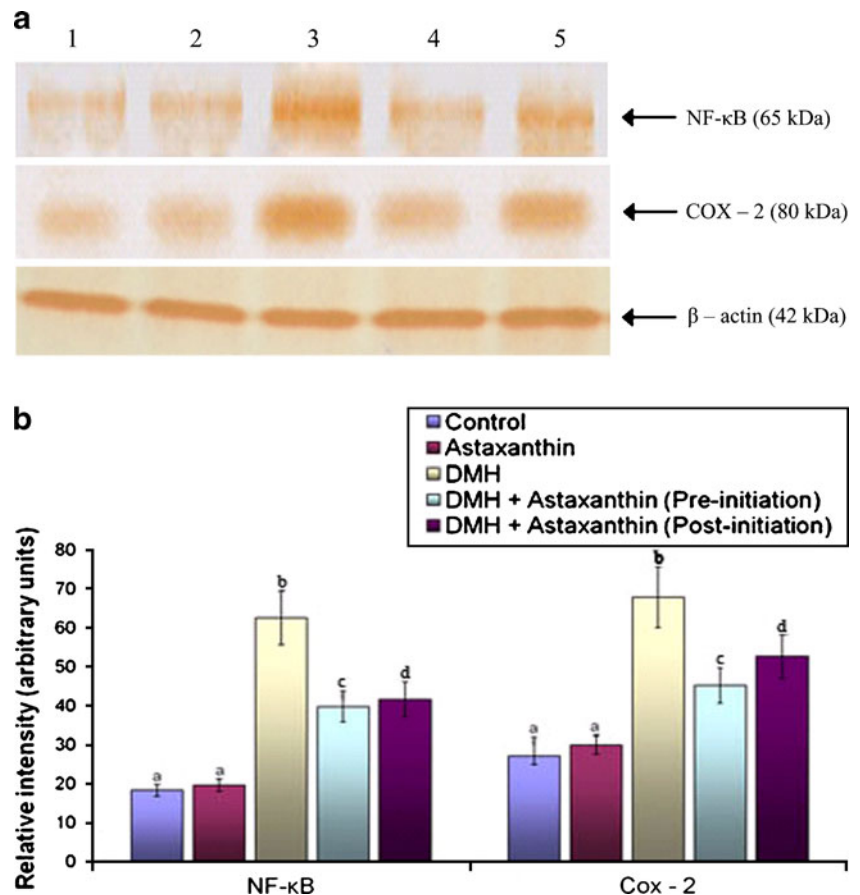


Fig. 3 Immunohistochemical analysis of COX-2 in the colon of control and experimental groups of rats. **A.** Control. **B.** AST alone treated group. **C.** DMH-induced group. **D.** DMH + AST (Pre-initiation) group. **E.** DMH + AST (Post-initiation) group. Arrows indicate COX-2 positive cells. **F.** Quantitative analysis of COX-2 expression. The number of stained (positive) cells per 20x field was

averaged across 20 fields for each rat. Hypothesis testing method included one-way analysis of variance (ANOVA) followed by least significant difference (LSD). Values are given statistically significant at $P < 0.05$; Values not sharing a common superscript letter (a–d) differ significantly

Fig. 4 Immunoblot analysis of NF- κ B and COX-2 in the colon of control and experimental groups of rats. **A.** Western blot analysis was performed with antibodies against NF- κ B, COX-2 and β -actin as indicated. Lane 1-Control, Lane 2-AST alone treated group, Lane 3-DMH-induced group, Lane 4-DMH + AST (Pre-initiation), Lane 5-DMH + AST (Post-initiation). **B.** Quantitative data expressing the corresponding protein levels was assessed using densitometry and is expressed in relative intensity (arbitrary unit). Hypothesis testing method included one-way analysis of variance (ANOVA) followed by least significant difference (LSD). Values are given statistically significant at $P < 0.05$; Values not sharing a common superscript letter (a–d) differ significantly



p65, rabbit polyclonal COX-2, Goat polyclonal IgG to rat MMPs-2/9, rabbit polyclonal Akt & ERK-2, and anti-mouse caspase-3], Overnight at 4°C. The membrane was then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 h. Protein antibody complexes were detected by the addition of diaminobenzidine (DAB) as a substrate.

Statistical analysis

All the grouped data were evaluated using SPSS/10.0 software. Hypothesis testing method included one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test. $P < 0.05$ was considered to indicate statistical significance. All the results were expressed as mean \pm S.D. for six rats in each group.

Results

AST reduces mast cell density in DMH-induced rat colon carcinogenesis

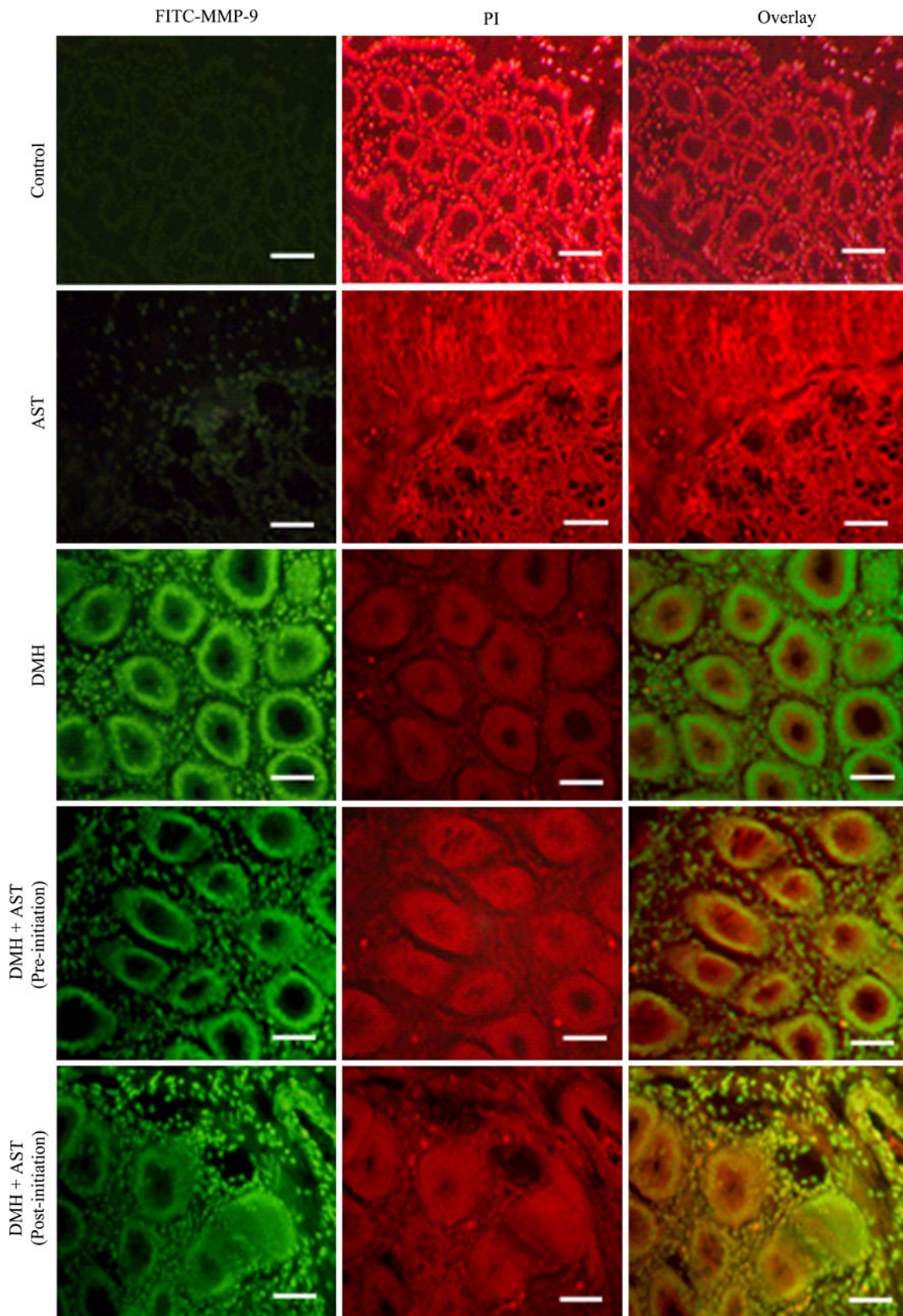
Mast cells are present in mucosa and submucosal tissue of gastrointestinal tract, which release active substances

leading to the growth of tumor tissues. The involvement of mast cells in the progression of cancer has been reported [32, 33] Fig. 1 shows the histochemical staining of mast cells stained with toluidine blue in the colon of control and experimental groups of rats. DMH-induced groups of rats (Fig. 1C) showed significantly higher ($P < 0.05$) number of mast cells (termed as mast cell density) when compared to control rats (Fig. 1A). Whereas both the astaxanthin administered rats (Pre and post initiation groups) showed a significant decrease ($P < 0.05$) in mast cell density when compared to DMH-induced rats (Fig. 1D and E).

AST attenuated DMH—induced inflammation

NF- κ B is an ubiquitous transcription factor in cells involved in inflammatory reactions, and exerts its effects

Fig 5 Immunofluorescence analysis of MMP-9 in the colon of control and experimental groups of rats. Tissue sections were immunostained with the anti-MMP-9 antibody and an FITC conjugated secondary antibody (green). Tissue sections were also counterstained with PI (red) to provide nuclear staining. (Scale bar-100 μ m). Slides were visualized under a fluorescent microscope (Nikon TE 300) using Excitation wavelength/Emission wavelength of 529 nm/620 nm for PI and 494 nm/525 nm for FITC



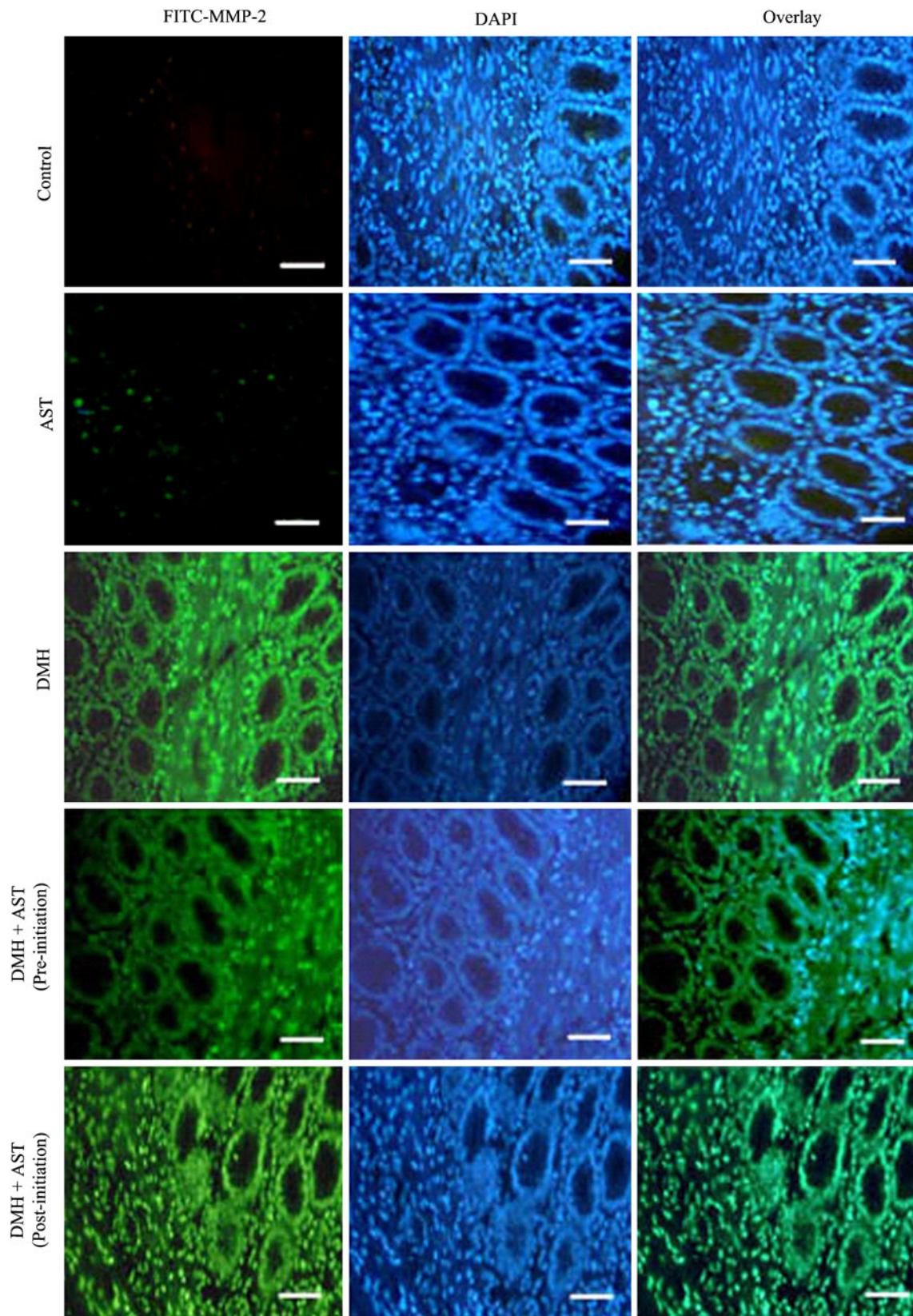


Fig. 6 Immunofluorescence analysis of MMP-2 in the colon of control and experimental groups of rats. Tissue sections were immunostained with the anti-MMP-2 antibody and an FITC conjugated secondary antibody (*green*). Tissue sections were also counter-

stained with DAPI (*blue*) to provide nuclear staining. (Scale bar-100 μ m). Slides were visualized under a fluorescent microscope (Nikon TE 300) using Excitation wavelength/Emission wavelength of 345 nm/456 nm for DAPI and 494 nm/525 nm for FITC

by expressing cytokines, chemokines, cell adhesion molecules and growth factors [34]. Activation of NF- κ B occurs via phosphorylation of I κ B α , resulting in the release and nuclear translocation of NF- κ B. In this study, control and AST alone treated groups of rats showed negligible expression of NF- κ B (Fig. 2A and B, respectively). DMH-administered rats showed an increased expression of NF- κ B-p65 (Fig. 2C) that was evident from increased immunohistochemical staining. DMH administered rats treated with AST (pre and post initiation) exhibited a decrease in NF- κ B expression as compared to DMH-induced rats (Fig. 2D & E). This finding suggests that AST administration could prevent NF- κ B activation, possibly by blocking the nuclear translocation of NF- κ B. Inhibition of NF- κ B by plant derived products has been documented earlier [35].

Figure 3 represents the immunohistochemical analysis of COX-2. Control rats show negligible expression of COX-2 (Fig. 3A). DMH administered rats showed an increased expression of COX-2 in rat colonic tissue (Fig. 3C) whereas, AST administered rats (Pre and post initiation) showed decreased expression of COX-2 when compared to

DMH administered rats (Fig. 3D and E). AST alone administered rats exhibited similar expression as that of control (Fig. 3B). Figure 4A represents the immunoblot analysis of NF- κ B (65 kDa) and COX-2 (80 kDa) in the colon of control and experimental groups of rats. The data suggests that DMH-induced animals (Fig. 4A, lane 3) showed increased expression of these proteins when compared to control group (Fig. 4A, lane 1). Treatment with AST to DMH-induced group resulted in a reduced expression of these proteins (Fig. 4A, lanes 4&5). AST alone treated group (Fig. 4A, lane 2) of rats showed similar pattern as that of control (Fig. 4A, lane 1). Quantitative data expressing the corresponding protein levels were assessed using densitometer and the results were expressed as relative intensity arbitrary unit (Fig. 4B).

AST treatment modulated the expressions of MMPs-2/9, PCNA, Akt and ERK-2 in DMH-induced group of rats

MMP-9 as a prognostic marker of colon carcinogenesis has been documented earlier [36]. In this study, the effect of AST in the regulation of MMP-9 expression in colonic

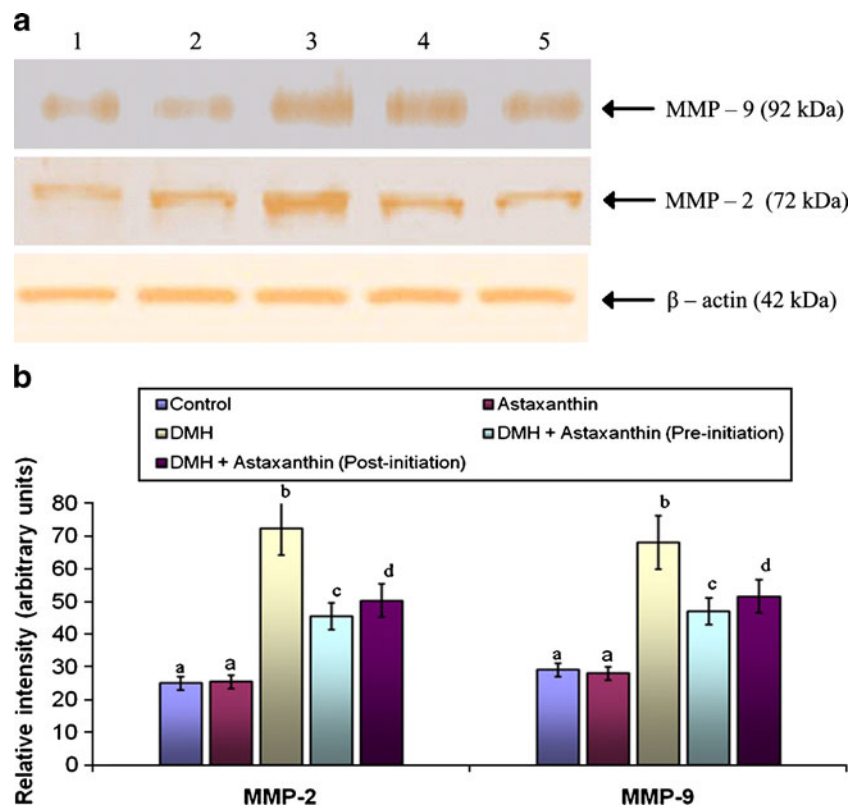


Fig. 7 Western blot analyses of MMPs-2/9 in the colon of control and experimental groups of rats. **A.** Western blot analyses were performed with antibodies against MMPs-2/9 and β -actin as mentioned in materials and methods. Lane 1-Control, Lane 2-AST alone treated group, Lane 3-DMH-induced group, Lane 4-DMH + AST (Pre-initiation), Lane 5-DMH + AST (Post-initiation). **B.** Quantitative data

expressing the corresponding protein levels was assessed using densitometry and is expressed in relative intensity (arbitrary unit). Hypothesis testing method included one-way analysis of variance (ANOVA) followed by least significant difference (LSD). Values are given statistically significant at $P < 0.05$; Values not sharing a common superscript letter (a–d) differ significantly

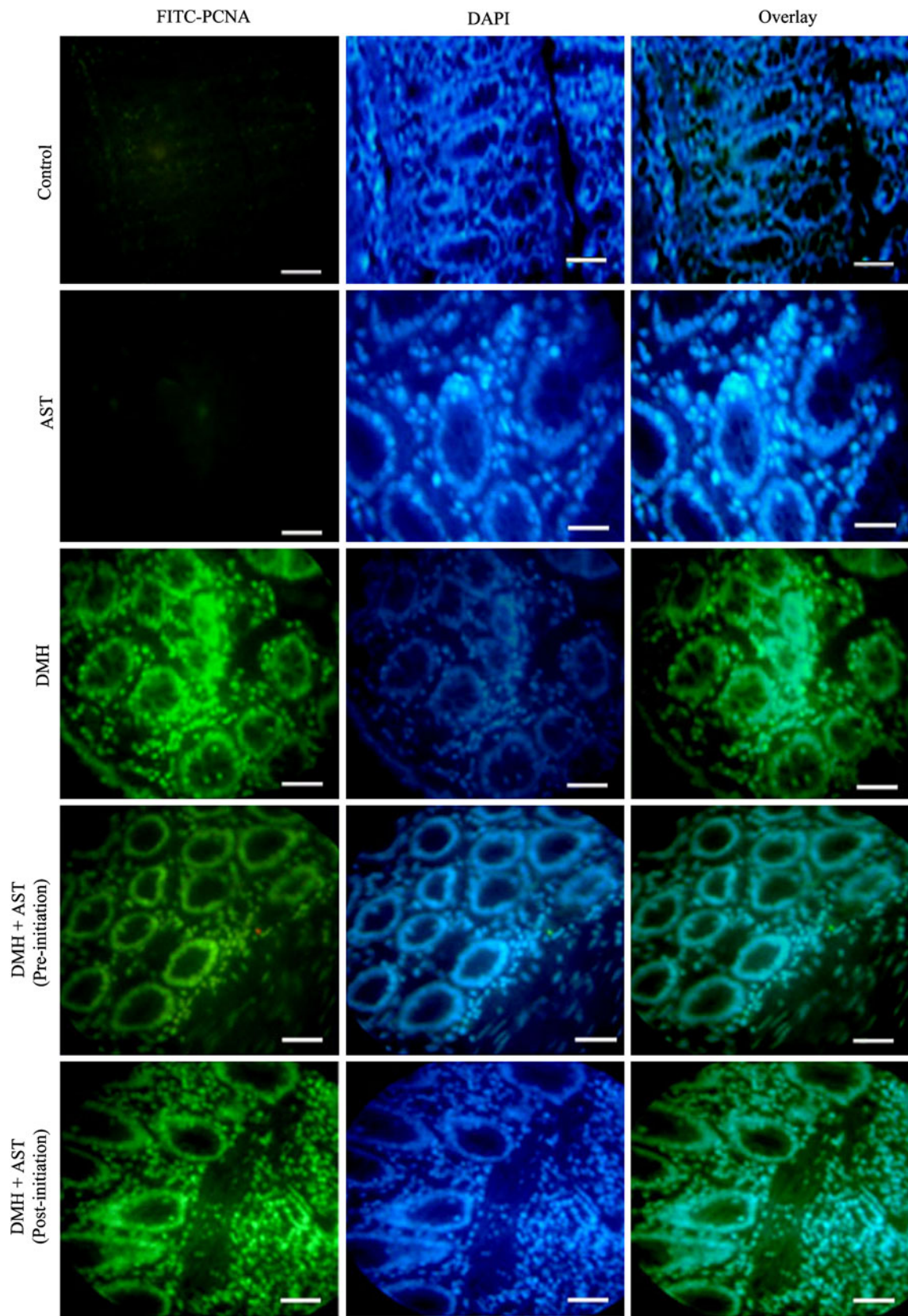


Fig. 8 Immunofluorescence analysis of PCNA in the colon of control and experimental groups of rats. Tissue sections were immunostained with the PCNA antibody and an FITC conjugated secondary antibody (green). Tissue sections were also counterstained with DAPI (blue) to provide nuclear staining. (Scale bar-100 μm). Slides were visualized under a fluorescent microscope (Nikon TE 300) using Excitation wavelength/Emission wavelength of 345 nm/456 nm for DAPI and 494 nm/525 nm for FITC

tissue sections of control and experimental groups were confirmed by immunofluorescence, counter stained with PI (Fig. 5). Control group of rats showed few positive expression of MMP-9, when compared to DMH administered rats which exhibited increased green fluorescence which signifies increased expression of MMP-9. The expression of MMP-9 was remarkably decreased in both the AST administered groups of rats (Pre and post initiation), which was evident from decreased staining. The expression in AST alone treated rats closely resembled the control (Fig. 5). The role of MMP-2 in colorectal carcinoma has been reported [37]. In this study, the expression of MMP-2 in control and experimental groups of rats were analysed by immunofluorescence. Control group of rats exhibited a few positive expression of MMP-2 (Fig. 6). The expression was significantly increased in DMH administered rats whereas the AST administered rats (Pre and post initiation) caused a significant descend in MMP-2. In order to confirm the relative protein levels in control and experimental groups, Western blot analysis of MMPs 2 and 9 were carried out. An increased levels of MMPs 2 and 9 (also known as gelatinases A and B, respectively) were observed in DMH-induced groups (Fig. 7A, lane 3) as compared to the control (Fig. 7A, lane 1). AST treated groups exhibited reduced expressions of these gelatinases A and B (Fig. 7A, lanes 4 and 5). Quantitative data expressing the corresponding protein levels were assessed using densitometer and were expressed as relative intensity arbitrary unit (Fig. 7B). From these results, it was clear that AST could significantly reduce the expressions of MMPs 2 and 9, which are prognostic markers of cancers.

PCNA is a cell cycle related protein that is maximally elevated in late G1 and S-phase of proliferating cells and its function has been demonstrated in colon cancer [38]. Fig. 8 show immunofluorescent analysis of PCNA in the colon of control and experimental groups of rats. Control rats exhibited negligible expression of PCNA, while DMH-administered rats showed increased expression of PCNA when compared to control rats, while AST treatment showed decreased expression of PCNA when compared to DMH-administered rats. The expression in AST alone treated rats closely resembled the control. ERK-2 and Akt plays a major role in cell survival [24–39]. To evaluate the role of ERK-2

during colon carcinogenesis, Immunofluorescence analysis of ERK-2 in control and experimental groups were performed using anti ERK-2 primary antibody (Fig. 9). Control group of rats exhibited meager expression of ERK-2, whereas in DMH-induced group prominent expression was noticed. Although, the expression of ERK-2 was not completely reduced upon treatment with AST, a remarkable reduction in the expression of ERK-2 was observed in AST treated groups (Pre and post initiation) of rats as compared to DMH induced group (Fig. 9). Further, the levels of Akt and ERK-2 were assessed by western blot analysis. (Fig. 10). A significant increase in the expression of ERK-2 was observed in lane 3. However, Westernblot analysis of the expression of total Akt in control and experimental groups of rats revealed only a slight modulation of protein levels.

Assessment of apoptosis

During apoptosis, exposure of phosphatidyl serine is an obvious event from the interior of the plasma membrane to the outer portion of the membrane. Thus phosphatidyl serine becomes available to bind to the annexin-V/FITC conjugate. Cells that are destined to apoptosis will stain positive for Annexin-V/FITC and negative for PI as early as 1 hr after stimulation, whereas normal viable cells in culture will stain negative [40]. Apoptosis cells will stain positive for Annexin-V/FITC and PI. DMH-induced and AST-treated groups of rat sections were stained with annexin-V/FITC and PI apoptosis kit to detect the apoptotic cells. (Fig. 11). An increased expression of annexin V (green fluorescence) was observed in AST-treated groups (pre and post initiation) of rats as compared with DMH-induced groups. A negligible quantity of positive apoptotic cells were observed in control and AST alone treated groups of rats (Fig. 11). Therefore, it is evident that AST-induces apoptosis in DMH-induced rat colon.

Internucleosomal fragmentation of DNA is a hall mark of apoptosis [41]. Fig. 12A represents the DNA fragmentation analysis of colon and experimental groups of rats. Lanes 1 and 2 depicts the control and AST alone groups which show intact DNA. Lane 3 exhibits an escalated DNA shearing and represents DMH induced groups. Lanes 4 and 5 astaxanthin treated groups (Pre and post initiation, respectively) exhibited increased DNA fragmentation, though a distinct ladder was not observed. The functional key component of apoptotic machinery is a proteolytic system involving a family of proteases called caspases. These enzymes are triggered in response to proapoptotic signals and culminate in cleavage of a set of proteins, resulting in disassembly of the cell leading to apoptosis [42]. Immunoblot expression of Caspase-3 in control and experimental groups of rats are shown in (Fig. 12B). AST treated groups (Pre and post intitation) of rats

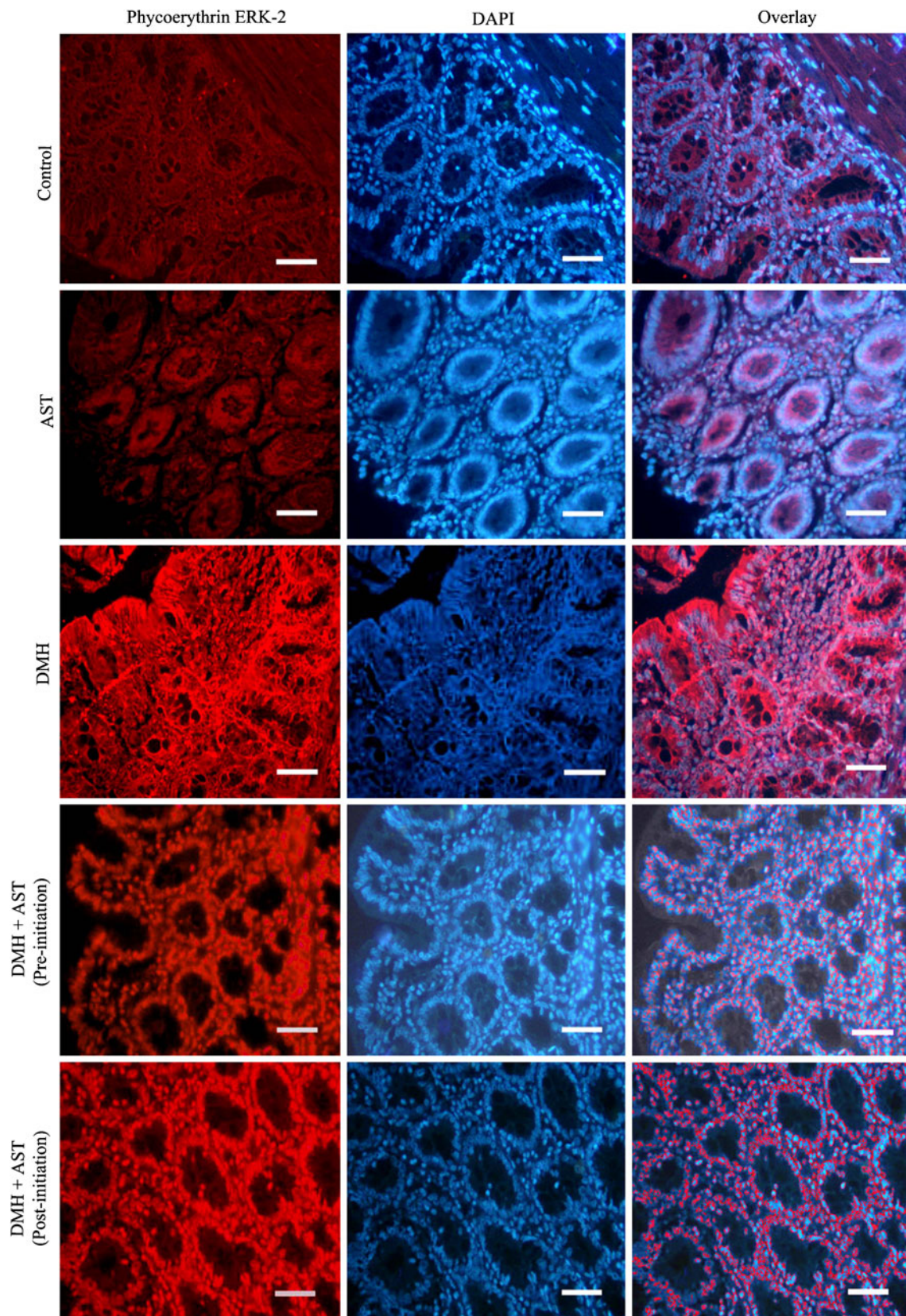
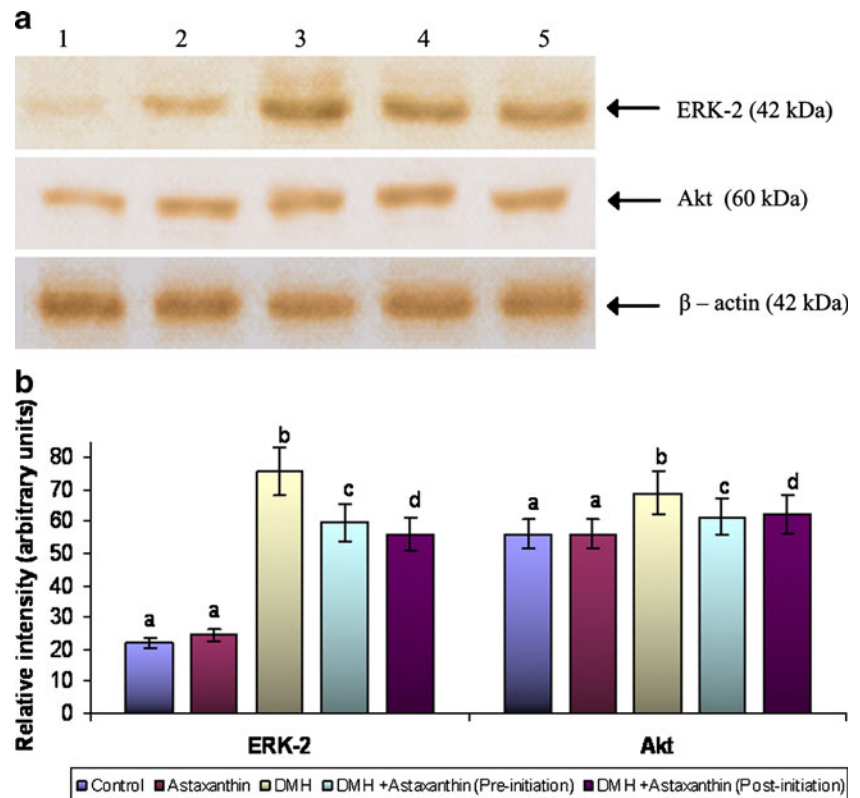


Fig. 9 Immunofluorescence analysis of ERK-2 in the colon of control and experimental groups of rats. Tissue sections were immunostained with the ERK-2 antibody and a phycoerythrin (PE) conjugated secondary antibody (*orange*). Tissue sections were also counterstained

with DAPI (*blue*) to provide nuclear staining. (Scale bar-100 μ m). Slides were visualized under a fluorescent microscope (Nikon TE 300) using Excitation wavelength/Emission wavelength of 345 nm/456 nm for DAPI and 488 nm/578 nm for PE

Fig. 10 Western blot analyses of ERK-2 and Akt in the colon of control and experimental groups of rats. **A.** Western blot analyses were performed with antibodies against ERK-2, Akt and β -actin as mentioned in materials and methods. Lane 1-Control, Lane 2-AST alone treated group, Lane 3-DMH-induced group, Lane 4-DMH + AST (Pre-initiation), Lane 5-DMH + AST (Post-initiation). **B.** Quantitative data expressing the corresponding protein levels was assessed using densitometry and is expressed in relative intensity (arbitrary unit). Hypothesis testing method included one-way analysis of variance (ANOVA) followed by least significant difference (LSD). Values are given statistically significant at $P < 0.05$; Values not sharing a common superscript letter (a–d) differ significantly



showed increased expressions of Caspase-3 (20 kDa) when compared to DMH-induced group. Control and AST-alone treated groups of rats did exhibit few expressions of caspase-3. Quantitative data expressing the corresponding protein levels were assessed using densitometer and is expressed in relative intensity arbitrary unit.

Discussion

As colorectal cancer becomes a serious health threat in many countries, a search has been carried out for phytochemicals that may show therapeutic potential to decrease the burden of this disease. Astaxanthin is a red pigment common to several aquatic organisms including algae, salmon, trout, and shrimp, the most common source of astaxanthin used in dietary supplements is from *Haematococcus* algae [43, 44]. The United States Food and Drug Administration approved astaxanthin as a nutraceutical agent [45]. In the present study, we evaluated the anti-inflammatory and apoptosis inducing effects of astaxanthin in colon cancer.

Chemoprevention, use of naturally occurring and synthetic agents, is an important and practical strategy for the management of cancer [46]. AST inhibiting the production of inflammatory mediators by blocking NF- κ B activation *in vitro* has been reported [47], though its effect on *in vivo*

system remains vague. To elucidate the transcriptional mechanisms underlying the anti-inflammatory effects, we further examined the effect of astaxanthin on the DNA-binding activities of NF- κ B in DMH-induced rat colon. NF- κ B is one of the prime components of the intracellular signaling pathways responsible for the up-regulation of pro-inflammatory proteins [48]. It has been reported that the DNA binding and transcriptional activities of NF- κ B in nucleus are easily influenced by the cellular redox status, and play a critical role in pro-inflammatory and carcinogenic events [49, 50]. We found that DMH induction apparently stimulates the DNA binding activity of NF- κ B and the expression of their subunits (p65), whereas these events were markedly inhibited by treatment with AST. Recently, we have reported that AST inhibited the proliferative activity in DMH-induced colon carcinogenesis. Our study is the first to demonstrate that AST suppresses NF- κ B *in vivo*.

COX-2 is induced during inflammation, up-regulated in adenomas and over-expressed in colon cancer [16, 51, 52]. It is possible that enzymatic action of COX-2, apparently one of the prostaglandins, alter cell growth, apoptosis, angiogenesis, or other steps that lead to cancer. We observed that DMH-induced rats expressed increased expression of COX-2. Increased levels of COX-2 lower the intracellular levels of free arachidonic acid, thereby preventing apoptosis, thus facilitating the progression of cancer. AST treated rats significantly inhibited the expression of COX-2, consequent-

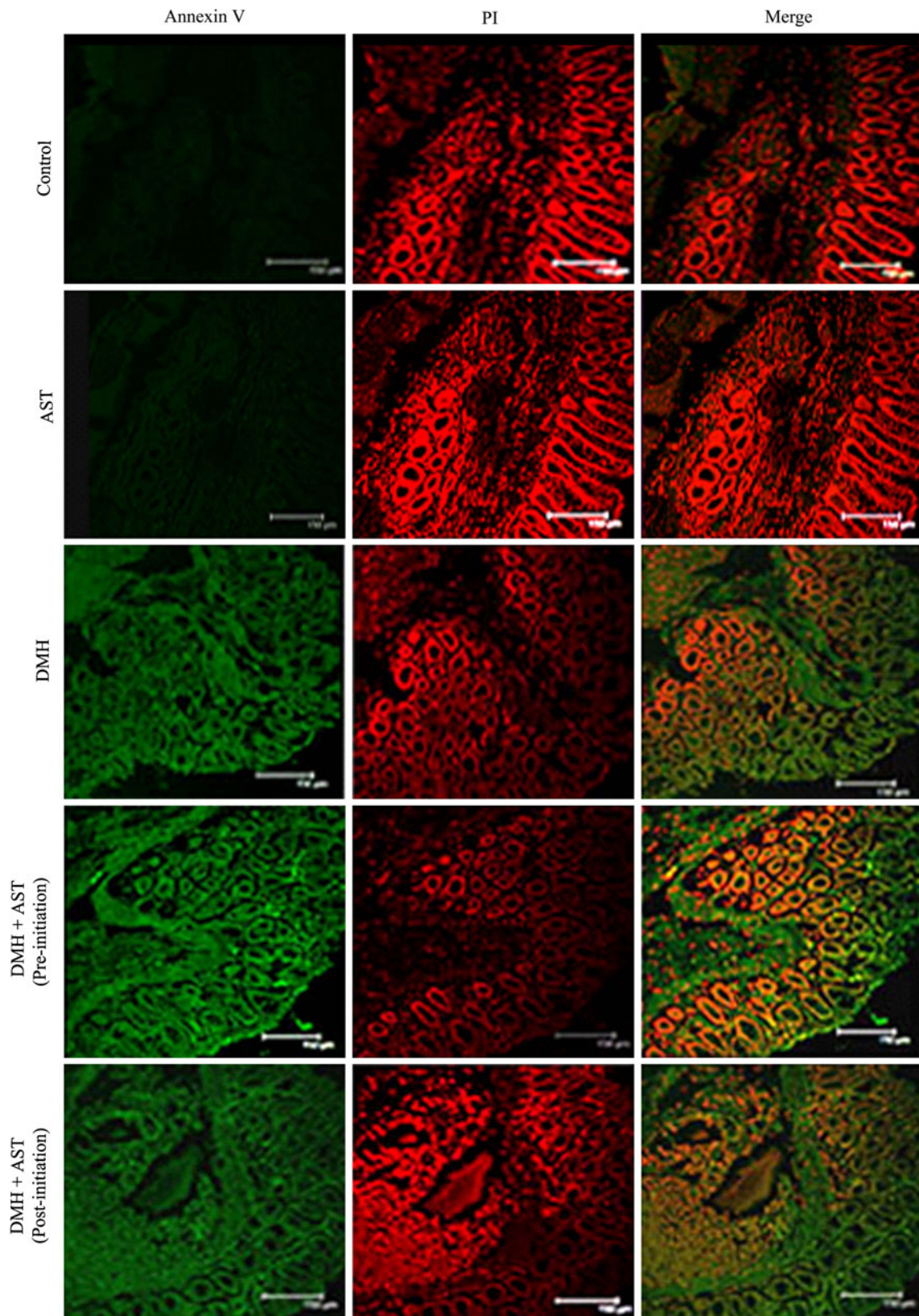


Fig. 11 Confocal microscopic analysis of apoptosis in the colon of control and experimental groups of rats. Tissue sections (scale bar - 75 μm) were analysed for apoptosis in a Annexin-V/FITC apoptosis detection kit as mentioned in materials and methods. Images of PI—red (excitation 529 nm; emission 550 nm) and annexin-V/FITC (excitation 488 nm; emission 520 nm) stained sections were visualized in a confocal microscopy (Leica TCS-SP2 XL)

ly maintaining increased levels of free arachidonic acid in the cells, which might have resulted in the suppression of carcinogenesis. PCNA is non-histone nuclear acidic protein expressed in the nuclei of proliferating cells during G_1 and S-phase of cell cycle. It is an important biomarker in gastrointestinal cancer [53, 54]. In this study, DMH administered rats exhibited increased expression of PCNA in the colon thereby indicating the hyper proliferative activity of tumor cells. The extent of carcinogenicity can be reduced by inhibiting the cellular proliferation. The higher expression of PCNA protein was closely related to increased proliferation, because it plays a vital role in nucleic acid metabolism as a part of the replication [54]. Decreased expression of this proliferative marker was clearly noted upon AST treatment. Carotenoids suppressing PCNA in oral carcinogenesis has recently been documented, which supports our study [55].

Mast cells contribute to the progression of various types of tumors [56]. Mast cells mediate tumor cell apoptosis through secretion of tumor necrosis factor or by production of reactive peroxides [57, 58]. In this study, AST-treatment

reduces mast cell infiltration in DMH-induced rat colon, there by triggering apoptosis, which is in agreement with the previous reports [59, 60]. MMPs contribute to the formation of a complex microenvironment that promotes malignant transformation and cancer progression [36, 61]. Beside their ECM remodelling functions, MMPs have cell signaling capabilities and can regulate the functions of bioactive molecules which are involved in tumour growth [62, 63]. An increase in the levels of MMP-2 and MMP-9 were observed in DMH-induced groups of rats. AST treatment decreased the levels of MMP-2 and MMP-9, which might be the reason for its anti-carcinogenic effect, because the local spreading is one of the primary reasons for mortality in colorectal cancer. In the present study, it can be speculated that this increased mast cells are responsible for the increased levels of MMP-2 and MMP-9 in the DMH induced rats. AST treatment resulted in decreased mast cell number thereby might have decreased the expressions of MMPs-2/9.

ERK are well established signaling components of Mitogen activated protein (MAP) kinases that regulates apoptotic signaling cascade [64] and documented reports denotes that Akt, NF- κB , MMP-9 and MMP-2 play important roles in promoting proliferation, migration, invasion, angiogenesis, and metastasis of cancer cells [65–68], which directly modulate the phospho-active levels of pro-apoptotic factors. Our results show that the expression of ERK-2 was critical in the development of colon cancer. In this study, AST

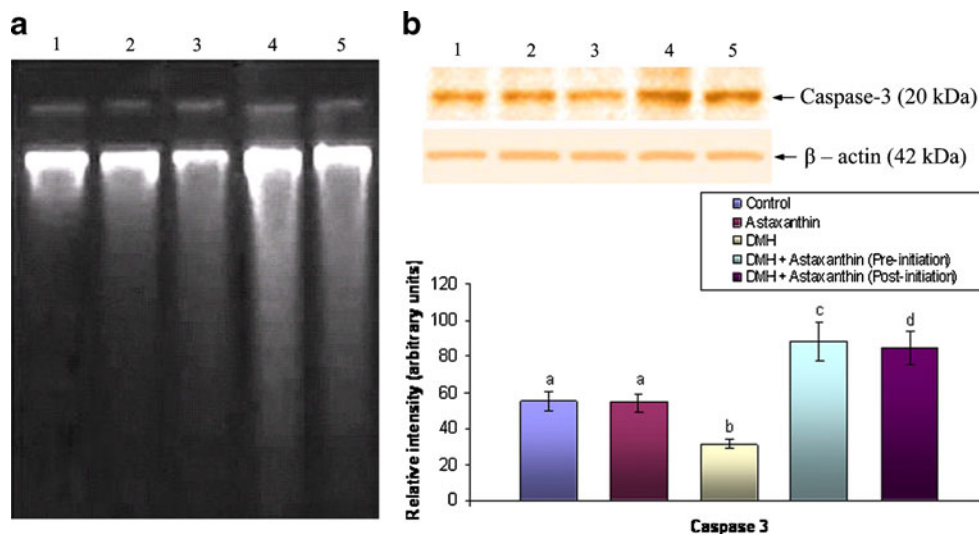
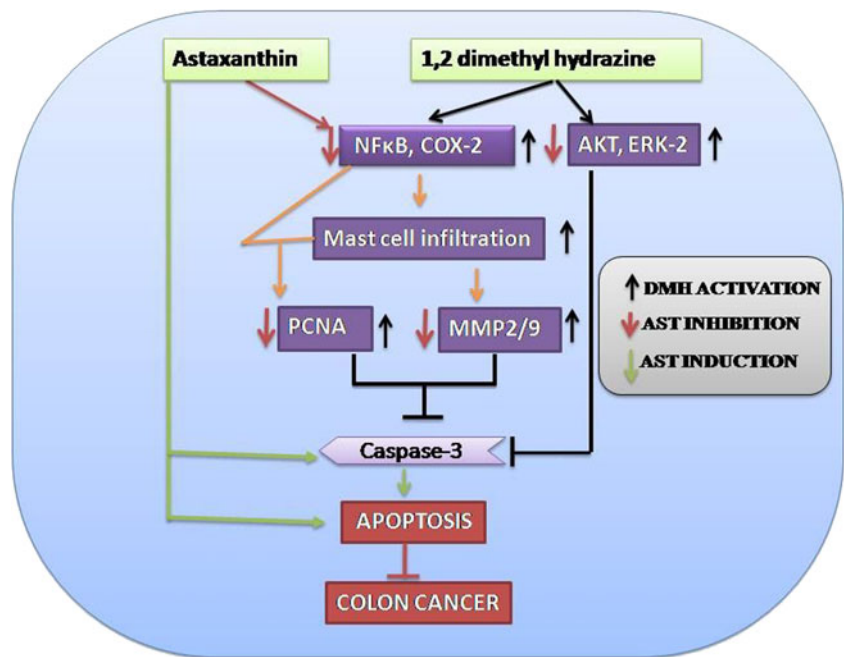


Fig. 12 Assessment of apoptosis by DNA fragmentation analysis and Western blot analysis of Caspase-3 in the colon of control and experimental groups of rats. **A.** Lane 1-Control, Lane 2-AST alone treated group, Lane 3-DMH-induced group, Lane 4-DMH + AST (Pre-initiation), Lane 5-DMH + AST (Post-initiation). **B.** Western blot analysis was performed with antibodies against caspase-3 or β -actin as indicated. Lane 1-Control, Lane 2-AST alone treated group, Lane 3-DMH-induced group, Lane 4-DMH + AST (Pre-initiation), Lane 5-

DMH + AST (Post-initiation). **B.** Quantitative data expressing the corresponding protein levels was assessed using densitometry and is expressed in relative intensity (arbitrary unit). Hypothesis testing method included one-way analysis of variance (ANOVA) followed by least significant difference (LSD). Values are given statistically significant at $P < 0.05$; Values not sharing a common superscript letter (a–d) differ significantly

Fig. 13 Schematic representation of the possible mechanism of action of AST during DMH induced colon carcinogenesis. DMH induction increased the expressions of NF κ B, COX-2, ERK and Akt. Increased expressions of these proteins lead to an increase in mast cell infiltration, which further triggers PCNA and MMPs2/9. AST reduces the expressions of these key proteins that are mediators of colon carcinogenesis and induces apoptosis in colon carcinogenesis



significantly modulated the expressions of ERK-2, however the effect of AST on Akt needs further investigation. Defects in the cascade of apoptosis-related events during neoplastic development could well affect the execution of apoptotic death and disrupt homeostasis regulation of the colonic tissue. Novel anticancer drugs induces apoptosis in cancer cells and apoptotic dysfunction leads to the progression of cancer [26]. Caspase-3 is the key executioner of apoptosis and its active form is essential in breaking cellular components connected with DNA repair and regulation. It has also been induced in various conditions, such as anticancer drug treatment [69, 70]. In this study, AST possesses anticancer effect by inducing apoptosis in DMH-induced rat colon. Certain plant derived components are known to induce apoptosis in colon cancers has been well documented, in agreement with this study [71, 72].

Taken together, our results demonstrate for the first time, that the apoptosis inducing effect of AST in DMH-induced rat colon carcinogenesis could be through the regulated expressions of NF κ B, COX-2, MMPs2/9, PCNA and ERK-2 (Fig. 13). However, the results of this study are limited and further exploration of tumor associated signaling cascades in depth are warranted to elucidate the mechanisms involved in the protective role of AST against colon cancer.

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