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ORIGINAL ARTICLE

Althea rosea Seed Extract Ameliorates 1,2-Dimethylhydrazine Induced Preneoplastic Lesions in Mouse Model of Colon Cancer by Modulating Oxidative Stress and Inflammation

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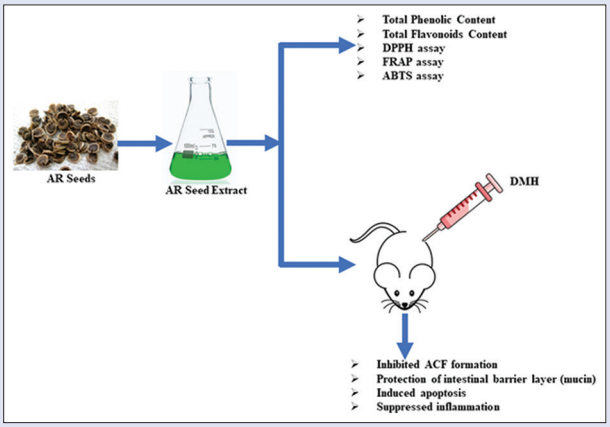
ABSTRACT

Background: Phytochemicals with strong antioxidant and anti-inflammatory properties are known to modulate the process of carcinogenesis. *Althea rosea* (AR) is an ornamental plant and is an integral part of traditional medicine for curing a wide range of inflammatory disorders such as asthma, inflammatory bowel diseases, and arthritis. Therefore, its potential as a chemopreventive agent in cancer needs to be evaluated using an appropriate animal model. **Materials and Methods:** In this study, different *in vitro* assays including total phenolic content, 1,1-diphenyl-2-picrylhydrazyl, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonate), and ferric reducing antioxidant power were used to evaluate the antioxidant capacity of AR seed extract. In addition, *in vivo* study at two different doses, i.e., 100 and 200 mg/kg body weight, was also conducted to analyze the chemopreventive potential of AR seed extract. The chemopreventive efficacy of AR seed extract was assessed by analysis of aberrant crypt foci (ACF), goblet cells/crypt, apoptotic index, and nuclear factor-kappa B (NF-κB) signaling pathway. **Results:** The results of *in vitro* assays suggested that AR seed extract exhibits a strong antioxidant potential. Administration of AR seed extract to 1,2-dimethylhydrazine group animals resulted in a marked reduction in ACF number, lymphocytic infiltration, and erosion of mucin layer from the intestinal epithelium. AR seed extract induced apoptosis in colonocytes as evident from the analysis of cleaved caspase-3, Bcl-2, and poly (ADP-ribose) polymerase 1/2 expression. Furthermore, treatment with AR seed extract inhibited the expression of NF-κB, a central mediator of chronic inflammation. The AR seed extract also ameliorated the damaging effects of oxidative stress by decreasing free radical generation and increasing the levels of enzymatic and non-enzymatic antioxidants. **Conclusion:** Taken together, these findings emphasized that AR seed extract could be considered as promising natural chemopreventive against colon carcinogenesis and should be further evaluated for the identification of active principle(s).

Key words: *Althea rosea,* apoptosis, colorectal cancer, inflammation, oxidative stress

SUMMARY

- Plant-based chemopreventive strategies are newfangled and have gained substantial public awareness in the recent times. Despite the piles of literature on numerous natural compounds for their anticancer properties, the pursuit of an ideal and effective chemopreventive agent is not yet accomplished. The current study is an attempt to address the chemopreventive potential of seeds derived from an ornamental plant, i.e., *Althea rosea* (AR) Linn. Both *in vitro* and *in vivo* studies have shown significant antioxidant potential of AR seed extract. Prior treatment with AR seed extract was capable to inhibit ACF formation by inducing apoptosis as indicated by caspase-3 levels and poly (ADP-ribose) polymerase cleavage. Inhibition of lymphocytic infiltration and nuclear factor-kappa B levels on administration of AR seed extract emphasizes its anti-inflammatory properties to resolve chronic inflammation. The chemopreventive properties of AR seed extract were attributed to its strong free scavenging capacity during early stages of colon cancer development. Collectively, results of the present study demonstrate that AR seeds are a rich source of natural antioxidants and regular consumption of these seeds can be an effective approach to combat colon carcinogenesis. Identification of bioactive principle(s) of AR seed extract is under progress which could be helpful in the development of chemopreventive/chemotherapeutics for colon cancer.



Abbreviations used: DMSO: Dimethyl sulfoxide; DMH: 1,2-Dimethylhydrazine; HBSS: Hank's buffered salt solution; TPC: Total phenolic content; TPTZ: 2,4,6-Tri (2-pyridyl)-s-triazine; FRAP: Ferric reducing antioxidant power; DPPH: 1,1-diphenyl-2-picrylhydrazyl; ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonate); ACF: Aberrant crypt foci; PBS: Phosphate-buffered saline; TBS: Tris-Buffered Saline; PAS: Periodic acid–Schiff; AB: Alcian blue; NF-κB: Nuclear factor-kappa B; Bcl-2: B-cell lymphoma 2.

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INTRODUCTION

Despite the remarkable progress in understanding the risk factors and critical events leading to neoplastic transformation, limited success has been achieved so far in the management of cancer. In addition, the standard therapeutic agents for colon cancer such as 5-fluorouracil, capecitabine, and cetuximab are not cost-effective and exhibit substantial side effects including cardiomyopathy, neutropenia, and thrombocytopenia.[1] Several epidemiological studies have highlighted the beneficial effects of regular consumption of fruits and vegetables in reducing the risk of various human ailments including colon cancer.[2] Recently, phytochemicals, the non-nutritive components of plant(s), have been attracting attention of the researchers worldwide for their colon cancer prevention properties.[3] In this context, chemoprevention seems to be an alternative approach for the management of colon cancer. Phytochemical analysis revealed the presence of a particular type of polyphenols, flavonoids, terpenoids, and alkaloids in these fruits, vegetables, and dietary components. Several phytochemicals such as curcumin,[4] resveratrol,[5] piperlongumine,[6] and piperine[7,8] have shown convincing anticancer potential in various *in vitro* and *in vivo* studies. These bioactive compounds target perturbed cellular processes such as apoptosis, cell cycle, cell proliferation, DNA repair, drug metabolism, activity of oncogenes, and tumor suppressor genes.[9,10] In addition, amelioration of carcinogen-mediated oxidative stress is one of the most common mechanisms by which phytochemicals exert anticancer effect. Several studies have shown that free oxygen radicals are among the critical determinants involved in cancer formation, invasion, and metastatic spread.[11] Free radicals react with different cellular components such as membrane lipids, proteins, and nucleic acids and thereby alter cellular homeostasis. In addition, reactive oxygen species also increase the expression of nitric oxide synthase which further elevates the cellular metabolic rates.[12] These species also react with protein tyrosine kinases and cause a second wave of lipid peroxidation which further activates a programmed death cascade. Lipid peroxidation is not only considered as a culprit for erroneous DNA replication which alters cell fate but is also responsible for the modulation of membrane characteristics.[13] Therefore, suppression of oxidative stress may play an important role in the chemoprevention of colon cancer.

Althea rosea (AR) Linn., popularly known as hollyhock, is an ornamental plant that belongs to Malvaceae family. It is widely grown in gardens and parks in Southern Europe and Asia. Traditionally, different parts of AR plant have been used for the treatment of various human ailments in folk medicine.[14] Several biological activities such as antiurolithiatic, diuretic, anti-inflammatory, antibacterial, hepatoprotective, analgesic, and cytotoxic activities have been reported for AR.[15,16] Recently, AR flower extract has also been demonstrated to exhibit antiproliferative properties in cancer cell lines by inhibiting cell proliferation as well as stemness of cancer cells.[17] As per our knowledge, no *in vivo* study has been reported till date demonstrating the chemopreventive potential of AR seed extract in colon cancer formation. Backdrop of the above, the present study was aimed to investigate the chemopreventive efficacy and elucidate the underlying mechanism of action of AR seed extract on 1,2-dimethylhydrazine (DMH)-induced preneoplastic lesions in Balb/c mice.

MATERIALS AND METHODS

Chemicals

N, N′-dimethylhydrazine dihydrochloride (DMH), bovine serum albumin (BSA), dithiothreitol (DTT), ammonium persulfate, N, N,N',N'-tetramethylethylenediamine, phenylmethanesulfonyl fluoride (PMSF), gallic acid, $NaNO_2$, $AlCl_3$, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,4,6-tris (2-pyridyl)-*s*-triazine (TPTZ), Hoechst 33342, propidium iodide dye, and $FeCl_3$ were purchased from Sigma Chemical Company (St. Louis, USA). Primary antibody against β-actin, cleaved caspase-3, nuclear factor-kappa B (NF-κB) (p65 subunit), and HRP-labeled secondary antibody raised in rabbit was purchased from Cell Signaling Technology (Beverly, USA). Primary antibody against poly (ADP-ribose) polymerase (PARP) ½ was procured from Santa Cruz Biotechnology (Minneapolis, USA). Acrylamide, bisacrylamide, polysorbate 20, Triton X-100, glycine, Tris ultra-pure grade, and isopropyl alcohol were obtained from MP Biomedicals (Strasbourg, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from HiMedia (Mumbai, India). Folin–Ciocalteu (FC) reagent was purchased from SISCO Research Laboratories (Pune, India).

Plant material

The seeds of AR were bought from local nursery at Chandigarh, India, and were authenticated by Dr. Anju Rao, Associate Professor, Department of Botany, Panjab University, Chandigarh.

Preparation of extract

AR seeds were dried in shade and powdered using mortar and pestle. The seed powder was then passed through sieve of 0.3-mm mesh size and was defatted using petroleum ether by Soxhlet extraction at 50°C for 24 h. The defatted powder was vacuum dried and was subjected to Soxhlet extraction again for 72 h using absolute ethanol as solvent. The solvent was evaporated using Hei-VAP rotary evaporator (Heidolph, Germany) to obtain crude extract. AR extract, thus obtained, was dissolved in normal saline for further *in vitro* assays and experimental procedures.

Phenols and flavonoids content in *Althea rosea* seed extract

Determination of total phenolic content

The total phenolic content in AR seed extract was estimated by FC method.[18] Briefly, AR seed extract was mixed with FC reagent in alkaline medium in dark for 2 h at 37°C and absorbance was measured at 765 nm. Gallic acid (0.1 mg/ml) was used as standard, and results were expressed in mg GAE/g of dry extract where GAE stands for gallic acid equivalents.

Determination of total flavonoids

Total flavonoid content was estimated by colorimetric method using aluminum chloride with some modifications.[19] Flavones and flavonols form stable acid complexes of pink color on addition of $AlCl_3$. Briefly, AR extract was mixed with 10% $AlCl_3$ and 5% $NaNO_2$ solution. The reaction was incubated for 5 min at room temperature, and the absorbance was measured at 510 nm. Quercetin (0.1 mg/ml) was used as standard for calibration, and the results were expressed as "mg QE/g of dry extract" where QE stands for quercetin equivalents.

Antioxidant properties of *Althea rosea* seed extract

Ferric reducing antioxidant power assay

The ability of AR seed extract to reduce ferric ions was measured using the method described by Benzie and Strain.[20] AR seed extract was mixed with ferric reducing antioxidant power (FRAP) reagent containing TPTZ, and the absorbance was read at 0 min and 30 min at

593 nm. Ascorbic acid (1 mM) was used as standard antioxidant, and the antioxidant capacity of the extract was expressed as "mM AAE/gram of dry extract where AAE stands for ascorbic acid equivalents.

2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonate) assay

ABTS assay was conducted as per the procedure described earlier with slight modifications.[21] Briefly, ABTS radical cations ($ABTS^{+}$) were produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate and stored in the dark at room temperature for 12–16 h before use. $ABTS^{+}$ solution was then diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. After addition of extract or ascorbic acid standard to 1 mL of diluted $ABTS^{+}$ solution, the absorbance was measured after 6 min at 734 nm using UV-VIS spectrophotometer (Shimadzu, USA). Results were expressed as "mM AAE/g of dry extract".

1,1-diphenyl-2-picrylhydrazyl assay

The free radical scavenging activity of AR seed extract was evaluated by DPPH assay according to a previously reported method.[22] Briefly, AR seed extract was mixed with 0.1 mM solution of DPPH. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Shimadzu, USA). Ascorbic acid was used as the standard (1 mM), and results were expressed in mM AAE/g of dry extract.

In vivo study

Animal ethical approval

The experimental protocols proposed for animal studies were approved by the Institutional Ethics Committee (Approval No. PU/IAEC/S/16/48). Male Balb/c mice weighing 20–25 g were procured from the Central Animal House of Panjab University, Chandigarh. The animals were housed in polypropylene cages bedded with sterilized rice husk and acclimatized for a week before the commencement of the study. All the experiments were conducted according to the Indian National Science Academy guidelines for the use and care of animals in scientific research. The animals were fed on standard mouse chow pellet diet (Ashirwad Industries, Punjab, India) and water *ad libitum*.

Experimental design

The animals were equally divided into 5 groups ($n = 25$)

- Group 1 (control)
  The animals of this group received a weekly injection of 1 mM EDTA (vehicle for DMH) for a period of four weeks.
- Group 2 (DMH treated)
  The animals were given weekly injection of DMH dissolved in EDTA (20 mg/kg body weight) for a period of 4 weeks.
- Group 3 (DMH treated + AR extract 1)
  The animals of this group received a dose of AR extract (100 mg/kg body weight) daily for a period of 14 days before the first injection of DMH and continued till the last dose of DMH.
- Group 4 (DMH treated + AR extract 2)
  The animals were given higher dose of AR extract, i.e., 200 mg/kg body weight, and rest of the treatment regimen was, as mentioned in Group 3.
- Group 5 (extract only).

The animals were given AR extract only (200 mg/kg body weight) daily for a period of 6 weeks. The dose of AR extract was based on an earlier study which reported its anticancer activity in mouse xenograft model at a dose of 200 mg/kg body weight.[17] All the animals were kept for a total period of 8 weeks and sacrificed by cervical dislocation.

Aberrant crypt foci analysis

The excised colons from each group were flattened on cardboard paper and fixed in 10% buffered formalin for 12–16 h. Next day, the colons were washed with phosphate-buffered saline (PBS) and stained by dipping in methylene blue (0.2%) for 2–3 min and subsequently photographed under microscope at ×4 magnification. The aberrant crypts were recognized by their abnormal size and slit-like opening compared to oval opening in disease-free animals. The images were acquired using a fluorescent microscope (Nikon Eclipse 80*i*) and analyzed using Northern Eclipse imaging Elements-D (NIS-D) software.

Histopathological examination

The colon was removed and washed with PBS, and 3–4 cm of distal colon was fixed in 10% buffered formalin. The colon tissue sections of 4–5 μm thickness were prepared and stained with standard hematoxylin and eosin staining method. Four random images were taken from each group and were evaluated by an authenticated pathologist without any prior knowledge of the experimental design.

Mucin production and goblet cell count

Alcian blue (AB) pH 2.5 and Periodic acid–Schiff's (PAS) reagent staining protocols were followed for the visualization of mucin layer and goblet cell number, respectively, in different groups. Photomicrographs were captured using a microscope attached with camera (Nikon Eclipse 80*i*) and analyzed with NIS-D software. Goblet cells were counted in each crypt for at least 10 crypts selected randomly per group and were expressed as average "goblet cells/crypt" in different groups.

Evaluation of apoptosis

Isolation of colonocytes

The colonocytes were isolated by the method of Sanders *et al.* (2004) with slight modifications.[23] The entire colon was cut longitudinally to expose lumen and placed in warm Ca^{2+} and Mg^{2+} free Hank's buffered salt solution (HBSS) containing 30 mM EDTA, 5 mM DTT, and 0.1% BSA. After 15 min shaking incubation at 37°C, the colon was transferred into warm HBSS containing 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 0.5% BSA, 10% FBS, and dispase (1.2 mg/ml) for 30 min. The mucosal side of the colon was then gently scraped and the cellular suspension was filtered through a nylon filter (70 μm) to obtain single-cell suspension. The isolated colonocytes were then centrifuged at 2,000 rpm for 10 min and counted using hemocytometer (Marienfeld, LK, Germany). Trypan blue exclusion method was used to assess the viability of isolated colonocytes.[24] A viability index of 85%–90% was kept as standard to perform experiment in the current study.

Morphological assessment of apoptotic cells

To assess the potential of AR seed extract to induce apoptosis, Hoechst 33342/propidium iodide staining method was performed. Briefly, approximately 1×10^6 freshly isolated colonocytes were incubated with 1 μM Hoechst dye for 30 min at room temperature. The cells were washed with PBS and then were incubated with 1 μM PI for 5 min at room temperature. The cells were allowed to immobilize on poly-L-lysine-coated glass slides for 5 min. The images from the same field were taken using fluorescent microscope (Nikon Eclipse 80*i*) for both red (propidium iodide) and blue fluorescence (Hoechst 33342). The images were then merged using NIS-D software. The cells with blue fluorescence were counted as normal while cells with pink fluorescence were counted as apoptotic cells. Four randomly selected fields from each group were analyzed to calculate apoptotic index.

Analysis of markers of apoptosis and DNA fragmentation

From Hoechst/PI staining results, it was evident that AR seed extract is capable of inducing apoptosis. Taking this forward, we decided to investigate the effect of AR extract on levels of key regulatory protein involved in apoptosis, i.e., active caspase-3 (17 kDa fragment) as well as DNA fragmentation (cleaved PARP) through Western blotting. Briefly, the distal end of the colon was homogenized in ice-cold lysis buffer[50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 2 mM DTT, 1 mM PMSF, 0.1% SDS, and 100 mM NaCl), 5 mM EDTA, and 0.2% EZBlock™ universal protease and phosphatase inhibitor cocktail (BioVision, USA)]. The homogenate was centrifuged at 12,000 rpm for 20 min, and the supernatant was collected as total cell lysate. The protein content was estimated by Bradford method.[25] A total of 50 µg of cell lysate from each sample was run on 12% SDS-PAGE and was electrotransferred to the PVDF membrane in an ice-cold buffer containing 25 mM Tris-HCl, 192 mM glycine, and 20% methanol for 1 h. Non-specific binding of antibodies was blocked by incubating the membrane with 3% BSA in TBS for 1 h at room temperature. After three consecutive washings with TBST, the membrane was probed with primary antibodies (caspase-3 [1:2000], PARP [1:2000], and β-actin [1:5000]) with gentle shaking for 3 h. The membrane was again washed in TBST and incubated with the respective HRP conjugated secondary antibodies for 1 h. The membrane was developed by pouring substrate (Clarity Western ECL Substrate, BIO-RAD, USA) on membrane in dark and was visualized using gel documentation system FluorChem M (ProteinSimple, USA), and densitometric analysis was performed using AlphaView software (Alpha Innotech, USA).

Effect of AR seed extract on inflammation

Activation of NF-κB signaling pathway is central to inflammation associated with neoplastic progression. Therefore, the expression of NF-κB (p65 subunit) was analyzed in the present study to assess the anti-inflammatory activity of AR seed extract. The expression of NF-κB (p65 subunit) was evaluated by Western blotting as per the protocol described earlier (NF-κB [1:2000] and β-actin [1:5000]).

Evaluation of oxidative stress in colon homogenate

The colon tissue was homogenized in ice-cold PBS and centrifuged at 4500 rpm for 15 min at 4°C. The supernatant was used to assess the levels of oxidative stress parameters such as Malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and glutathione reductase (GR).[26-31]

Toxicity profile

The liver and kidney toxicity caused either by carcinogen exposure or AR seed extract administration was assessed by estimating levels of serum aspartate transaminase (AST), serum alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine. For this purpose, fresh blood was collected from retro-orbital plexus of mice and placed in slanting position for 1 h. The clotted blood was centrifuged at 2000 g for 15 min. The clear serum was used for the assessment of all the toxicity markers using commercially available kits (Reckon Diagnostics Pvt. Ltd., Baroda, India).

Statistical analysis

The results were expressed as mean ± standard deviation (S.D). The differences between the groups were assessed by one-way analysis of variance (ANOVA) using Graphpad Prism 7 software package for windows. *Post hoc* testing was performed for intergroup comparisons using the Tukey's multiple comparison test. A value of $P < 0.05$ was considered to indicate a significant difference between groups.

RESULTS

In vitro studies

AR seed exhibited high phenol and flavonoid contents

Total phenolic content in AR extract was 6.74 ± 0.44 mg and was expressed as gallic acid equivalent in mg per g of AR seed ethanolic extract. Total flavonoid content was 31.25 ± 0.19 mg quercetin equivalent per gram of AR extract [Table 1].

Althea rosea seed extract exhibited free radical scavenging activity

AR seed extract exhibited the high potential to scavenge free radicals as evident both by DPPH and ABTS assays. The value was 26.4 ± 0.56 mM AAE/g dry extract using DPPH assay and 86.14 ± 12.42 mM AAE/g dry extract with ABTS assay [Table 1]. The reduction potential of AR seed extract was determined using FRAP assay. FRA*P* value of AR seed extract was 2.02 ± 0.04 mM AAE/g dry extract, suggesting that the extract exhibits strong reduction potential [Table 1].

In vivo studies

Effect of Althea rosea extract on average gain in body weight

The initial body weights of mice were measured at the commencement of the study and thereafter on weekly basis. The average gain in body weight was represented as a difference between final and initial body weight of animals after the completion of treatment period. No significant difference was observed in the gain in body weight of animals among different groups.

AR extract inhibited aberrant crypt foci morphology

Aberrant crypt foci (ACF) are characterized as preneoplastic lesions and are generally quantified as a measure of chemopreventive efficacy of compounds. No ACF were observed in the control animals or the animals treated with extract only. A number of ACFs were observed in the colon of DMH-treated animals along with the abnormal epithelial cell morphology. However, treatment with AR seed extract at both the doses, i.e., 100 and 200 mg/kg body weight, resulted in evident improvement in the epithelial cell arrangement and morphology, and no established ACFs were observed in comparison to DMH-treated animals [Figure 1].

Histopathological examination

Crypt architecture

The histopathological examination showed a normal colonic epithelium with intact mucosal and submucosal features in the control animals. Disrupted crypt architecture with increased lymphocytic infiltration was observed in DMH-treated animals. Treatment with AR seed extract, both at the dose of 100 and 200 mg/kg body weight, improved the crypt architecture. Long-term administration of AR seed extract even at 200 mg/kg body weight revealed no aberrations in colon architecture [Figure 2].

Table 1: Free radical scavenging activity, total phenolic, and flavonoid content of *Althea rosea* seed extract

Assay	Result
Total phenolic content	6.74±0.44 mg GAE/g extract
Total flavonoid content	31.25±0.19 mg quercetin/g extract
DPPH assay	26.4±0.56 mM AAE/g extract
ABTS assay	86.14±12.42 mM AAE/g extract
FRAP assay	2.02±0.04 mM AAE/g extract

The values are expressed as mean±SD of *n*=5 observations. SD: Standard deviation; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; ABTS: 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); FRAP: Ferric reducing antioxidant power; GAE: Gallic acid equivalent, AAE: Ascorbic acid/g extract

Mucin depleted foci and goblet cell count

Staining of histopathological sections for mucins with PAS and AB depicted that AR seed extract has a remarkable effect on mucin production and goblet cell count. In AB staining, depleted mucin layer can be observed in the DMH group and DMH + Extract 2 group, whereas intact mucin layer can be observed in control, DMH + Extract1 and Extract only groups [Figure 3]. Treatment with DMH even in preneoplastic stage showed a significant increase in goblet cell count [Figure 4]. Treatment with both the doses of extract resulted in a highly significant decrease in goblet cell count pointing to the protective effect of the extract. Treatment with only extract did not have any significant effect on goblet cell count.

Effect of *Althea rosea* seed extract on oxidative stress

As AR seed extract showed strong antioxidant potential *in vitro*, it was interesting to elucidate this activity at *in vivo* platform. MDA is the final aldehydic product of lipid peroxidation that quickly reacts with biomolecules such as lipids, proteins, nucleic acids, and carbohydrates and perturbs cellular homeostasis. A significant increase in MDA and nitrite levels in DMH-treated group as compared to control animals depicts the generation of free radicals [Table 2]. In addition, there was also a significant reduction in SOD, CAT activity, GSH levels, and an increase in GR activity in this group compared to the control group which collectively contributed to the development of oxidative stress in this group [Table 2]. On the other hand, administration of AR extract to DMH-treated animals resulted in a significant reduction in MDA and nitrite levels. Furthermore, a significant increase in the activity of SOD and CAT, GSH levels, and a decrease in GR activity was observed in DMH + AR extract as compared to the DMH group indicating antioxidant properties of the extract. Overall, the results of *in vitro* antioxidant activity assays were also validated by *in vivo* studies.

Effect of AR extract on cancer biomarkers

Apoptosis

Hoechst 33342 and propidium iodide double-staining method is a conventional method to determine the apoptotic index. Hoechst 33342 binds to A-T base pairs in DNA and helps in the identification of normal and apoptotic cells on the basis of morphology, while propidium iodide is impermeable to live cells and therefore identifies the dead cells. Treatment with DMH resulted in a significant increase in percentage of apoptotic cells (11.35 ± 3.32%) as compared to the

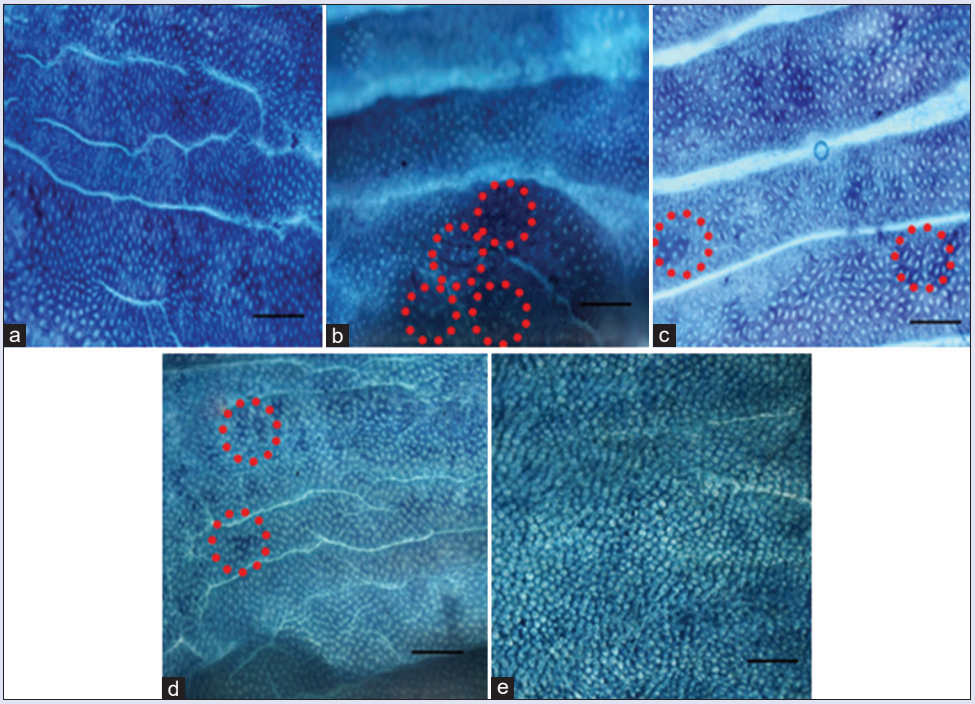


Figure 1: Light photomicrographs of the colon tissue of different groups stained in methylene blue. Dotted circles are indicating the aberrant crypt foci. (a) Control; (b) 1,2-dimethylhydrazine group; (c) 1,2-dimethylhydrazine + extract 1; (d) 1,2-dimethylhydrazine + extract 2, (e) Extract only. Scale bar: 50 µm

Table 2: Evaluation of oxidative stress-related parameters in different groups

Parameters	Control	DMH	DMH + extract 1	DMH + extract 2	Extract only
LPO (nmoles of MDA formed/min/mg of protein)	0.667±0.03	2.07±0.20	1.10±0.42	1.77±0.37	0.803±0.106
Nitrite (µmoles of nitrite formed/mg protein)	1.60±0.04	3.14±0.11	2.46±0.11	2.51±0.13	2.19±0.12
SOD (units/min/mg of protein)	14.43±1.31	3.31±1.29	10.66±1.02	8.24±0.99	12.75±2.70
CAT (µmoles of H_2O_2 decomposed/min/mg of protein)	201±19.43	115.9±22.27	168±7.9	160.10±34.57	166.7±14.12
GSH (µmoles of GSH formed/min/mg of protein)	2.23±0.105	0.69±0.05	1.18±0.23	1.05±0.25	1.85±0.33
GR (nmoles of NADPH oxidized/min/mg of protein)	1.67±0.27	3.34±0.19	2.06±0.47	3±0.52	1.87±0.21

Values are mean±SD of three observations in each group. $P<0.001$ versus control, $P<0.01$ versus control, $P<0.001$ versus DMH, $P<0.01$ versus DMH, $P<0.05$ versus DMH group, $P<0.001$ versus control. DMH: N, N'-dimethylhydrazine dihydrochloride; SD: Standard deviation; LPO: Lipid peroxide; MDA: Malondialdehyde; SOD: Superoxide dismutase; CAT: Catalase; GSH: Reduced glutathione; GR: Glutathione reductase; NADPH: Nicotinamide Adenine Dinucleotide Phosphate Hydrogen

control group (4.8 ± 0.84%) [Figure 5]. Prior treatment with AR seed extracts (100 and 200 mg/kg body weight) resulted in a significant increase in apoptotic cells as compared to DMH-treated animals, indicating induction of apoptosis by AR seed extract. We also observed an increase in apoptotic cells on treatment with AR extract. To further substantiate the results on apoptotic morphology, we analyzed the expression of active caspase-3 and PARP cleavage [Figure 6]. On treatment with DMH, there was no significant alteration in the levels of active caspase-3 and cleaved PARP as compared to control animals. However, treatment with AR extract at lower dose (100 mg/kg b. w.) led to a significant increase in the levels of active caspase-3 (17 kDa) and cleaved PARP (total/cleaved). Treatment with AR extract only did not alter any of the parameters significantly.

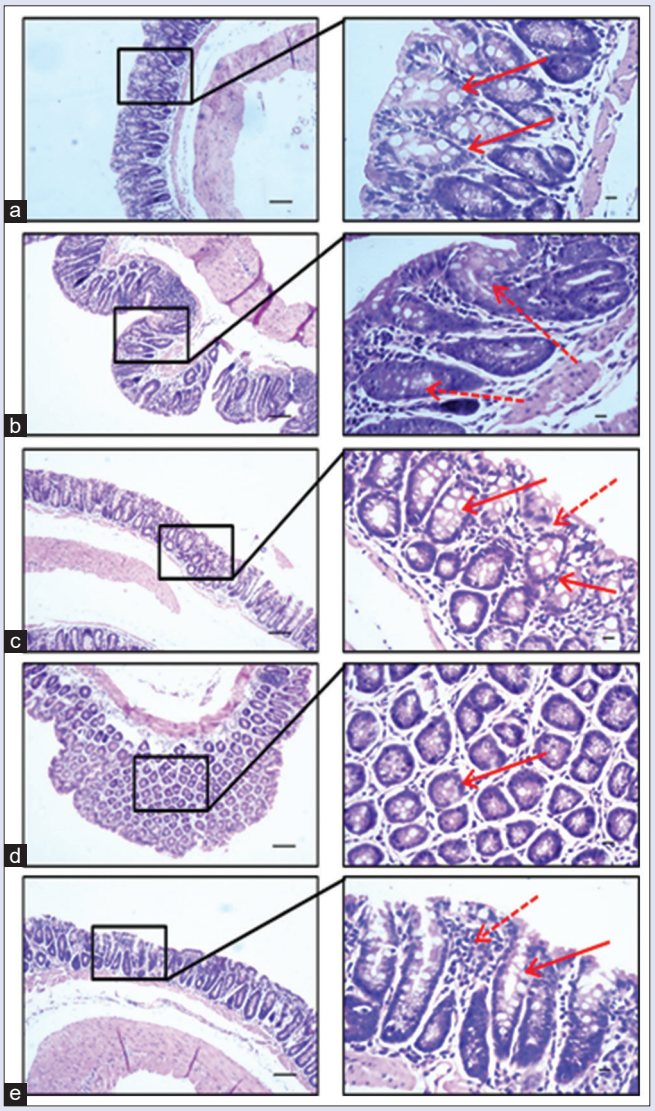


Figure 2: Light photomicrographs of hematoxylin- and eosin-stained colon sections of different groups. (a) Control; (b) 1,2-dimethylhydrazine group; (c) 1,2-dimethylhydrazine + extract 1; (d) 1,2-dimethylhydrazine + extract 2; (e) Extract only. Scale bar: 50 µm. ——→ Indicates disrupted crypt architecture. - - - -> Indicates normal crypt architecture

Anti-inflammatory effect of AR seed extract

As inflammation and cancer are intricately linked and many of the plant products exhibit chemopreventive property due to their anti-inflammatory nature, therefore, we decided to investigate the effect of AR seed extract on NF-κB expression. NF-κB is a transcription factor and its overexpression leads to the upregulation of a myriad of cellular components called mediators of inflammation. These mediators further orchestrate the process of inflammation to assist neoplastic growth. In the current study, there was a significant increase in NF-κB (p65 subunit) expression in DMH-treated animals which indicated activation of NF-κB signaling pathway in this group. Conversely, we observed a significant reduction in the levels of NF-κB (p65 subunit) on treatment with AR extract to DMH-treated animals [Figure 7]. Inhibition of NF-κB (p65 subunit) expression was found to be similar with both the doses (100 and 200 mg/kg bw). However, we also observed an increase in NF-κB (p65 subunit) expression on the administration of AR extract at 200 mg/kg body weight in animals treated with extract only.

Effect of Althea rosea seed extract on biochemical markers

To evaluate the toxicity caused by AR extract administration on liver and kidney if any, we decided to measure the relevant biochemical markers, i.e., AST, ALT, BUN, and creatinine levels. We noticed a difference in the levels of the above-mentioned biochemical markers among different groups, but the difference was not statistically significant and all the values were in the biochemical reference range [Table 3]. The results indicate that AR seed extract even at a dose of 200 mg/kg body weight did not have any potential toxic effects.

DISCUSSION

Cancer prevention strategies have garnered worldwide attention and have been the major focus of scientific exploration to combat the ever-increasing burden of cancer. A wide range of phytochemicals derived from fruits, vegetables, spices, and dietary and herbal supplements have been proven to exhibit chemopreventive potential by pleiotropic mechanisms.[32,33] The present study was designed to evaluate the chemopreventive potential of ethanolic extract of AR seed extract in DMH-induced preneoplastic lesions.

Colon cancer is a complex and multistage process that involves a series of pathological changes ranging from characteristic microscopic mucosal lesions such as ACF to malignant tumors.[34] ACF are generally considered as earliest detectable colonic lesions that are associated with

Table 3: Toxicity profile of *Althea rosea* extract

Parameters	Normal range	Control	DMH	DMH + extract 1	DMH + extract 2	Extract only
SGOT/AST (U/L)	54-298	106.08±10.0	127.29±7.5	96.26±1.3	94.58±6.2	106.08±15.1
SGPT/ALT (U/L)	17-77	52.15±6.2	68.068±3.7	60.11±10.1	60.11±2.5	50.12±5.8
Serum urea (mg/dl)	38-58	37.16±0.8	50.84±1.1	46.47±0.5	51.33±0.4	43.12±1.2
BUN (mg/dl)	8-33	17.35±0.3	23.74±0.4	18.05±0.1	21.70±0.2	20.13±0.5
Creatinine (mg/dl)	0.2-0.9	0.63±0.1	1.49±0.1	0.94±0.1	0.78±0.1	0.65±0.1

Values are mean±SD of three observations in each group. $P<0.01$ versus control group, $P<0.05$ versus DMH group, $P<0.01$ versus DMH + extract 1. AST: Aspartate aminotransferase; SGOT: Serum glutamic-oxaloacetic transaminase; SGPT: Serum glutamic-pyruvic transaminase; SD: Standard deviation; BUN: Blood urea nitrogen; DMH: N, N'-dimethylhydrazine dihydrochloride; ALT: Alanine aminotransferase

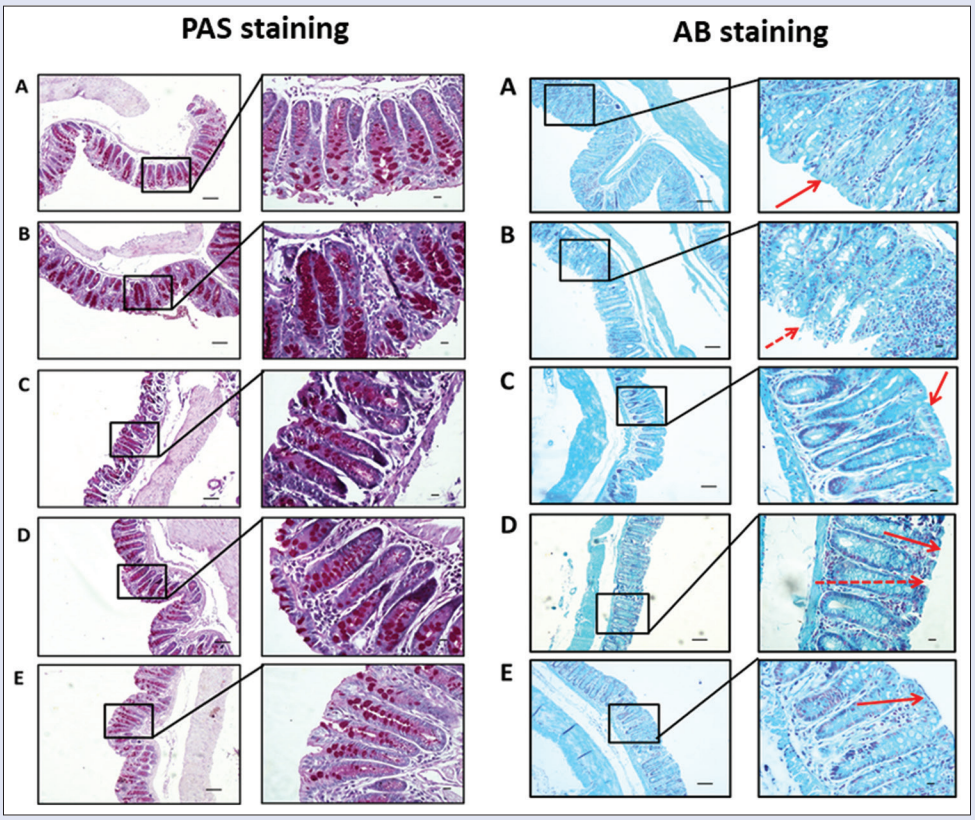


Figure 3: Light photomicrographs of the colon tissue of different groups stained with PAS and alcian blue. (A) Control; (B) 1,2-dimethylhydrazine group; (C) 1,2-dimethylhydrazine + extract 1; (D) 1,2-dimethylhydrazine + extract 2, (E) Extract only. Scale bar: 50 µm. ⟶ Indicates normal and intact mucin layer. - - - -> Indicates depleted mucin layer

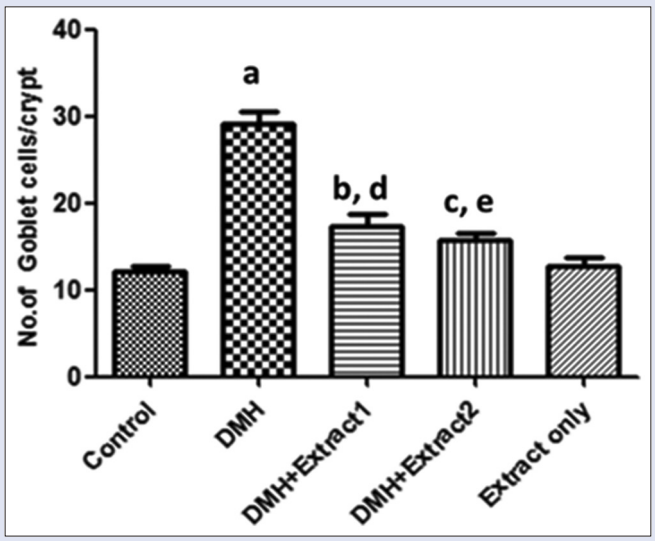
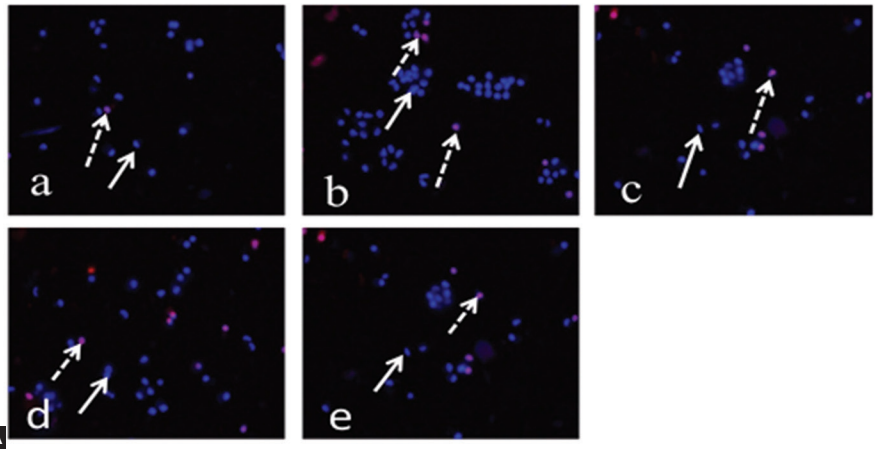


Figure 4: Graphical representation of the goblet cell count/crypt in different groups. Values are mean ± standard deviation of three observations in each group. [a]$P < 0.001$ versus control, [b]$P < 0.01$ versus control, [c]$P < 0.05$ versus control, [d]$P < 0.05$ versus 1,2-dimethylhydrazine, [e]$P < 0.001$ versus 1,2-dimethylhydrazine

increased risk of colorectal cancer development.[35] Due to morphological and genetic similarities in the development of ACF in rodents and humans, these preneoplastic lesions are used in cancer screening and evaluation of the protective effect of various chemopreventive agents. In addition to ACF, the depletion of mucin from the colon epithelial layer is also a recognized feature of CRC initiation.[36] In this study, we used a known colon carcinogen, DMH, to initiate the process of carcinogenesis and formation of preneoplastic lesions in the colon. DMH is metabolized to azoxymethane that causes methylation of DNA bases in the colon epithelial layer resulting in the development of dysplastic aberrant crypts, adenoma and adenocarcinoma, the classical features of colon cancer.[37,38] We also observed ACF lesions, derangement of epithelial cells, lymphocytic infiltration, and altered crypt architecture in DMH-treated animals in the current study. However, treatment with AR seed extract resulted in improved crypt architecture, reduction in ACF, and lymphocytic infiltration. Collectively, these features indicated the protective properties of AR seed extracts against DMH-induced colonic damage.

Mucins are high molecular weight glycoproteins secreted by intestinal epithelial cells, particularly goblet cells, and provide the first line of defense against pathogens and toxins.[39] Based on histochemical staining, mucins can be classified into neutral and acidic types (including sulfo- and sialomucins) and can be differentiated based on their staining with PAS and AB, respectively. Mucin evaluation in colorectal

A



B

GROUPS	LIVE CELLS (%)	APOPTOTIC CELLS (%)
Control	95.2 ± 0.84	4.8 ± 0.84
DMH	88.65 ± 3.32	11.35 ± 3.323
DMH + Extract 1	72.5 ± 6.15 [a, d]	27.5 ± 6.15 [a, d]
DMH + Extract 2	75.7 ± 3.25 [b, e]	24.3 ± 3.25 [b, e]
Extract only	89.9 ± 3.53 [c]	10.1 ± 3.53 [c]

Figure 5: Effect of *Althea rosea* seed extract on apoptotic index by Hoechst 33342 and PI dual staining method. (A) Merged photomicrographs of colonocytes stained with Hoechst 33342 and PI; a: Control, b: 1,2-dimethylhydrazine, c: 1,2-dimethylhydrazine + extract 1, d: 1,2-dimethylhydrazine + Extract 2, e: Extract only. Faint blue color indicated live cells and pink color indicated apoptotic cells. (B) Percentage of both live and apoptotic cells in different groups. Values are mean ± standard deviation of three observations in each group. [a]$P < 0.001$ versus control, [b]$P < 0.001$ versus control, [c]$P < 0.01$ versus control, [d]$P < 0.01$ versus 1,2-dimethylhydrazine, [e]$P < 0.05$ versus 1,2-dimethylhydrazine. ⟶ Indicates live cells. - - - -> Indicates apoptotic cells

cancer is considered a valuable prognostic feature in colon cancer. An increased mucin production in colon cancer is generally correlated with poor prognosis. It has been reported that mucins play a role in tumor progression, invasion and metastasis, tumor cell survival, and escape from host immune surveillance.[40] In the present study, DMH treatment resulted in a significant increase in goblet cell number as evident from PAS staining. There was a marked disruption in the mucin layer along with inflammatory cells in colonic sections in DMH-treated animals. In addition, there was a significant increase in neutral mucins. Mucin histochemistry is an indirect evidence of the establishment of carcinoma sequence and supports our hypothesis of early neoplastic lesions. An increase in neutral mucins in colon cancer patients has been reported to be associated with poor prognosis and survival.[41] Treatment with extract at both the doses resulted in comprehensive reversal of the mucin histochemistry which is suggestive of the protection conferred by the extract.

DMH administration causes the production of free radical oxygen species that eventually cause oxidative stress.[42] The toxic effect of DMH was further substantiated by the observations in the present study. Our data indicated the free radical scavenging capacity of AR seed ethanolic extract both *in vitro* and *in vivo*. The *in vitro* antioxidant studies showed that free radical scavenging capacity of AR seed extract as assessed by DPPH, FRAP, and ABTS assays was even higher than standard antioxidants including Vitamin C, gallic acid, and quercetin. Administration of DMH led to a significant increase in MDA and NO levels. MDA is an indicator of lipid peroxidation that damages mitochondrial membrane components such as polyunsaturated fatty acids and membrane-specific proteins resulting in loss of mitochondrial integrity.[43] DMH treatment has also been shown to enhance iNOS expression and subsequently NO production.[44] The damaging effects of MDA and NO ultimately perturb the crypt architecture and redox homeostasis of the colon.[45] In the present study, AR seed extract was able to reduce oxidative stress as indicated by a significant reduction in MDA and NO levels. Our results corroborated well with Kim *et al*. demonstrating the protective effect of coenzyme Q_{10} against DMH-induced precancerous lesions in Sprague Dawley rats.[46]

Reduced glutathione is one of the major intracellular antioxidant that scavenges free radicals on oxidation to disulfide form and thereby contributes to cellular homeostasis.[47] DMH treatment has been reported to perturb the glutathione pool and a reduction in reduced glutathione levels.[48] Plant-based bioactive compounds have been reported to combat DMH-mediated depletion of cellular antioxidants including reduced glutathione, SOD, and CAT activity. Similar observations were recorded in the current study where treatment with AR seed extract led to the restoration of GSH levels as compared to the DMH group, with a compensatory decrease in the GR activity, thereby ameliorating

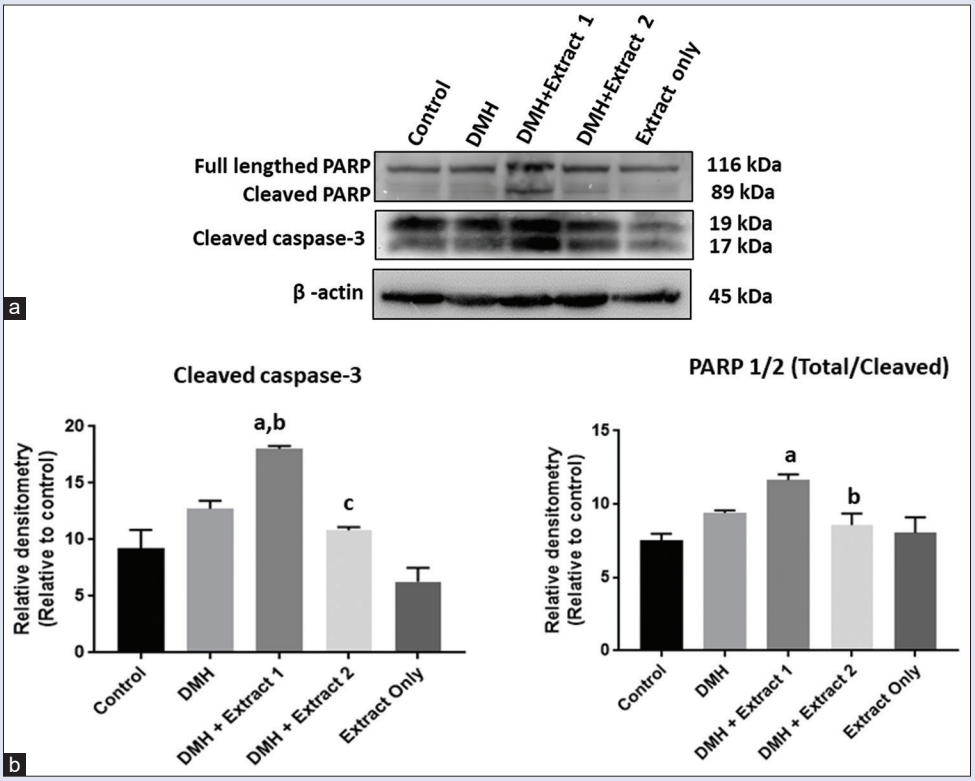


Figure 6: Effect of *Althea rosea* seed extract on apoptosis. (a) Expression of marker proteins of apoptosis, i.e., caspase-3 and PARP in different groups by Western blotting. Each band is representative for three independent experiments. For the cropped images, samples were run in the same gels under the same experimental conditions and processed in parallel; (b) Densitometric analysis of protein levels relative to internal control β-actin. Values are mean ± standard deviation of three observations in each group. [a]$P < 0.01$ versus control group, [b]$P < 0.05$ versus control group, [c]$P < 0.05$ versus 1,2-dimethylhydrazine group, [d]$P < 0.01$ versus 1,2-dimethylhydrazine group, [e]$P < 0.05$ versus 1,2-dimethylhydrazine + extract 1 group

the oxidative effect of DMH on glutathione pool.[49] In addition to glutathione, we also assessed the activity of the major antioxidant enzymes, i.e., SOD and CAT. We observed a diminished activity of SOD and CAT enzymes in the colon homogenate of DMH-treated animals which would further exacerbate the redox imbalance. Treatment with AR seed extract increased SOD and CAT activities, thereby reducing the free radical-mediated damage to cellular components such as nucleic acids, mitochondria, plasma membrane, and other cellular organelles. Furthermore, restoration of SOD and CAT activity and strong free radical scavenging activity demonstrated the way by which AR seed extract exerted its chemopreventive effect.

Apoptosis is a crucial process characterized by sequential morphological and intracellular changes including membrane blebbing, activation of caspases, and chromosomal DNA fragmentation.[50] Different apoptotic signaling cascades culminate on the activation of caspases which ultimately execute cell death in a programmed manner. In this study, analysis of apoptosis induction by AR seed extract was evaluated by quantifying the percentage of apoptotic cells as well as active caspase-3 levels in different groups which clearly indicated that AR seed extract induced apoptosis. Recently, flower extract of AR has been shown to capable to induce apoptosis in hepatocellular carcinoma HepG2 cell line.[51] The promising anticancer activity of AR flower extract was attributed to the presence of high flavonoid content. Noteworthy, we did observe high phenolic content as well as anti-neoplastic activity of AR seed extract in the current study.

DNA fragmentation triggered in response to apoptotic signal is an important event in programmed cell death. PARP plays the central role in the repair of damaged DNA by participating in different DNA repair mechanisms including base excision repair, nucleotide excision repair, and single-strand base repair. PARP is also a well-known target protein of active caspase-3, and its cleavage into two fragments of 89 kDa and 24 kDa is considered to be a signature of apoptosis. The larger fragment with greatly reduced catalytic activity is liberated from the nucleus, resulting in increased DNA fragmentation and thereby increased apoptosis.[52] In this study, DMH treatment did not cause any significant change in PARP cleavage compared to control animals which indicated negligible DNA fragmentation or apoptosis in these animals. However, a significant increase in the expression of cleaved fragment of PARP protein, i.e., 89 kDa, was noticed on treatment with AR seed extract in DMH-treated animals. Collectively, these data confirm that AR seed extract induced apoptosis; however, the effect was more pronounced with a lower dose of AR seed extract (100 mg/kg body weight).

Numerous studies have established the central position occupied by NF-κB as a major transcription factor which regulates cell proliferation, apoptosis, angiogenesis, invasion, and metastasis.[53] In the present study, DMH treatment led to a significant increase in NF-κB levels, suggestive of an increase in tumor-promoting inflammation in the colon. Enhanced lymphocytic infiltration observed in the colon sections of these animals further supported the apparent increase in NF-κB levels. Treatment with AR seed extract with both doses, i.e., 100 and 200 mg/kg body weight,

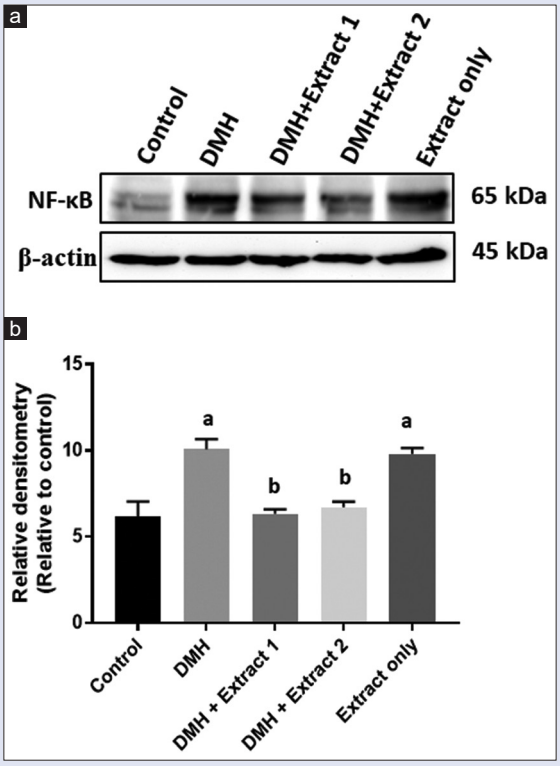


Figure 7: Effect of *Althea rosea* seed extract on inflammation using Western blotting. (a) Expression of nuclear factor-kappa B in different groups. Each band is representative for three independent experiments. For the cropped images, samples were run in the same gels under the same experimental conditions and processed in parallel. (b) Densitometric analysis of nuclear factor-kappa B protein levels relative to internal control β-actin. Values are mean ± standard deviation of three observations in each group. [a]$P < 0.01$ versus control group, [b]$P < 0.05$ versus control group, [c]$P < 0.05$ versus 1,2-dimethylhydrazine group, [d]$P < 0.01$ versus 1,2-dimethylhydrazine group, [e]$P < 0.05$ versus 1,2-dimethylhydrazine + Extract1 group

resulted in a significant reduction in NF-κB expression suggesting anti-inflammatory properties of AR seed extract. Recently, Sharma *et al.* have demonstrated the chemoprevention of DMH-induced colon cancer with morin, a plant flavonoid by suppressing NF-κB expression. The current study has also described the chemopreventive potential of AR seed extract on DMH-induced lesions in the mouse colon.

CONCLUSION

This study clearly indicated a significant potential of AR seed extract in colon cancer prevention. Our results demonstrate that AR seed extract exerts its chemopreventive effect by scavenging free radicals and inhibiting the formation of preneoplastic lesions in the colon. The study also emphasized that AR seeds are a rich source of polyphenols and its incorporation in diet can be an effective strategy for the prevention of colon cancer. In addition, identification and evaluation of active principle(s) of AR seed extract would be helpful in unraveling its mechanism of action and may provide lead compounds for drug development for the treatment of colon cancer.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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