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Original Research Article (Experimental)

Unveiling antioxidant and anti-cancer potentials of characterized *Annona reticulata* leaf extract in 1,2-dimethylhydrazine-induced colorectal cancer in Wistar rats

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ABSTRACT

Background: Colorectal cancer (CC) is the third most common cancer in the world. *Annona reticulata* (AR) also known as bullock's heart, is a traditional herb. AR leaf extract was initially investigated for its anti-bacterial, anti-inflammatory, anti-malarial, anti-helminthic, anti-stress, and wound healing properties. Only a few *in vitro* cancer studies have been conducted on AR. Although few studies have linked AR leaf extract to many cancers, comprehensive studies addressing regulation, biological functions, and molecular mechanisms leading to CC pathogenesis are clearly lacking.

Objectives: The present study aimed to explore the antioxidant and anti-cancer potentials of AR leaf extract in CC.

Materials and methods: The MTT assay was used to test the anti-proliferative activity of AR leaf extract *in vitro* on the HCT116 cell line. Qualitative and quantitative phytochemical characterization was carried out using gas chromatography: mass spectrometry (GC–MS). 1,2-dimethylhydrazine (DMH) was used to establish CC model in female Wistar rats. The acute toxicity of AR leaf extract was tested in accordance with OECD guidelines. Aberrant Crypt Foci (ACF) count, organ index, and hematological estimations were used to screen for *in vivo* anti-cancer potential. The antioxidant activity of colon homogenate was determined.

Results: The alcoholic leaf extract (IC50, 0.55 μg/ml) was found to be more potent than the aqueous extract. Using GC–MS, a total of 108 compounds were quantified in the alcoholic leaf extract. The LD 50 value was found to be safe at a dose of 98.11 mg/kg of body weight. AR alcoholic leaf extract significantly ($p < 0.05$) decreased ACF count and normalized colon length/weight ratio. AR leaf extract increased RBC, hemoglobin and platelets levels. The AR alcoholic leaf extract reduced the DMH-induced tumors and significantly ($p < 0.05$) increased the activity of endogenous antioxidant enzymes such as catalase, reduced glutathione, superoxide dismutase, and decreased the lipid peroxidase activity. AR leaf extract reduced the inflammation caused by DMH and helped to repair the colon's damaged muscle layers.

Conclusion: Based on the findings from the present study, it can be concluded that the alcoholic leaf extract of AR has antioxidant and anti-proliferative properties and can aid in the prevention of CC development and dysplasia caused by DMH.

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1. Introduction

Colorectal cancer (CC) is the third most common cancer in the world. In developing countries, the incidence and mortality rate of CC are rapidly increasing. According to the Globocan 2020 report, the incidence and mortality rate in Asia are 49.6% and 51.4%, respectively, with a 5 years prevalence of 1,436,272 (47.2%) (https://gco.iarc.fr/). The prevalence of this cancer is expected to rise to 60% by 2030 [1]. Approximately 41% of all CC occurs in the proximal colon, 22% in the distal colon, and 28% in the rectum [2]. Alcohol consumption, smoking, obesity, and meat consumption have all been identified as risk factors for CC. In addition, genetic and environmental factors play a role in the etiology of CC [3]. This cancer typically begins as a single mutation in a cell and progresses until it is detectable or as a polyp, at which point the polyp can develop into a life-threatening cancer [4]. Surgery, chemotherapy, radiotherapy, hormone therapy, immunotherapy, and newer targeted therapies are used to diagnose CC. Despite the fact that these treatments improve the outcome. There were some drawbacks and side-effects recorded; side-effects were associated with the use of chemotherapies, such as oxaliplatin (dysesthesias and renal dysfunction) or irinotecan (nausea, alopecia, and bone marrow suppression), fluorouracil (stomatitis, diarrhea, neutropenia), etc. Even if treatments allow for long survival, long-term side-effects such as gastrointestinal problems, urinary incontinence, sensory neuropathy, and sexual dysfunction have been increasing for many years. People rely heavily on traditional medicines in addition to modern medicines.

Global herbal medicine is one of the most important aspects of traditional medicine, and it is also used in cancer treatment. Approximately 70% of the world's population uses medicinal plants as a promising potential source of the bioactive anti-cancer molecule. When vinca alkaloids were discovered in the 1950s, it became possible to extract anti-cancer drugs from plants [5]. Plant-derived phytochemicals such as terpenoids, glycosides, phenolics, flavonoids, and alkaloids exhibit meticulous physiological results. These effects often include positive beneficial effects [6]. In most cases, herbal treatment does not depend on the individual, age, or gender.

Annona reticulata L. (AR) is one such herbal source for anti-cancer activity, belonging to Annonaceae family. It is also known as bullock's heart, custard apple, netted custard apple, wild-sweetsop (English) and locally popular as 'Ramphal' in Karnataka, India. Traditionally, the plant has been used to treat bacterial infection, parasite and worm infestations, cardiac problems, dysentery, epilepsy, ulcer, hemorrhage, constipation, dysuria, as insecticide and in fever. It is also used as an anti-inflammatory, anti-malarial, anti-helminthic, anti-stress, wound healing agent, and in the treatment of diabetes, diarrhea, and dysentery [7,8]. An *in vitro* study stated that the ethanolic root extract of AR has strong anti-proliferative activity against human cancer cell lines such as HeLa, K-562, A-549 and MDA-MB [9]. Methanolic leaf extract of AR inhibited the growth of MCF-7, A549, SCC9, and HCT116 cells in a dose-dependent manner. The extract is reported to show cell cycle arrest at the G2/M phase in A549 and MCF-7 cells [10]. Another study found that AR methanolic extract was cytotoxic to colon and liver cancer cells and may have anti-cancer properties [11]. A study identified the phytochemical constituent of methanolic leaf extract of AR. They emphasized the presence of important phytochemicals such as amino acids, alkaloids, glycosides, flavonoids, steroids, proteins, triterpenoids, and phenolic compounds. The same study discovered that AR has anti-cancer activity against hepatoma (HEPG2) and human lung cancer (Hop65) cell lines [12]. According to a recent review of AR, the isolated constituent catechin and methanolic extract of AR demonstrated excellent free-radical scavenging activity as well as antioxidant activity. Acetogenins are abundant in the Annonaceae plant family. The presence of acetogenins may be responsible for AR methanol extract's anti-cancer properties [13]. Ganesh et al. investigated the acute toxicity of AR leaf extract on Swiss albino mice and found that the leaf extract is non-toxic and non-allergic, with no significant toxic effect observed. They also found that AR leaf extract 2000 mg/kg body weight is safe for oral administration [14]. Although few studies have linked AR to many cancers, comprehensive studies addressing regulation, biological functions, and molecular mechanisms leading to CC pathogenesis are clearly lacking. Thus, investigations are necessary to understand the mechanism action of AR in CC cell lines. *In vivo* studies are also needed to understand the potential impact of AR. As a result, the current study was designed to investigate the anti-cancer effect of AR leaf extract on the 1,2-dimethylhydrazine (DMH) induced CC rat model, which might relate pharmacological significance to the ethnobotanical claims of the local population.

2. Materials and methods

The current research has been approved by the Institutional Animal Ethics Committee (IAEC), Kasturba Medical College, Manipal Academy of Higher Education (MAHE) Manipal. The AR leaf was used in this study and it was collected in Parkala, Udupi district, Karnataka, India, in January 2018. The plant was authenticated by Ms. Usharani S Suvarna, Associate Professor and Head of Department of Botany, Mahatma Gandhi Memorial (MGM) College, Udupi, Karnataka, India (MGMC/2018-19/1). The availability of the plant name has been checked and confirmed with https://mpns.science.kew.org/

2.1. Reagents and chemicals

DMH has been purchased from TCI Chemicals, Chennai, India. CC cell line, Human colon cancer cell line (HCT116) was procured from NCCS, Pune and sub-cultured according to the standard protocol (http://www.atcc.org/).

2.2. Preparation of extract

The leaves were completely washed and dried under the shade and coarsely powdered using a blender. The powder was packed into two different thimbles within the Soxhlet extractor/apparatus. It was then macerated with aqueous and absolute alcohol in two different Soxhlet apparatus. Extraction/cycles were performed until the solvent became colorless. Once the extraction was completed, the solvent was removed and placed in a lyophilizer to remove the moisture content. The dried extract was stored in a desiccator until use.

2.3. Cell culture

HCT116 cells were maintained in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS). In presence of 5% CO_2, the cells were grown at 37 °C in a humidified incubator. Every 2–3 days, the media was replaced with fresh media. Once it reached the confluency of 80–90%, cell was trypsinized and seeded in different plates for the various experiments. After 24hr, the media was removed, and cells were exposed to different concentrations of AR for 48hr.

2.4. Cell viability study by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

To determine cell viability, an enzyme-based colorimetric assay was used. The MTT assay is most commonly used to estimate the cytotoxic potential of the crude extract, which can be determined using the IC50 value. Initially, 0.1×10^5 HCT116 cells/well were

seeded into a 96-well sterile tissue culture plate and allowed to attach for 24hr. Cells were then exposed to various concentrations of AR extract and incubated at 37 °C for 48 hr. After incubation, 20 μl of MTT reagent (Stock 5 mg/ml in PBS and pH −7.4) was added to the cells and incubated for 4hr at 37 °C. The medium and MTT were then aspirated, and to stabilize the formazan crystals, 100 μl of 100% DMSO was added to each well, and the plate absorbance was measured at 540 nm.

2.5. Phytochemical characterization

2.5.1. Qualitative analysis

To identify the preliminary active phytochemicals in AR's alcoholic leaf extract. The test was performed using standard procedures [15,16].

2.5.2. Quantitative analysis

The phytochemical investigation of alcoholic leaf extract of AR has been performed using Shimadzu gas chromatography: mass spectrometry (GCMS-QP2010S) instrument. The GCMS conditions were, RTX5 column with a length of 30 m and internal diameter was 0.25 mm, and the film thickness was 0.25 micron. The column oven initial 60 °C, injection mode was split, and its ratio was 4.0. Ion sources were 200 °C and temperature at the interface was 280 °C. For analysis, 1 μl of the sample was injected and the flow rate of 1 ml/min was maintained. MS values from the range of 40–500 m/z were identified. For compound recognition and conformation, the obtained MS spectrum was aligned with the NIST library. The study was performed at Analytical Research and Metallurgical Laboratories Pvt. Ltd. (ARML), Bengaluru, India.

2.6. Animal procurement

Female Wistar rats inbred at Central Animal Research Facility (CARF), MAHE, Manipal. Animals were maintained at temperature 23 ± 3 °C, 14:10 hrs dark and light cycle for adaptation to the experimental environment, and controlled humidity conditions. The animals were housed in sterile polypropylene cages with bedding made of fully clean/sterile paddy husk. Animal care and handling have been carried out in accordance with the guidelines put forth by the Institutional Animal Ethics Committee (IAEC), KMC-Manipal, MAHE-Manipal who approved the study conducted and the clearance certificate was provided with numbers: **IAEC/KMC/40/2017**.

2.7. Acute toxicity study

In order to assess the safety and to determine a safe dose of a drug, an acute toxicity analysis was carried out in compliance with recommendations of the Organization for Economic Co-operation and Development (OECD No. 425). The fractions to be tested were given to female Wistar rats who had fasted overnight. The leaf extract was given as a suspension in 0.25 percent carboxymethyl cellulose (CMC), and the dosage was determined based on the bodyweight of Wistar rats. After administration, the animals were constantly monitored for 30 min, 1 hr, 4 hr, 24 hr, and up to 14 days. The animals were then examined for gross behavioral changes using Irwin's table and a tolerable drug dose was chosen based on acute toxicity (LD50) [16].

2.8. Carcinogen preparation

The methods for inducing carcinogens with DMH are well established and have already been published. DMH was dissolved in 1 mM EDTA and the pH was adjusted to 6.5 with 1 M sodium hydroxide. Before each dosing, the DMH solution was freshly prepared. The carcinogen was injected intraperitoneally (i.p) once a week for the first 10 weeks at a dosage of 20 mg/kg and 30 mg/kg for the next 10 weeks during the 20-week induction period [16,17].

2.9. Experimental setup

The female Wistar rats were divided into five groups, each with six rats.

Group I: Healthy control = Healthy control.

Group II: DMH control = Disease control (DMH control).

Group III: Standard = Disease and 5-Fluorouracil (10 mg/kg, i.p).

Group IV: Treatment 1 = Disease and treatment with AR-5mg/kg, p.o.

Group V: Treatment 2 = Disease and treatment with AR-10 mg/kg, p.o.

2.10. Experimental procedure

Following carcinogen induction, group III received the standard drug 5-Flurouracil (10 mg/kg, i.p) once a week, while, groups IV and V received AR leaf extract 5 mg/kg and 10 mg/kg, p.o. daily respectively for 21 days [16,17].

2.11. Aberrant crypt foci (ACF) count

The experimental animals were euthanized, and a portion of the colon was dissected, opened into a longitudinal section, and washed with saline. The specimen was then fixed flat on filter paper for 24 h with 10% of formalin buffered. The colon was then stained for 5 min with 0.1% of methylene blue dye and washed with PBS, specimens were observed under a microscope with a magnification of 40× and ACF numbers were counted and measured as number/cm^2 and the images were taken under a microscope.

2.12. Colon length/weight ratio

The dissected entire colon length was measured in centimeters (cm) and weighted in grams, and the ratio was calculated as follows:

Ratio of colon length/weight = length by cm/weight in grams

2.13. Spleen and liver index

The spleen and liver, two major organs, were weighed. The organ index was calculated based on the animal's body weight:

Organ index (liver/spleen) = (organ weight in grams/animal body weight in grams)

2.14. Hematological estimation

Blood was collected from the euthanized animals via retro-orbital sinus puncture and 500 μl of blood was collected in a microcentrifuged tube containing 50 μl of 10% EDTA as an anticoagulant. A veterinary blood cell counter was used to test the blood samples for hematological parameters (Model: PCE-210VET, ERMA INC, TOKYO).

2.15. Estimation of antioxidant markers

Following the 21st dose of treatment, all animals were euthanized and the colon was isolated and weighed. A 10% colon tissue

homogenate was prepared using a homogenizer and ice-cold potassium chloride (150 mM) (Model: Yamato L.S.GL.H-21, Japan). The homogenized colon has been used for estimation antioxidants such as catalase (CAT) [18], reduced glutathione (GSH) [19], superoxide dismutase (SOD) [20], and lipid peroxidase (LPO/MDA) [21]. The methods for estimating antioxidants are well-established and have already been published.

2.16. Histopathological study

After the end of the study, the colonic tissue was dissected out and stored in 10% formalin, and tissue blocks were prepared. A thin section of tissue was stained with hematoxylin and eosin stain, and images were captured using a 4× magnification microscope, and samples were examined.

2.17. Statistical analysis

GraphPad Prism 5.03 Version (Graph Pad Software Inc., La Jolla, CA, USA) was used for data analysis and One-way variance analysis (ANOVA) was used to compare results between groups, followed by Tukey's post hoc test for statistically significant result. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cell viability study by MTT

AR aqueous and alcoholic leaf extracts were tested for antiproliferative activity against HCT116 cell line at different concentrations. The IC50 values of aqueous and alcoholic leaf extract of AR on HCT116 was found to be >7.25 μg/ml and 0.55 μg/ml respectively. The AR alcoholic leaf extract was found to be more potent than aqueous extract.

3.2. Phytochemical characterization

3.2.1. Qualitative analysis

The alcoholic leaf extract tested positive for flavonoids, terpenoids, saponins, tannins, quinine, carbohydrate, and steroids but negative for phenols and glycosides (Table 1).

3.2.2. Quantitative analysis

The GC–MS method was used to quantify the contents of AR's alcoholic leaf extract. A total of 108 compounds were identified from the large percentage area of components listed in Supplementary Table 1.

GC–MS profiling of the extracts revealed the presence of a total of 108 peaks (Fig. 1). Based on peak area (%), 50 major compounds were listed in Supplementary Table 1. These compounds reported to exhibit important biological functions. Some of major components such as gamma-Sitosterol (15.86%), Phytol (7.64%), Stigmasterol (7.25%), Ergost-5-en-3-ol, (3 beta)- (5.88%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (5.46%), 9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)- (5.10%), Stigmast-4-en-3-one (4.94%), Tetrapentacontane (4.35%), l-(+)-Ascorbic acid 2,6-dihexadecanoate (4.34%), were detected (Supplementary Table 1).

3.3. Acute toxicity study

Acute toxicity of AR alcoholic leaf extract was tested in Wistar rats and the LD50 value was found to be safe at a dose of 98.11 mg/kg of body weight, with no lethality or serious visible toxicity. Based on these findings, 1/20th and 1/10th of the LD50 which corresponds to 5 mg/kg and 10 mg/kg respectively, of alcoholic leaf extract were chosen as treatment doses.

Table 1
Qualitative analysis of alcoholic leaf extract of AR.

S. No	Phytoconstituents	**Result**
01	Terpenoids	Present
02	Flavonoids	Present
03	Phenols	Absent
04	Saponins	Present
05	Tannins	Present
06	Quinine	Present
07	Glycosides	Absent
08	Carbohydrates	Present
09	Steroids	Present

3.4. Tumor development

The tumors were developed in the colon region after 20 weeks of intraperitoneal DMH induction, and the tumor size was reduced after treatment with alcoholic leaf extract of AR (Fig. 2).

3.5. ACF count

DMH treatment showed ACF production in the colon (302.83 ± 27.83). Standard (153.7 ± 23.8) and AR treatment groups 5 mg/kg (113.7 ± 13.15) and 10 mg/kg (113.2 ± 11.02) showed a significant ($p < 0.05$) decrease in ACF counts as compared to DMH Control group (Fig. 3).

3.6. Colon length/weight ratio

There was a significant ($p < 0.05$) reduction in the colon length/weight ratio in the DMH (7.51 ± 0.44) control group as compared to healthy group (10.62 ± 0.76). Standard (10.86 ± 0.37) and AR treatment 10 mg/kg (11.18 ± 0.63) significantly ($p < 0.05$) normalized the length/weight ratio of the colon as compared to disease control (Fig. 4).

3.7. Spleen and liver index

There were no major variations observed between treated and control groups (Table 2).

3.8. Hematological examination

DMH control showed a significant ($p < 0.05$) decrease in RBC and hemoglobin (Hb) levels as compared to normal group. Standard and AR leaf extract showed a significant ($p < 0.05$) improvement in RBC, Hb and platelets, whereas there was no considerable difference was observed in the WBC count (Table 3).

3.9. Catalase activity (CAT)

DMH control group (4.22 ± 0.655) showed a significant ($p < 0.05$) reduction in CAT activity as compared to healthy group (19.3 ± 1.80). Standard (14.63 ± 2.434) and AR leaf extract showed a significantly ($p < 0.05$) increased in the CAT activity in both treatment groups of 5 mg/kg (14.02 ± 2.57), and 10 mg/kg (16.22 ± 2.54) groups as compared to DMH group (Fig. 5).

3.10. Reduced glutathione (GSH)

GSH level was found to be decreased in DMH control (1.830 ± 0.362) group when compared to healthy control

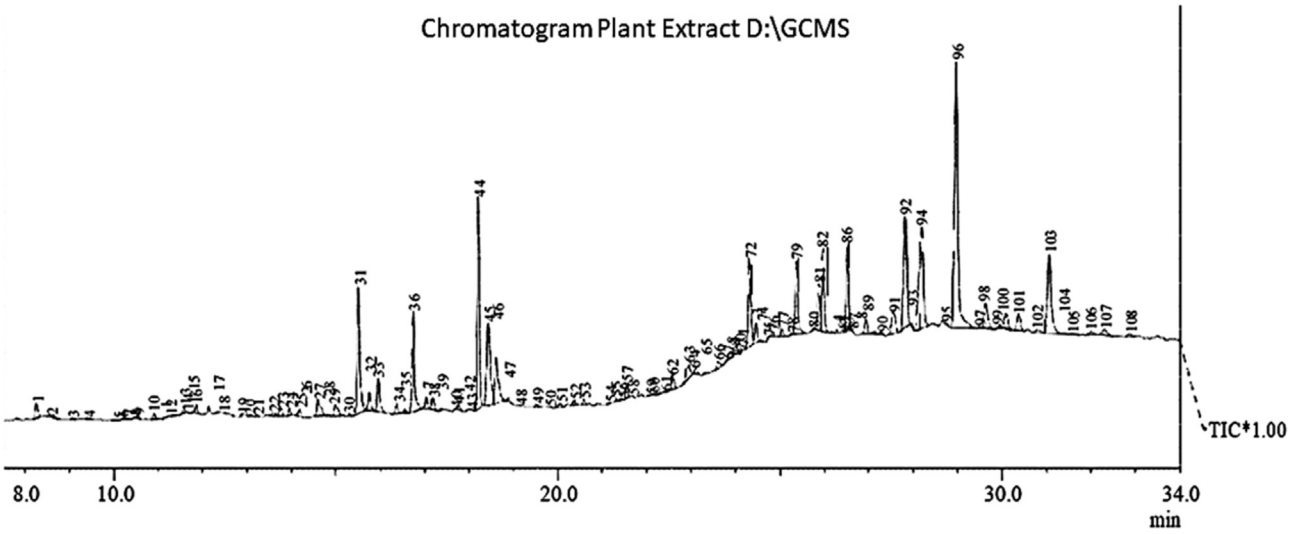

Fig. 1. GC–MS chromatogram for alcoholic leaf extract of AR. Legend: GC–below in MS chromatogram, contents from the alcoholic leaf extract of AR. A total of 108 compounds were recorded and represented in peaks. The X-axis represents R. time and Y-axis the percentage of area.

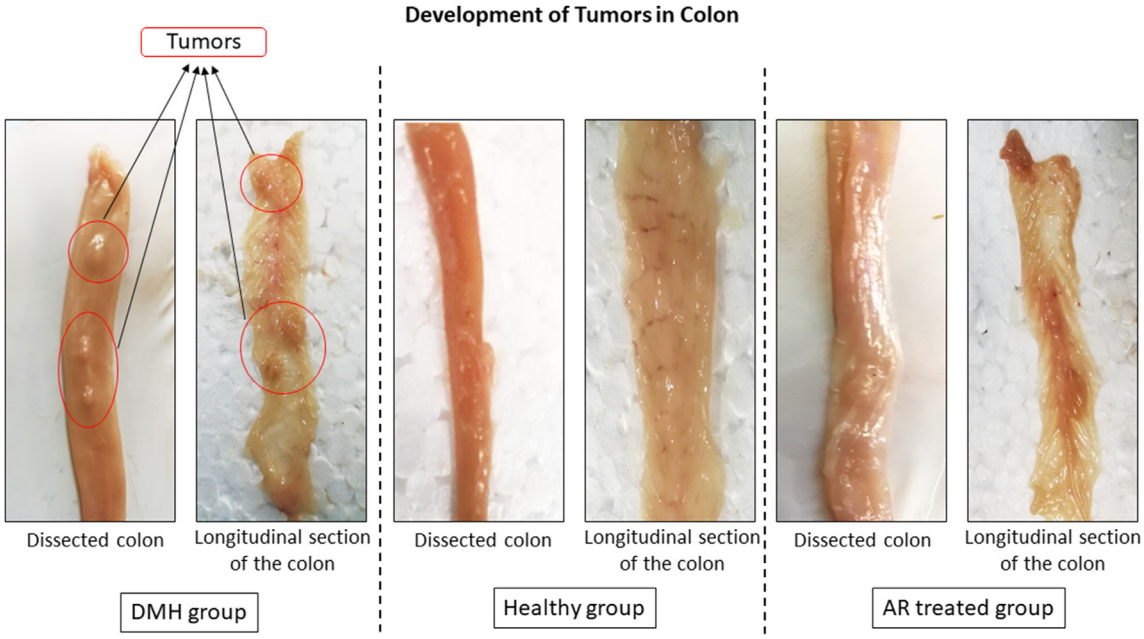

Fig. 2. Showing the development of tumors in various groups. Legend: DMH induced tumors in the colon region, AR leaf extract inhibited tumor formation, and the healthy group had no tumors.

(4.04 ± 0.429). Standard (4.23 ± 0.302) and AR leaf extract showed significantly increased in GSH activity in both treatment groups 5 mg/kg (3.34 ± 0.236), and 10 mg/kg (3.95 ± 0.522) compared to DMH control group (Fig. 6).

3.11. Superoxide dismutase (SOD)

DMH (0.58 ± 0.055) control group showed a significant ($p < 0.05$) reduction in SOD activity as compared to healthy control (1.11 ± 0.059). Standard (1.11 ± 0.125) and AR leaf extract 5 mg/kg (1.20 ± 0.102) and 10 mg/kg (1.15 ± 0.087) showed a significant ($p < 0.05$) increases in SOD activity as compared to DMH control group (Fig. 7).

3.12. Lipid peroxidase (LPO)

DMH (1167.6 ± 159.70) group showed a significant ($p < 0.05$) increases in LPO activity as compared to healthy group (404.0 ± 28.30). Standard (676.5 ± 72.6) and both AR leaf extract 5 mg/kg (590.3 ± 62.03) and 10 mg/kg (730.1 ± 39.97) showed a significant ($p < 0.05$) reduction in the LPO activity as compared to DMH control group (Fig. 8).

3.13. Histopathological examination

DMH induction caused the severity of inflammation, hyper-proliferative cells and damage of muscle layers of the colon in DMH group (Fig. 9(c) & (d)) as compared to healthy group (Fig. 9(a) &

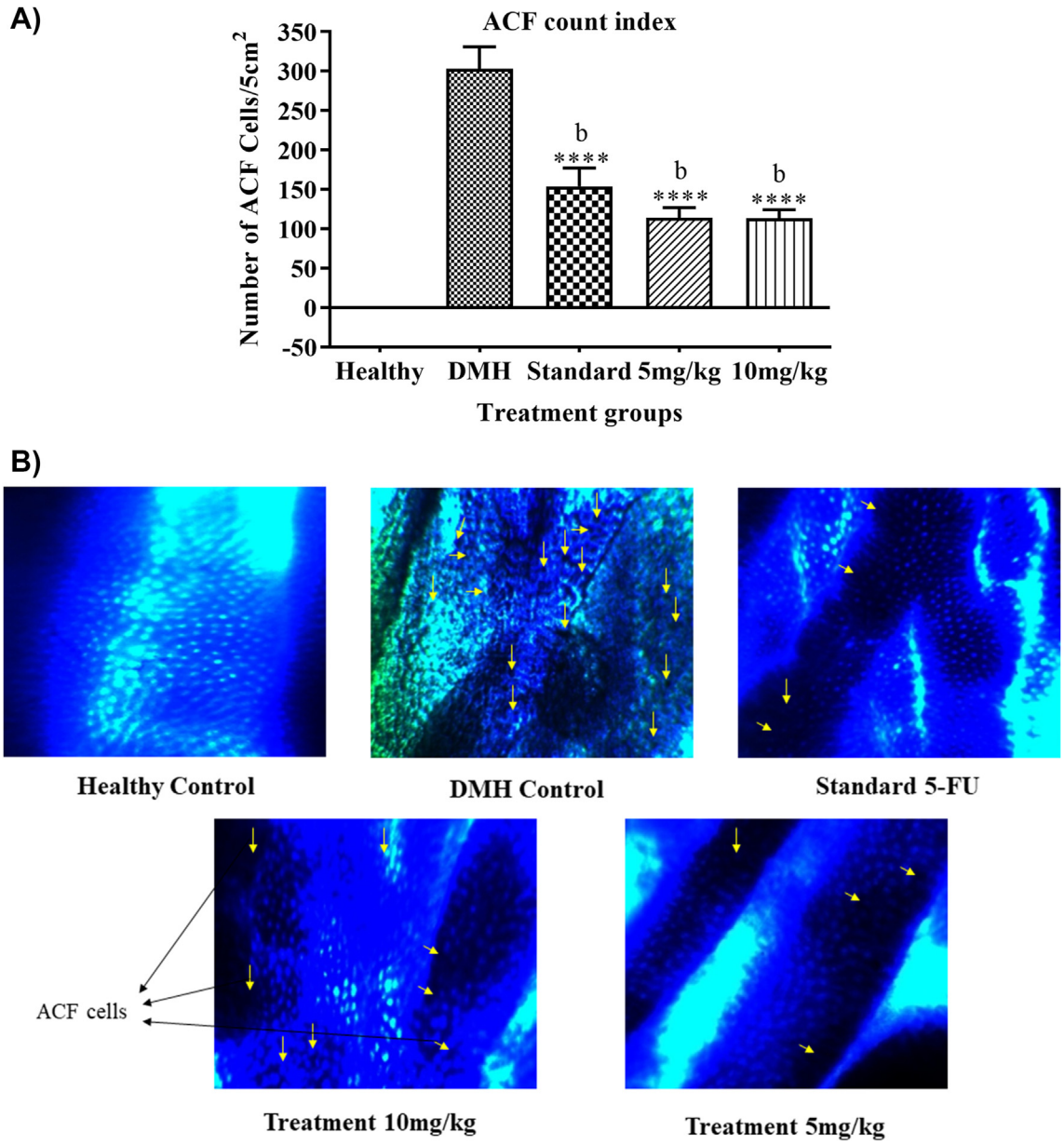

Fig. 3. A) Graph represents the effect of AR alcoholic leaf extract on the ACF count index in different groups. B) ACF pathology figures. Legend: all the values are mean ± SEM of six samples, where $^{a***}p < 0.001$ compared to Healthy (no ACF formation observed in healthy animals), $^{b***}p < 0.001$ compared to DMH control.

(b)). The alcoholic leaf extract of AR decreased the inflammation formed due to DMH and recovering of damaged muscle layers was observed in both AR 5 mg/kg (Fig. 9(g) & (h)), and 10 mg/kg treated groups (Fig. 9(i) & (j)) compared to the DMH control group (Fig. 9(c) & (d)).

4. Discussion

Studies have shown that AR has anti-bacterial, anti-viral, and anti-cancerous properties. Suresh et al. showed that AR root extract inhibited the growth of human cancer cell lines A-549, K-562, HeLa, and MDA-MB [22]. AR leaf extract exhibited cytotoxicity against HT-29 cell lines indicating that it is a potent anti-cancer agent [23]. AR also showed anti-cancer activity against HCT116, MCF7, A549, and SCC9 cell lines [10]. Our current study also demonstrated the cytotoxicity of alcoholic leaf extract of AR against HCT116 cell lines. Biological active phytochemicals such as saponins, flavonoids, steroids, terpenoids, tannins, quinine, and carbohydrates have been shown to have anti-cancer, antioxidant, and anti-microbial activity [24–35]. In this study, we also confirmed the presence of phytochemical constituents like saponins, flavonoids, terpenoids, tannins, quinine, carbohydrates, and steroids in the alcoholic leaf extract of AR through quantitative analysis performed by GC–MS. From the major percentage area of the components listed, a total of 108 compounds were identified. The identified compounds have anti-cancer activity against a variety of cancers such as Gamma-Sitosterol, which has been shown to have anti-cancer activity against the human MCF-7 and A549 cell lines [36]. Phytol-inhibited cell proliferation and promoted autophagy in MCF-7 and human gastric adenocarcinoma AGS cells [37]. Other constituents observed were stigmasterol chemopreventive activity in cancer [38], Ergost-5-en-3-ol [anti-cancer in gastric, lung, and ovarian cancer [39–42]], 3,7,11,15-Tetramethyl-2-hexadecen-1-ol [anti-cancer [43]], 9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-compound

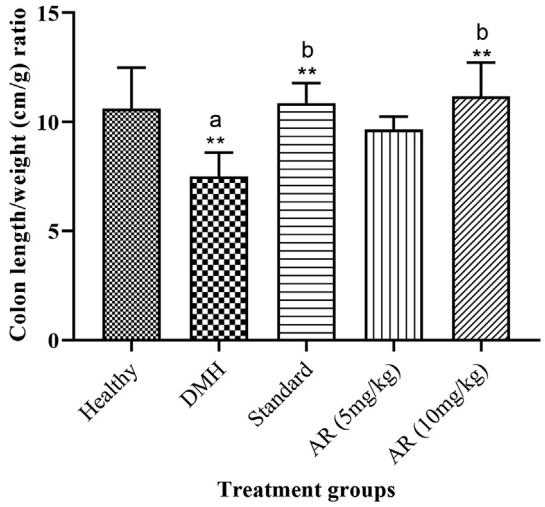

Fig. 4. Effect of AR leaf extract on colon length/weight ratio. Legend: values are expressed as mean ± SEM of six samples, where $^{a**}p < 0.01$ compared to healthy control, $^{b**}p < 0.01$ compared to DMH control.

Table 2
Showing the effect of AR leaf extract AR on the spleen and liver index.

Treatment groups	Spleen index (Mean ± SEM)	Liver index (Mean ± SEM)
Healthy control	0.38 ± 0.016	0.028 ± 0.000
DMH control	0.42 ± 0.022	0.078 ± 0.047
Standard (5FU)	0.37 ± 0.015	0.030 ± 0.001
AR 5 mg/kg	0.38 ± 0.011	0.028 ± 0.001
AR 10 mg/kg	0.044 ± 0.074	0.035 ± 0.004

Legend: Values are expressed as Mean ± SEM of five samples.

(cytotoxic on human epidermoid larynx carcinoma (Hep-2) cell line) [44], stigmast-4-en-3-one [anti-cancer effect on the MG-63 cell line [45]].

To investigate the safety of AR in animals, acute oral toxicity was performed, where LD50 of the extract was found to be 98.5 mg/kg body weight. Thus, 1/20th and 1/10th doses of the extract's LD50 value were chosen as treatment doses which were 5 mg/kg and 10 mg/kg respectively in CC model in animals. The lowest LD50 indicated toxic nature of the extract.

ACF is known to be the earliest neoplastic lesion in the progression of CC, and it is one of the biomarkers used to diagnose CC [46]. Patients with an elevated number of adenomas have also reported a large amount of ACF [47]. Studies indicated that genetic alteration in CC may contribute to the development of malignant tumors [48]. According to the researchers, individuals with adenomas or CC have significantly increased histological progression, ACF number, and size. In our study, we observed an increase in the number of ACFs in the DMH-treated group compared to the AR-treated group and a significant ($p < 0.05$) decrease in ACF in all treated groups. Bodyweight is an important

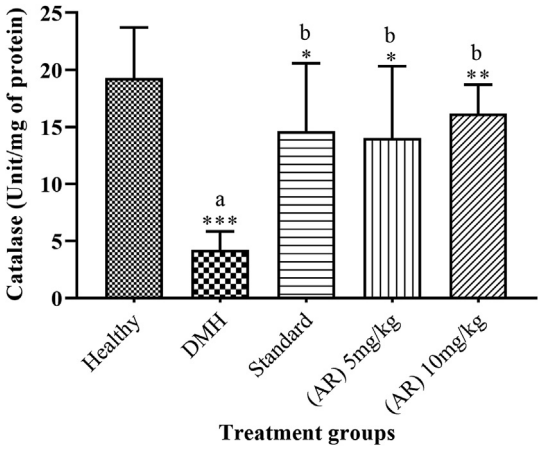

Fig. 5. Impact of AR leaf extract on CAT activity. Legend: values are expressed as mean ± SEM of six samples, where $^{a***}p < 0.001$ compared to Healthy control, $^{b*}p < 0.05$ compared to DMH control, $^{b**}p < 0.01$ compared to DMH control.

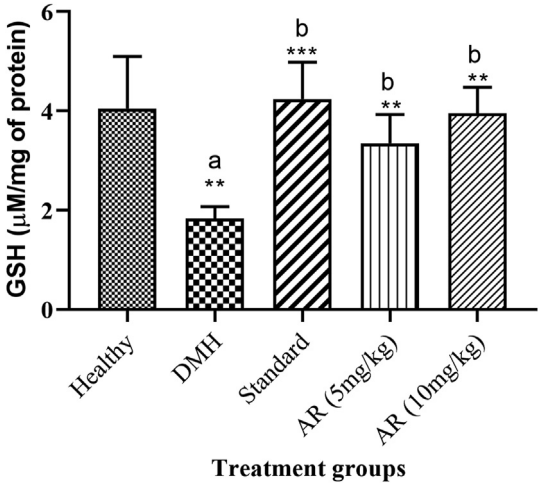

Fig. 6. Impact of AR leaf extract on GSH activity. Legend: values are expressed as mean ± SEM of six samples, where $^{a**}p < 0.05$ compared to Healthy control, $^{b***}p < 0.001$ compared to DMH control, $^{b**}p < 0.01$ compared to DMH control.

marker of CC in rats. Several studies have reported a change in the bodyweight of the CC rat; significantly lower colon length of azoxymethane (AOM) and dextran sulfate sodium (DSS)-inducing CC mice as compared to healthy mice [49]. Wistar rats with DSS-induced colitis had a lower length/weight ratio of the colon [50]. The development of adenomas and adenocarcinomas reduces the length/weight ratio of the colon. The length/weight ratio of the colon is reduced in the DMH induced CC model [16]. In this study, we observed that DMH significantly reduced the length/weight ratio of the colon in the DMH control group when compared to

Table 3
Showing the effect of AR leaf extract AR on haematological parameters.

Treatment groups	WBC (cells/mm$^{3)}$	RBC (cells/mm^3)	% Hb (g/dl)	Platelets (cells/μl)
Healthy control	3800 ± 385.86	12,281,667 ± 1,106,068	13.55 ± 0.125	82666.67 ± 7760.298
DMH control	4066.6 ± 668.99	11,402,667 ± 1,271,870	10.36 ± 1.563^a	123333.3 ± 5605.553^a
Standard (5FU)	3675 ± 151.84	13,071,667 ± 492604.2	12.85 ± 0.313^b	136666.7 ± 4825.856^b
AR 5 mg/kg	3400 ± 598.88	14,188,333 ± 922239.4^b	12.77 ± 0.326^b	145666.7 ± 5211.099^a
AR 10 mg/kg	3500 ± 186.18	14,336,667 ± 525048.1^b	12.94 ± 0.431^b	143666.7 ± 6747.016^a

Legend: All values are expressed in Mean ± SEM, where $^ap < 0.05$ compared to healthy control, $^bp < 0.05$ compared to DMH control.

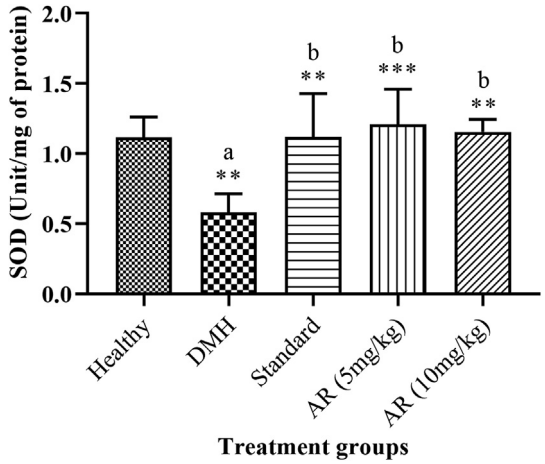

Fig. 7. Impact of AR leaf extract on SOD activity. Legend: values are expressed as Mean ± SEM of six samples, where $^{a**}p < 0.01$ compared to Healthy control, $^{b**}p < 0.01$ compared to DMH control, $^{b***}p < 0.001$ compared to DMH control.

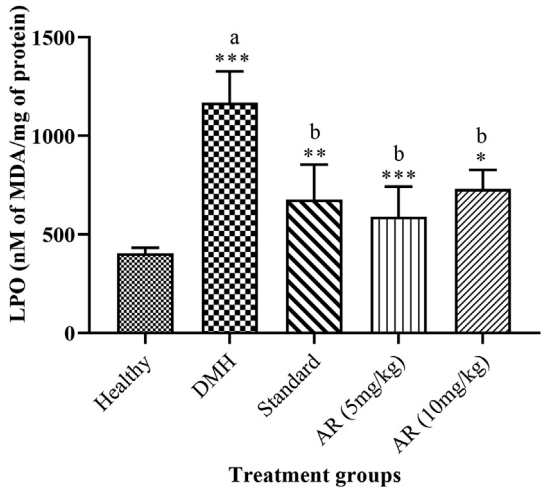

Fig. 8. Impact of AR leaf extract on LPO activity. Legend: values are expressed as mean ± SEM of six samples, where $^{a**}p < 0.01$ compared to healthy control, $^{b*}p < 0.05$ compared to DMH control, $^{b**}p < 0.01$ compared to DMH control.

the healthy group and that alcoholic leaf extract of AR significantly ($p \leq 0.05$) increased the length/weight ratio of the colon at both doses of 5 mg/kg and 10 mg/kg compared to the DMH control.

Tumor growth, manifest pathological changes in the organism, and prognostic factors for disease development and treatment all cause changes in blood values. Thus, a study of the macro-functional status of blood cells in rats during DMH-induced CC revealed a link between the hemolysis mechanism and oxidative stress. As a result, oxidative products are increased and antioxidant enzymes such as SOD, GSH, and CAT activity in cells are altered [51]. Lee Y-J et al. found a positive correlation between WBC and CC, indicating that an enhanced level of WBC increases the risk of CC incidence and mortality in both men and women [52]. According to studies, RBC lysis occurs during tumor development, and microscopic bleeding has been observed in the colon and related digestive tract in CC, which may be a reason to lower RBC levels in CC [53]. In the current study, it was observed that the RBC level in the DMH control group decreased while the WBC level increased when compared to the healthy group whereas, alcoholic leaf extract of AR lowers the WBC levels and significantly ($p \leq 0.05$) increased RBC levels in both doses of 5 mg/kg and 10 mg/kg when compared to the DMH control group. In a recent study, erythrocytes and Hb levels were measured in CC patients and anemia, often normocytic, followed by microcytic conditions was also observed. The Hb count was reduced due to a tumor, and low blood Hb was observed as a result of systemic inflammation [54]. In the current study, we observed that there is a significant decrease in Hb count at $p < 0.05$ in the DMH control group (10.36 ± 1.563) compared to the healthy control (13.55 ± 0.125) and the treatment with AR leaf extract showed a significant increase in Hb count at both doses of 5 mg/kg (12.27 ± 0.326) and 10 mg/kg (12.28 ± 0.431) $p < 0.05$ compared to the DMH group. Cancer cell and platelet interactions have been the subject of extensive research for many years. Recent research has linked a high platelet count and thrombocytosis to a poor prognosis in many cancer patients, including those with colorectal cancer [55]. A study reported that platelet count and plateletcrit (Pct) were increased in CC patients compared to healthy subjects [56]. Xu-Dong Rao et al. have stated that increased platelet counts are a negative association predictor of survival in both primary CC and resectable colorectal liver metastases [57]. Our current study shows that the DMH group has higher platelet levels (123333.3 ± 5605.5) than the healthy group (82666.6 ± 7760.2), but there is no significant difference between the AR treated groups and the DMH group.

Histopathological results showed that DMH caused severe inflammation, hyperproliferative cells, and muscle layer damage in the DMH control group compared to healthy control. Treatment with alcoholic leaf extract of AR reduces the inflammation caused by DMH and improves muscle damage recovery at doses of 5 mg/kg and 10 mg/kg compared to the DMH control.

CAT, SOD, and GSH are endogenous enzymes with antioxidant activity that help the body maintain normal free radical levels. These antioxidants play an important role in maintaining the body's health. These enzyme activities decrease as cancer progresses, resulting in an increase in free radical levels [58]. The antioxidant defence system in CC tissue is determined by SOD activity. The SODs convert superoxide radicals into hydrogen peroxide (H_2O_2) and a molecule of oxygen (O_2), whereas CAT degrades H_2O_2 into O_2 and water. Two lethal species, superoxide radical and hydrogen peroxide, are converted into water [59].

Many studies have found that CAT activity decreases in DMH-treated rats during the development of CC [60–62]. Another study found that CAT is losing its ability to protect against free radical damage [63]. Our current study also showed a strong resemblance to the previous study, with a significant decrease in CAT activity ($p \leq 0.05$) in DMH control compared to a healthy control group, whereas AR with both doses of 5 mg/kg and 10 mg/kg showed a significant increase in CAT activity ($p \leq 0.05$). Many studies have found that DMH reduces the activity of antioxidants such as SOD and CAT [64–67]. Several studies have also found significantly higher levels of MDA in DMH-treated rats [64,65]. In our study, we found that the activity of SOD and CAT was significantly reduced in DMH-treated rats compared to other groups, while MDA activity was significantly higher in DMH-treated colon tissue. Following treatment with AR leaf extract, SOD, CAT, and MDA activity returned to normal in comparison to the healthy control groups, but there was no significant difference in GSH activity.

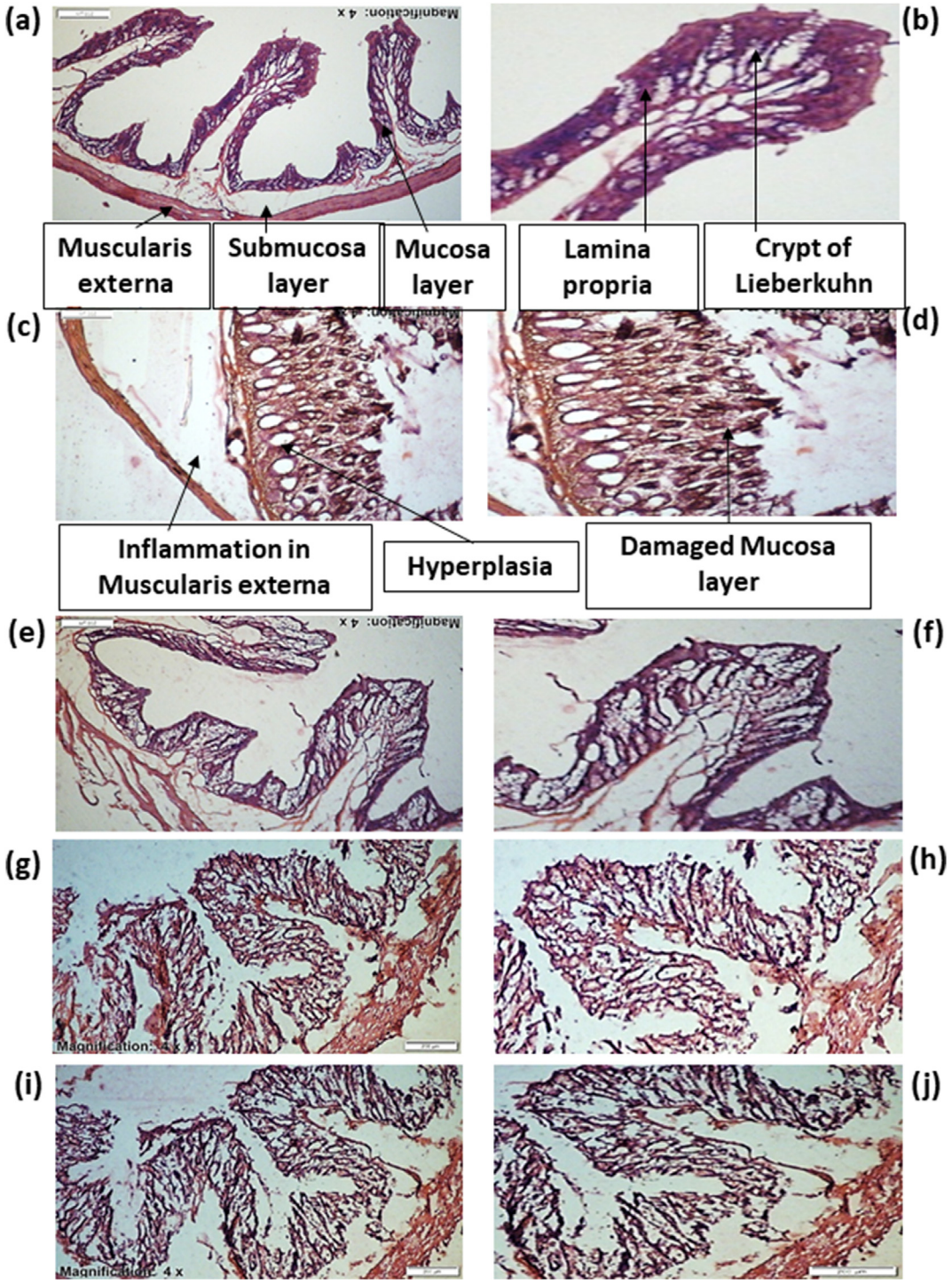

Fig. 9. Histopathological examination of colon. Legend: (a) & (b) are healthy control group, (c) & (d) are DMH control group, (e) & (f) are standard- 5flurouracil, (g) & (h) are treatment with 5 mg/kg and (i) & (j) are treatment with 10 mg/kg. Objective used 4×.

5. Conclusion

The current study was carried out to investigate the anti-cancer efficacy of AR alcoholic leaf extract. The AR extract had the greatest anti-proliferative activity against the human HCT116 cell line, which may be due to the presence of major phytochemical constituents such as flavonoids, triterpenoids, gamma-sitosterol, phytol, stigmasterol, ergost-5-en-3-ol, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, 9-Octadecenoic acid, and 1,2,3-propanetriyl ester. The *in vivo* study provided evidence for the protective effect of AR alcoholic leaf extract in the DMH-induced CC rat model. The leaf extract reduced the DMH-induced tumors and increased the activity of endogenous antioxidant enzymes such as CAT, SOD, and GSH, while decreasing MDA levels. AR alcoholic leaf extract was effective in reducing DMH-induced inflammation and dysplasia. Thus, based on the current study's findings, it can be concluded that

the alcoholic leaf extract of AR has antioxidant and anti-proliferative properties and can aid in the prevention of CC development and dysplasia caused by DMH. AR extract has shown to be effective against CC. Further research on the isolation of specific components of the leaf extract responsible for the anti-cancer activity can be conducted to increase specificity.

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Conflict of interest

None.

Authors contributions

Vijetha Shenoy Belle and Nitesh kumar conceived the study, reviewed the manuscript and approved submission. Nadeem Khan G performed the study and written the manuscript. Abhijna Ballal and Divya Datta helped during study performing and writing the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaim.2021.05.010.

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