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Original Article

Chemopreventive effect of myrtenal on bacterial enzyme activity and the development of 1,2-dimethyl hydrazine-induced aberrant crypt foci in Wistar Rats

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ABSTRACT

Colon cancer remains as a serious health problem around the world despite advances in diagnosis and treatment. Dietary fibers are considered to reduce the risk of colon cancer as they are converted to short chain fatty acids by the presence of anaerobic bacteria in the intestine, but imbalanced diet and high fat consumption may promote tumor formation at different sites, including the large bowel via increased bacterial enzymes activity. The present study was conducted to characterize the inhibitory action of myrtenal on bacterial enzymes and aberrant crypt foci (ACF). Experimental colon carcinogenesis induced by 1,2-dimethylhydrazine is histologically, morphologically, and anatomically similar to human colonic epithelial neoplasm. Discrete microscopic mucosal lesions such as ACF and malignant tumors function as important biomarkers in the diagnosis of colon cancer. Methylene blue staining was carried out to visualize the impact of 1,2-dimethylhydrazine and myrtenal. Myrtenal-treated animals showed decreased levels of bacterial enzymes such as β-glucuronidase, β-glucosidase, and mucinase. Characteristic changes in the colon were noticed by inhibiting ACF formation in the colon. In conclusion, treatment with myrtenal

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provided altered pathophysiological condition in colon cancer-bearing animals with evidence of decreased crypt multiplicity and tumor progression.

1. Introduction

Colorectal cancer is a major cause of death in developed countries with an increased rate of morbidity and mortality [1]. The incidence rate continues to rise as people change their lifestyles and food habits [2]. In this regard, substance-use disorders are associated with numerous medical, psychiatric, psychological, spiritual, economic, social, family, and legal problems, creating a significant burden for affected individuals, their families, and society [3]. Insidious factors such as alcohol, smoking, sedentary lifestyle, aging, and diet are the most important exogenous promoters of colon cancer [4]. Supporting this, Chang [5] investigated the mechanisms by which alcohol damages immune defense function to identify therapeutic targets for effective treatment of alcoholic patients with severe bacterial infection. Davie [6] stated that dietary fibers are considered to reduce the risk of colon cancer as they are converted to short chain fatty acids by the presence of anaerobic bacteria in the intestine, which prevents the invasion of pathogenic bacteria [7]. However, imbalanced diet and high fat consumption is known to alter this protective metabolism of the intestinal microflora, and reduce the host immune response by increased bacterial enzyme activity [8]. Thereby β-glucuronidase activity in the intestine plays a major role in the generation of toxic and carcinogenic metabolites that may promote tumor formation at different sites, including the large bowel [9]. In that way, 1,2-dimethylhydrazine (DMH), a procarcinogen, is metabolized by β-glucuronidase enzymes along with its metabolic pathway in the liver and released as a carcinogen in the colon epithelium to induce colon cancer in experimental animals [10]. A variety of fungi and bacteria synthesize the major component enzyme, β-glucosidase, which can synergistically convert cellulose into glucose for the enzymatic depolymerization of cellulosic material [11]. Enzymatic hydrolysis of cellulose is a major limitation in lignocellulose's conversion to glucose, as β-glucosidase activity causes accumulation of aglycones [12]. Robertson et al [13] proved that hydrolytic activity of the bacterial enzyme mucinase acts on the destruction of mucins or their prosthetic polysaccharides by the bacteria. The intestinal mucinous layer also experiences an autolytic enzyme hydrolysis, leading to altered permeability of the mucosal membrane. Nitroreductase is involved in the reduction of nitrogen-containing compounds including nitro functional group members such as dinitrotoluene, nitrobenzenes, and nitropyrenes to amines. A number of these proteins are described as oxidoreductases and are primarily found in bacterial lineages that exhibit toxic, mutagenic or carcinogenic activities [14,15].

Colon cancer has been postulated as a complex and multistage process that includes a series of pathological alterations ranging from discrete microscopic mucosal lesions, such as aberrant crypt foci (ACF), to malignant tumors [16]. ACF are generally considered the earliest identifiable macroscopic lesion within the colonic mucosa that may be associated with risk of future neoplastic development [17]. Due to morphological and genetic similarities in ACF development between rodents and humans, this preneoplastic lesion may be used in cancer screening and prevention studies for the detection of risk factors and protective factors associated with colon cancer progression [18]. Among various biomarkers, ACF and mucin-depleted foci are recognized on the surface of cancer-predisposed colons of rodents and humans [19]. Therefore, an experimental model of colon cancer was induced with DMH, a chemical carcinogen known to cause colon cancer with a reproducible experimental *in vivo* system for studying sporadic forms of colon carcinoma [20]. Colon carcinogenesis induced by DMH is histologically, morphologically, and anatomically similar to human colonic epithelial neoplasms with ACF, as one of the early morphological changes on the DMH-stimulated colonic mucosal surface in rodents [21], containing crypts that are larger in diameter and stain deeper with methylene blue than normal crypts, are considered as a reliable surrogate biomarker of colorectal cancer. Hence chemoprevention trials with colorectal ACF set as the primary endpoint may have some advantages [22].

Monoterpenes found in the essential oils of numerous species of plants including mints, cherries, and celery seeds have shown chemopreventive and therapeutic activity in rodent colon tumor models [23]. They are nonnutritive dietary components found in the essential oils of citrus fruits and other plants. A number of these dietary monoterpenes have antitumor activity. For example, d-limonene, which comprises > 90% of orange peel oil, has chemopreventive activity against rodent mammary, skin, liver, lung, and forestomach cancers. Similarly, other dietary monoterpenes have chemopreventive activity against rat mammary, lung, and forestomach cancers when fed during the initiation phase. The monoterpenes have several cellular and molecular activities that could potentially underlie their positive therapeutic index. Among naturally occurring oxygen-containing monoterpene derivatives, myrtenal is one of the most widespread compounds distributed in the Asteraceae family. It is a ubiquitous constituent of the essential oils of flowers, stems, and leaves that possess antimicrobial and antifungal activities [24]. Biological interest on the isoprenoid structure of myrtenal was found to suppress the proliferation of carcinogenic processes [25]. Wattenberg et al [26] explained the action of chemopreventive agents during the initiation phase of carcinogenesis to prevent the interaction of chemical carcinogens with DNA, by modulating carcinogen metabolism to less toxic forms to prevent the outgrowth of initiated cells. Thus,

monoterpenes would appear to act through multiple mechanisms in the chemoprevention and chemotherapy of colon cancer. In particular, the specific mechanism of monoterpenes inhibit the post translational isoprenylation of cell growth-regulatory proteins such as Ras that could alter signal transduction and result in altered gene expression leading to downregulation of proliferating cancer cells.

2. Methods

2.1. Reagents

DMH and myrtenal were purchased from Sigma Chemical Company (St. Louis, MO, USA). All the other chemicals used in this study were of analytical grade available commercially.

2.2. Experimental animals

Experiments were carried out with 5 week old male Wistar rats procured from central animal house facility, Dr. A.L.M. Postgraduate Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai, India. They were maintained in the controlled environmental conditions of temperature and humidity on alternative 12-hour light–dark cycle, noise level maintained below 85 dB, and had unrestricted access to standard diet consisting of 24% protein, 4.5% fat, and 4% fiber. The experiment was sanctioned and approved by the Institutional Animal Ethical Committee (IAEC No.01/13/2013).

2.3. Experimental design

The experimental animals were divided into four groups, each group with six animals. Group 1: Control animals fed with standard diet and pure drinking water. Group 2: Animals were administered with 20 mg/kg body weight of DMH, in 1mM EDTA, pH adjusted to 6.5 with 1mM NaOH and subcutaneously injected once a week for 15 weeks. Group 3: Animals were treated with myrtenal (230 mg/kg body weight) with corn oil as vehicle for 30 weeks by intragastric administration. Myrtenal treatment was started 1 week prior to the first dose of 20 mg/kg body weight of DMH (as in Group 2) and continued till end of the experimental period. Group 4: Animals were treated with myrtenal (230 mg/kg body weight) for 30 weeks by intragastric administration to assess the cytotoxicity if any, induced by myrtenal, and rats were referred as drug control.

After the end of the experimental period, the rats were fasted overnight and anesthetized using diethyl ether and sacrificed by cervical decapitation. A portion of colon was used for histopathological studies and remaining tissue was homogenized in 0.1M Tris–HCl buffer pH 7.4 and centrifuged; the supernatant was used for biochemical studies.

2.4. Colon analysis

Colons were excised from experimental rats, and were blotted dry and opened longitudinally, with the inner surface examined for visible macroscopic lesions. Tumor incidence (percentage of animals with tumors) and multiplicity (mean counted tumors per animals) were determined for the colons. Immediately following sacrifice, colons were removed and washed with ice-cold saline, and colon homogenates (10%) were prepared in ice cold TBS (50mM Tris and 150mM NaCl; pH 7.2) then centrifuged at 10,000*g* for 10 minutes at 4°C and were stored as aliquots at or below −20°C for subsequent assays.

2.5. Identification of ACF

The colon was detached, flushed with 0.9% saline, opened from cecum to anus, divided into three segments and fixed flat between two pieces of filter paper in 10% buffered formalin. Microscopic slides were placed on top of the filter paper to ensure that the tissue remained flat during fixation. After 24 hours in buffered formalin, the colon was stained with 0.2% methylene blue as described by Bird and Good [27]. It was then placed mucosal side up, again on a microscopic slide and observed under a light microscope. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, significantly increased distance from laminae to basal surface of cells, and the easily discernible pericryptal zone. Crypt multiplicity was determined as the number of crypts in each focus and was categorized as containing one, two, three, four, or more aberrant crypts/focus.

2.6. Measurement of bacterial enzyme activity

The colon was flushed gently with saline, cut open longitudinally, and placed on a flat surface. Mucosal samples were collected by gently scraping the colonic mucosal layer using microscopic slides. The collected samples were transferred into preweighed tubes containing 0.1M phosphate-buffered saline (pH 7.0) and sonicated for 30 seconds. Fecal and mucosal samples were centrifuged at 2000*g* for 5 minutes, and aliquots of supernatant were used immediately.

Beta-glucuronidase activity was measured by the method of Freeman [28] (1986). It consists of supernatant enzyme with 0.02M phosphate buffer adjusted to pH 7.0, 0.1mM EDTA, and 3.0mM p-nitrophenyl-b-D-glucopyranoside. The mixture was incubated at 37°C for 20 minutes. The reaction was arrested with 0.2M glycine buffer (pH 10.4) and the amount of p-nitrophenol released was read at 540 nm with a spectrophotometer. All reactions were linear with respect to concentration and incubation time to 45 minutes. The amount of p-nitrophenol liberated was determined by comparison with a standard nitrophenol curve.

Beta-glucosidase activity was measured by the method of Freeman [28]. The mixture of samples and substrate (p-nitrophenyl-b-D-glucoside) were incubated with 37°C for 60 minutes. After incubation, 0.2M Na_2CO_3, was added to arrest the reaction. The released p-nitrophenol was measured at 400 nm. All reactions were linear with respect to concentration and incubation time to 60 minutes. The amount of p-nitrophenol liberated was determined by comparison with a standard nitrophenol curve.

Beta-galactosidase activity was measured by the method of Freeman [28]. The mixture contained 3mM p-nitrophenyl-b-D-galactopyranoside, a known volume of sample and incubated at 37°C for 15 minutes. Then the reaction was terminated by the addition of 0.25M Na_2CO_3. Release of p-nitrophenol was measured spectrophotometrically at 405 nm.

The amount of p-nitrophenol liberated was determined by comparison with a standard nitrophenol curve.

Mucinase activity was determined by the method of Shiau and Chang [29]. The assay mixture contained 0.2M porcine gastric mucin with a known amount of fecal suspension made up to 1 mL with distilled water. The mixture was then incubated at 37°C for 25 minutes. The amount of reducing sugar was measured by the method of Nelson [30] at 520 nm. Values are expressed as mg of glucose liberated/min/mg protein.

Nitroreductase activity was measured by the method of Bratton and Marshall [31]. The assay mixture contained 1.5mM p-nitrobenzoic acid, 0.1M of phosphate buffer, and a known amount of sample. The reaction was arrested by the addition of 20% trichloroacetic acid and centrifuged. P-aminobenzoic acid released was measured at 550 nm. The amount of transformed substrate was determined by comparison with a standard curve.

Sulfatase activity was measured by the method of Rowland et al [32]. The reaction mixture contained 0.02M phosphate buffered saline, 1mM EDTA, 1mM p-nitrocatechol sulfate, and a known amount of enzyme suspension. The reaction was arrested with 0.01M NaOH, and the amount of p-nitrocatechol liberated was read spectrophotometrically at 492 nm. All the reactions were linear with respect to concentration and incubation time to 60 minutes. Protein content of fecal and mucosal samples was measured by the method of Lowry et al [33].

2.7. *Statistical analysis*

Values are expressed as mean ± standard deviation. The results were statistically evaluated using one-way analysis of variance (ANOVA) by SPSS student version 10.0 (SPSS, Chicago, IL, USA), followed by Tukey's multiple comparison method to compare means of different groups. The mean difference is significant at the 0.05 levels.

3. Results

3.1. *Colon tumor analysis: Tumor incidence*

Tables 1 and 2 depict the DMH-induced colon tumor development and crypt multiplicity respectively. DMH-induced Group 2 rats exhibited 100% tumor development throughout the colon. Myrtenal treatment in Group 3 resulted in the decrease of tumor incidence percentage. Similarly, in Table 2, crypt multiplicity results show that Group 2 rats had more crypts ranging from one, two, three, and four crypts in the DMH-induced group. Crypt multiplicity was controlled and seen to be reduced in myrtenal-treated Group 3 rats compared to Group 2. Control and myrtenal alone-treated groups showed no significant difference.

3.2. *Effect of myrtenal on bacterial enzymes*

Figs. 1 and 2 show the activity of fecal and mucosal biotransforming enzyme activity of control and experimental rats. The DMH-treated rats in Group 2 showed increased bacterial enzyme activity compared to control, whereas the activities of the bacterial enzymes were found to revert back to near normal upon myrtenal administration in Group 3 rats compared to Group 2 DMH-induced colon cancer rats.

3.3. *Effect of myrtenal and the assessment of ACF*

Methylene blue staining (Fig. 3) shows the formation of crypts in DMH-treated Group 2 rats with normal structure in Group 1 control animals. The intensity of staining was found to decrease in myrtenal-supplemented rats in Group 3 when compared with Group 2 animals. Normal structure was identified in Group 4 myrtenal alone-treated rats.

4. Discussion

Monoterpenes have shown chemopreventive and therapeutic activity in colon tumor models and also in clinical studies on cancer patients [34]. Several patients with refractory colon cancer and metastatic colorectal cancer have shown evidence of its antitumor activity [34]. In addition, monoterpenes have been shown to inhibit both the growth of tumors and the post-translational isoprenylation of cellular proteins, including *ras* [35]. Intense research on nitrilimine cycloaddition with monoterpenes, such as myrtenal, produced 4,5-dihydropyrazole-containing skeletons of biological interest, as the mentioned isoprenoids can suppress the proliferation of cancer cells [36]. Dietary supplementation of myrtenal is thought to reduce colon cancer, since the colon is considered to have bacterial enzymes which transforms the xenobiotic substances to direct-acting genotoxins [37] and detoxification products formed in the liver are reactivated in the gut by bacterial enzymes [38]. Similar to the activation of DMH in the liver followed by intestinal bacterial enzymes to produce carcinogenic effect on the colon epithelium reported by Reddy et al [39].

Secondary bile acids produced from bacterial actions in the colon undergoes glucuronidation, which is involved in

Table 1 – Effect of myrtenal on 1,2-dimethyl hydrazine induced tumor incidence percentage.[a]

Groups	No. of rats	No. of tumor-bearing rats	Tumor incidence (%)[a]	Total No. of tumors	No. of tumors/tumor bearing rats
1	6	0	0	0	0
2	6	6	100	20	3.33
3	6	1	16.66	2	2.0
4	6	0	0	0	0

[a] (Number of tumor bearing rats/total number of rats in each group) × 100.

Table 2 – Effect of myrtenal on 1,2-dimethyl hydrazine induced aberrant crypt foci (ACF) formation in rats.[a]

Experimental groups	Total No. of ACF/colon	1 crypt	2 crypts	3 crypts	4 crypts
1	0	0	0	0	0
2	56 ± 3.35	12 ± 0.98	18 ± 0.81	16 ± 0.81	10 ± 0.75
3	30 ± 3.06*	7 ± 0.63*	8 ± 0.54*	9 ± 0.89*	6. ± 1.0*
4	0	0	0	0	0

Values are represented as mean ± standard deviation for six rats in each group.
*denotes the significant difference between the major two groups.
[a] $p < 0.05$ compared to 1,2-dimethyl hydrazine treated group. Number of total ACF and ACF with one, two, three, and four crypts/focus at the end of the experiment.

xenobiotic metabolism of bilirubin by the activity of β-glucuronidase that catalyzes hydrolysis of β-glucuronic acid and liberates unconjugated chemical carcinogens on the walls of the colon epithelium [40]. Increased β-glucuronidase activity noticed in DMH treated rats shows the metabolic degradation of the carcinogens. At this juncture, the β-glucuronidase activity was found decreased upon myrtenal treatment which may due to the anti-inflammatory property of myrtenal. Cytosolic β-glucosidase functions in major metabolic pathways that occur in the cytosol in animals such as glycolysis and gluconeogenesis, but it is predominantly found in liver, kidney, and intestine, and efficiently hydrolyzes β-D-glucoside [41]. It is also involved in the hydrolysis of plant glycosides to produce agylocones that could be carcinogenic upon its increased activity as in the case of DMH-treated rats. The supplementation of myrtenal has been found to decrease its activity, which may be due to its protective role against colon cancer via its inhibitory action against β-glucosidase.

Beta-galactosidase is a lysosomal exoglycosidase involved in the catabolism of glycoconjugates by sequential release of β-linked terminal galactosyl residues which degrade pericancerous matrix favoring tumor growth, invasion, and metastatic propagation in the intestine [42]. The elevated β-galactosidase levels were brought back to near normal in myrtenal-treated rats as the tumors completely regressed. Increased mucus shedding by an enzyme capable of destroying mucin, with contribution from multiple enzymes is an important factor elaborating the degradation of mucosal layer on the intestinal layer and represents a potential target on the colon epithelium which permits measurement of mucin degradation by release of reducing sugars [43]. Myrtenal treatment was found to reduce the enzyme mucinase by decreasing colonic mucosal cell microsomal enzyme activity that plays a role in the metabolism of many drugs by inhibiting the transformation of precarcinogens into colon carcinogens [44].

A wealth of evidence supports that cell transformation and evolution through the different stages of malignancy is accompanied by modifications in sulfate expression and structure including changes in 6-O-sulfation [45]. Increased

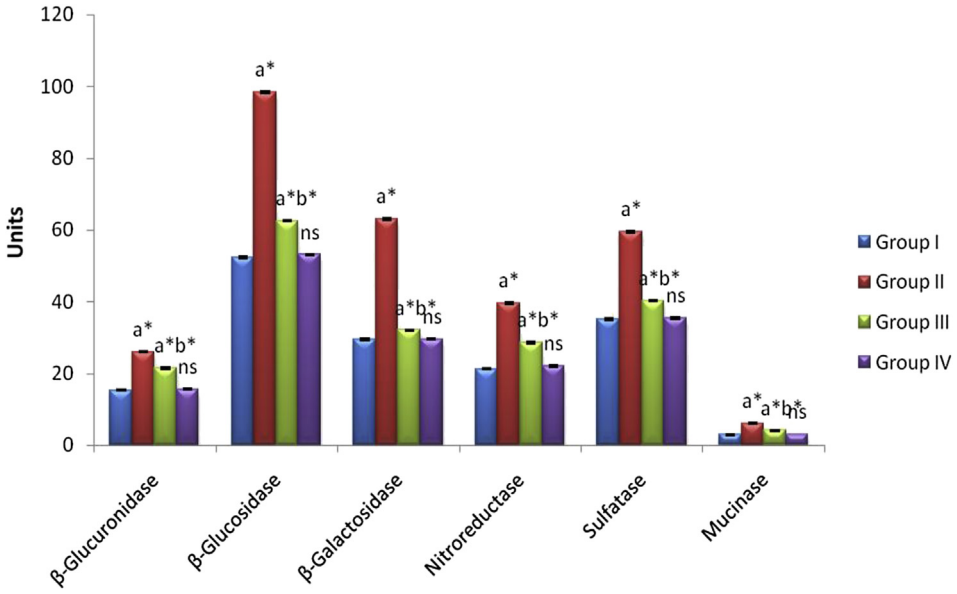

Fig. 1 – Effect of 1,2-dimethyl hydrazine and myrtenal treatment on fecal biotransforming enzymes in rats. Results are expressed as mean ± standard deviation for six animals. [a] Groups 2, 3, and 4 compared with Group 1. [b] Group 3 compared with Group 2. *$p < 0.05$. Units: β-glucuronidase, β-galactosidase = mg of p-nitrophenol liberated/min/g protein; β-glucosidase = mg of p-nitrophenol liberated/min/g protein; Mucinase = mg of glucose liberated/min/mg protein; Nitroreductase = μmol of p-aminobenzoic liberated/min/g protein; Sulfatase = μmol of p-nitrocatechol liberated/min/g protein. ns = not significant.

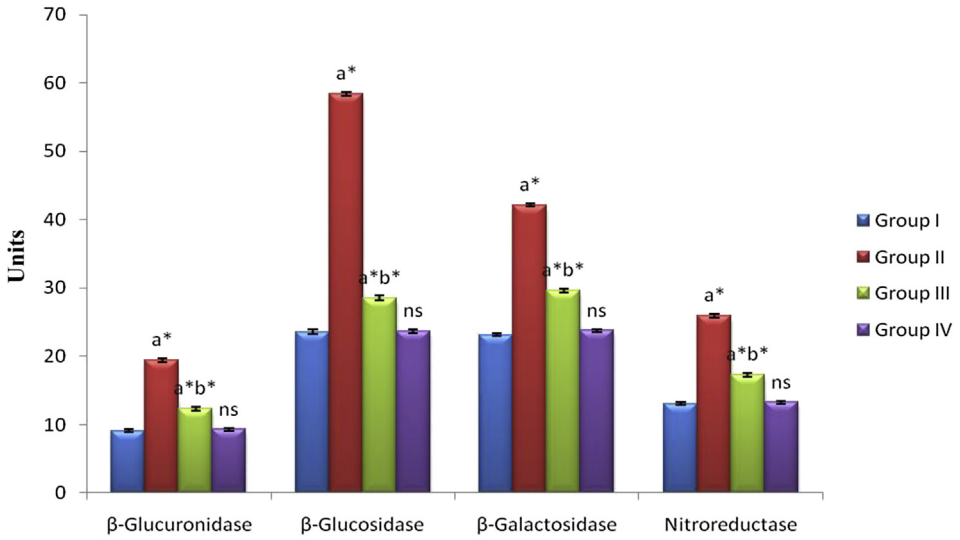

Fig. 2 – Effect of 1,2-dimethyl hydrazine and myrtenal treatment on mucosal biotransforming enzymes in rats. Results are expressed as mean ± standard deviation for six animals. [a] Groups 2, 3, and 4 compared with Group 1. [b] Group 3 compared with Group 2. *$p < 0.05$, ns = not significant. Units: β-glucuronidase, β-galactosidase = mg of p-nitrophenol liberated/min/g protein; β-glucosidase = mg of p-nitrophenol liberated/min/g protein; Nitroreductase = μmol of p-aminobenzoic liberated/min/g protein.

levels of sulfatase and nitroreductase were found in DMH-treated groups due to the bacterial lineages, but myrtenal supplementation reduced the activity of sulfatase and nitroreductase, which may be due to the tumor-suppressing activity through inhibition of pro-oncogenic growth factors. In conclusion, the increased activity of bacterial enzymes by DMH-induced groups failed to proceed as myrtenal plays some key role in interrupting the energy requirement of tumor tissues and leads to the suppression of tumor growth and producing inhibitory mechanism against enhanced bacterial activity in the intestine.

ACF are benign adenomatous polyps that progress into an advanced adenoma with high-grade dysplasia that later can lead to an invasive cancer [46]. ACF refer to the abnormal

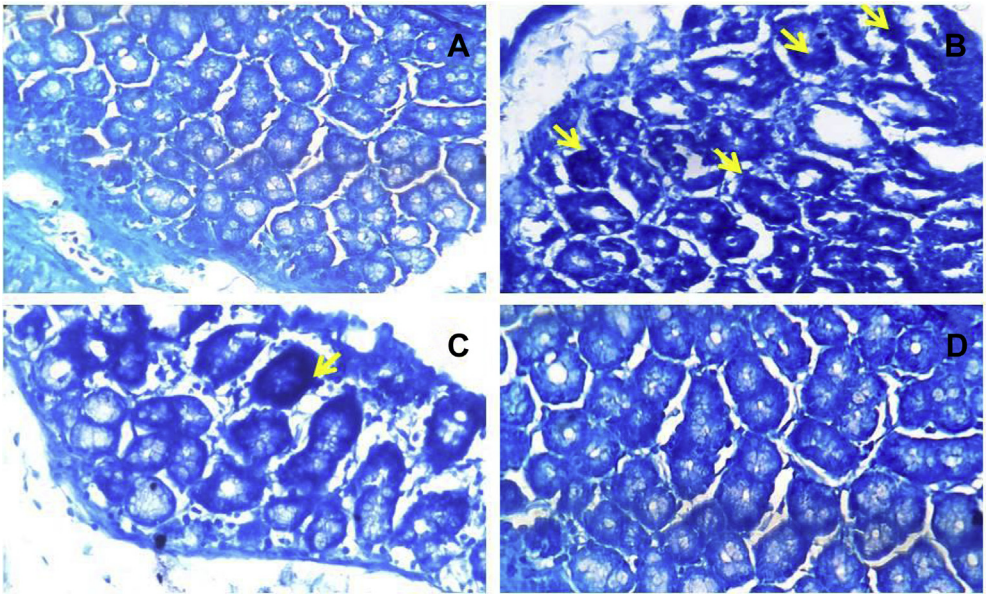

Fig. 3 – Aberrant crypt foci analysis by methylene blue staining in control and experimental animals. (A) Control with normal epithelial lining. (B) 1,2-dimethyl hydrazine induced crypts with elongated lamellae and multiple crypts with deeper staining of methylene blue. (C) 1,2-dimethyl hydrazine + myrtenal treated group with transformation of the cancer cells with few crypts to normal architecture. (D) Myrtenal alone with normal morphology.

change of normal foci. Greaten of foci, epithelium thickening, and foci with several or multiple mutations gathered together in a focal distribution are characteristics of ACF [47]. In both rodents and humans, the pathogenesis of colorectal cancer induced by carcinogens produces ACF, the precancerous lesions of colorectal cancer, which are considered to be good biological markers for evaluating the effect of drugs that are used to prevent and control the formation of colorectal cancer in rats [48]. ACF analysis done according to the method described by Bird [49] produced results with increased size, a significantly increased distance from the lamina to the basal surface of the cells, darker stain, and importantly four or more aberrant crypts/foci were categorized as multicrypts. The increases in ACF count and number of polyps were used to evaluate the extent of damage to the colon caused by DMH [50]. Myrtenal supplementation has been shown to decrease the number of crypts multiplicity and improved morphological and structural changes, along with few dysplastic colonocytes, and congested sinusoids. As reported by Karthik Kumar et al [51], the decrease in the level of β-glucuronidase activity in the colon may be directly linked with the formation of colonic crypts relating a correlation with β-glucuronidase and crypt multiplicity. In our study the myrtenal-treated animals showed no characteristic change in the colon, which clearly indicates the anticancer activity of myrtenal against DMH-induced colon cancer in Wistar rats.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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