

Limonin Modulated Immune and Inflammatory Responses to Suppress Colorectal Adenocarcinoma in Mice Model

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Abstract

Purpose. Inflammation and compromised immune responses often increases colorectal cancer (CRC) risk. The immune-modulating effects of limonin on carcinogen/inflammation-induced colorectal cancer (CRC) were studied in mice.

Methods: Male Balb/c mice were randomly assorted into three groups (n=6): healthy control, non-treated CRC-induced (azoxymethane/dextran-sulfate-sodium AOM/DSS) control, and CRC-induced+50 mg limonin /kg body-weight. The CRC development were monitored via macroscopic, histopathological, ELISA and mRNA expression analysis.

Results: Limonin downregulated inflammation (TNF- α , *tumor necrosis factor- α*), enhanced the adaptive immune responses (CD8, CD4, and CD19), and upregulated antioxidant defense (Nrf2, SOD2) mRNA expressions. Limonin reduced serum malondialdehyde (MDA, lipid peroxidation biomarker), prostaglandin-E2 and histopathology inflammation scores, while increasing reduced-glutathione (GSH) in the CRC-induced mice. Limonin significantly (p<0.05) increased T cells (CD4 and CD8) and B cells (CD19) in spleen tissues. The CD335 (natural killer cells) were increased in the CRC-induced mice and limonin treatment restored it to normal levels suggesting reinstatement to normal colon conditions.

Conclusion: Limonin apparently mitigated CRC development, by ameliorating adaptive immune responses (CD8, CD4, and CD19), reducing inflammation (serum prostaglandin-E2; TNF- α , innate immune responses), oxidative stress and enhancing the endogenous anti-oxidation defense reactions (GSH) in the CRC-induced mice.

Introduction

Colorectal cancer (CRC) is amongst the top three killer cancer worldwide, second most common cancer for men (10.9% of the total cancer cases) and third in women (9.5% of the total cancer cases) (Arnold et al. 2017). Chronic inflammatory bowel disease increases CRC risk (Axelrad et al. 2016). Colitis-associated-cancer (CAC) is a serious inflammatory bowel disease (IBD) complications which can progress from the chronic inflamed mucosa to dysplasia and ultimately, colorectal cancer. Surgery, neoadjuvant chemotherapy and radiotherapy are the most common therapy used with many painful and undesirable side effects. The FDA approved chemotherapeutical drug for CRC includes Capecitabine, Fluorouracil (5-FU), Irinotecan, Oxaliplatin, Trifluridine/tipiricil. Although chemotherapy is the foremost selected therapy, it usually causes nausea, vomiting, diarrhea, neuropathy, mouth sores, fatigue, hair loss, increased infection risks and often extend the lifespan for only a short time.

Dietary limonin was reported to significantly reduce the incidence of colonic adenocarcinoma in azoxymethane-initiated CRC-induced rats (Tanaka et al. 2001). Limonin was also reported to cause apoptosis and inhibit colon adenocarcinoma cells proliferation (Chidambara Murthy et al. 2011). Limonin was previously reported to have anti-cancer and preneoplastic lesions suppressive effects *in vivo* (Shimizu *et al.*, 2015). To the best of our knowledge, there is to date not much animal study reports on the

immuno-modulatory effects of limonin against colorectal cancer development. Previously, Tanaka et al., (2001) studied the effects of citrus limonoids obacunone and limonin on azoxymethane (AOM)-induced colon tumorigenesis in male F344 rats. In this study, we examined the effects of limonin on inflammation, mRNA expressions and immune related responses in colorectal adenocarcinoma mice using AOM/dextran sulfate sodium (DSS) for the adenocarcinoma induction. This study provides indications that dietary limonin suppressed colon adenocarcinoma, at least in part by enhancing immune, anti-inflammatory and endogenous anti-oxidant responses in Balb/c mice.

Materials And Methods

Chemicals and drugs

Azoxymethane (AOM) and dextran sulfate sodium (DSS) salt (Mr ~40,000) were purchased from the Sigma-Aldrich (St. Louis, MO, USA). Limonin (95% purity) were obtained from **Xi'an Xin Sheng Bio-chem Co.,Ltd.** (Xi'an city, Shaanxi province, China).

Animals

Five weeks old male Balb/c mice (25-30 g) were purchased from the Animal Resource Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia (Serdang, Selangor, Malaysia), given commercial rat chow (Gold Coin, Malaysia) and tap water *ad libitum*, in plastic cages (3 mice/cage) with a 12-h light-dark cycle at room temperature. Male mice were used because males are more prone to CRC than females, and females may be influenced by fluctuating hormones or estrus cycle. The animal study protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (UPM/IACUC/AUP-R069/2017).

Experimental design

Mice were divided into three groups (n=6) G1: healthy control, G2: non-treated CRC-induced (Azoxymethane/Dextran Sulfate Sodium AOM/DSS) control, G3: CRC-induced + 50 mg Limonin /kg body-weight. The dose for limonin was chosen based on previous studies (Shimizu et al., 2015). (The CRC was induced by a single intraperitoneal injection of azoxymethane AOM (10 mg/kg body weight) to the mice in groups 2 and 3. After a week of AOM injection, mice received 2% dextran sulfate-sodium (DSS) in the drinking water for 7 days, followed by regular drinking water for recovery thereafter (Tanaka et al. 2000). Limonin were completely homogenized in distilled water (0.25 mg/ml) and given as drinking water to G3. The average daily Balb/c mice drinking water intake was 0.16 ml/g body weight or 5±1 ml/mouse/day. The similar (insignificantly different) progressive body weight gains indicated that the mice consumed about the same amount of food and water throughout the 20 weeks experimental duration after the CRC-induction (AOM/DSS). Oral gavage was not used to avoid any additional stress to the mice, which may increase their mortality. Limonin did not seem to affect the water or food intake of the mice in the treatment group.

Mice were weighed monthly and the disease activity index (DAI) was scored based on the calculated sum of the individual scores of stool consistency and blood in stool as follows: stool consistency score = 0: normal, 2: loose, 4: diarrhea; blood in stool score = 0: normal, 2: reddish, 4: bloody (Li, Shen and Luo, 2016). Following an intraperitoneal injection of ketamine: xylazine (100 mg/kg:10 mg/kg), the mice were sacrificed by exsanguination (intracardiac puncture) 17 weeks post-treatment. The intracardiac puncture allowed for sufficient blood to be collected for the subsequent biochemical analysis. The blood was allowed to clot for 30 min, centrifuged at 3000 g for 10 min at 4°C, and the serum stored at -20°C until analyses. Colon and spleen were collected and either stored at -80°C or fixed in 10% formalin for further use.

Macroscopy and histopathology

The colon of each mouse was cut, cleaned, colon length measured and tumor incidence/numbers was counted. The colon length was measured using a ruler, measured and recorded to the nearest cm. The tumor incidence (%) was determined as the percentage of mice having at least one tumor when examined under the microscope. Colon samples were fixed in 10% buffered formalin for 24 h and handled through automated programmed tissue processing machine. The tissue was embedded in paraffin and tissue blocks sectioned at thickness of 5 µm and stained with hematoxylin and eosin (H&E) for light microscopic examination. Due to the short CRC developmental period, only a few tumors were present in each AOM/DSS mouse. The inflammation score was graded as previously described (Saadatdoust et al. 2015). The inflammation thickness ranged from 0 to 3 (0 = no inflammation, 1 = mucosa, 2 = mucosa plus submucosa and 3 = transmural). The thickness of an inflammation is measured throughout the layers of the colon wall, such as the mucosa, sub-mucosa or transmural tissues. The inflammation score was calculated based on the average scores from 0 to 3 (0 - no inflammation; 1 – mild (infiltration of inflammatory cells into the mucosa); 2 – moderate (infiltration of inflammatory cells into the mucosa and submucosa); 3 – severe (infiltration of inflammatory cells into the transmural layer).

Enzyme-linked immunosorbent (ELISA) assay

The serum malondialdehyde (MDA), reduced glutathione (GSH) and prostaglandinE2 (PGE2) levels were determined using commercial ELISA kits (Elabscience Biotechnology, Wuhan, China) following the manufacturer's protocol.

RNA extraction and RT-qPCR analysis

Total RNA from large intestine of the mice was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany). Briefly, ~20 mg of large intestine was disrupted in liquid nitrogen utilizing mortar and pestle and homogenized in 600 µL of Buffer RLT. The homogenate was transferred to a new tube and centrifuged for 3 min at full speed. The supernatant was transferred to a new tube, added 70% ethanol, mixed with pipetting, and passed through RNeasy mini column (Qiagen, Germany). The column was washed with wash buffer and contaminating DNA was digested on the column with DNase 1 (Qiagen, Hilden, Germany). RNA was eluted off the column using 30 µL of RNase free water. The RNA yield was

determined by measuring absorbance at 260 nm and purity was assessed according to the ratio of absorbance readings at 260 nm to 280 nm using the Nano Drop™ ND-1000 (Thermo Fisher, USA).

Total RNA of each sample (772 ng) was reverse transcribed with RT2 First Strand kit (Qiagen, Hilden, Germany). Quantitative PCR for selected genes (Table S2) was performed according to Custom RT2 Profiler PCR array using RT2 SYBR Green qPCR Mastermix (Qiagen, Hilden, Germany). Thermal cycling and fluorescence detection were performed using a CFX96 Touch qPCR System (Bio-Rad, California, USA). The average relative mRNA expressed for each experimental group (n=3) was calculated using $2^{-(\Delta \text{Ct})}$ normalized to β -actin, since β -actin was the most stable gene compared to the other house-keeping genes analyzed in this study. Fold change ($2^{(-\Delta \Delta \text{Ct})}$) were calculated by dividing the average normalized gene expression ($2^{(-\Delta \text{Ct})}$) in the test group with the average normalized gene expression ($2^{(-\Delta \text{Ct})}$) in the control healthy group. The target genes analyzed, and primer design are as in Supplementary Materials (Table S2).

Flow cytometry (immunophenotyping) analysis

The spleen was prepared by passage through a 70 μm cell strainer to obtain single-cell suspensions. The cell suspension was washed with 5 mL of phosphate buffered saline (PBS) and 5 mL of complete DMEM medium (10% FBS and 1% penicillin/streptomycin). The cell suspension was centrifuged at 500 g, 4°C for 5 minutes. Then, the pellet was washed with 3 mL of PBS, followed by incubation with 3 mL of ACK lysis buffer (0.15 M NH_4Cl , 1.0 mM KHCO_3 , 0.1 mM EDTA) for 3 minutes and centrifuged at 500g for 5 minutes. The single-cell suspension was washed with BSA solution twice and centrifuged at 300 g, 4°C for 5 minutes. The BSA (fetal bovine serum albumin) is a nutrient, stabilizer and surfactant in cell cultures, to protect enzymes/proteins and to prevent their adhesion to the surfaces of reaction vessel walls.

The cell pellets were stained with 5 μl of monoclonal antibodies specific for mouse (CD4+, CD8+ and CD19+) on ice for 20 minutes. The antibodies were from BD Pharmingen™ and they were (i) PerCP-Cy™5.5 Rat Anti-Mouse CD4, Cat No: 550954, Concentration: 0.2 mg/ml, Isotype: Rat DA, also known as DA/HA IgG2a, κ ; (ii) APC Rat Anti-Mouse CD8a, Concentration: 0.2 mg/ml, Isotype: Rat LOU, also known as Louvain, LOU/C, LOU/M IgG2a, κ , Cat No: 553035, (iii) PerCP-Cy™5.5 Rat Anti-Mouse CD335 (NKp46), Concentration: 0.2 mg/ml, Isotype: Rat IgG2a, κ , Cat No: 560800; (iv) PE Rat Anti-Mouse CD19, Concentration: 0.2 mg/ml, Isotype: Rat LEW, also known as Lewis IgG2a, κ , Cat No 557399.

The spleen cells were washed with 1 mL of BSA solution and centrifuged at 300 g for 5 minutes. The cell pellets were suspended in BSA solution and analysed by BD LSRFortessa™ Cell Analyzer (BD Biosciences, San Jose, USA) and BD FACS Diva™ software (BD Biosciences, San Jose, USA).

Statistical analysis

All animals in all treatment groups were included in the data collection and analysis are presented as the mean \pm S.D, unless otherwise stated (Please refer to the raw data provided as Supplementary materials).

Comparisons between CRC and CRC + limonin were analyzed by *t*-test. Multiple group comparisons were performed by one-way ANOVA followed by Duncan post hoc test. Data was analyzed by SPSS 22.0 software and $p < 0.05$ was considered significant. Correlations were evaluated using the Spearman test and using best curve fit online software at <https://mycurvefit.com/>.

Results

Pre-clinical animal model macroscopic observations and histopathology of colon

The limonin was given through the drinking water to reduce handling and stress to the mice throughout the experiment. The CRC-induced mice showed significantly ($p < 0.05$) lower net weight gains than the normal control mice (Fig. 1a). Supplements with limonin improved the body weight gains. The near normal weight gains indicated that they consumed similar amount of food and water, indicating that limonin did not affect the appetite of the mice. The CRC-induced mice showed serious signs of colitis, (diarrhea, weight loss and bloody stool) indicating chronic inflammation in the colon. The disease activity index (DAI) score for bloody stools and consistency was highest in the non-treated CRC- induced mice and the treatment with limonin significantly ($p < 0.05$) reduced DAI and tumor incidence / number (Figure 1b and 1c). The sizes of the tumor were too small to be measurable.

The oxidative stress biomarker MDA was significantly elevated in the CRC-induced mice, and the limonin significantly attenuated the serum MDA levels ($p < 0.05$) (Figure 1d). The colon tissues had precancerous changes including gland distortion, ulceration of the mucosa, abundant inflammatory cell infiltration, high grade dysplasia, necrotic debris, loss of goblet cells, and severe inflammation. In mice treated with limonin, these lesions were either absent or significantly reduced. The histopathology results showed that the limonin suppressed CRC-development (Fig 2 a-c). The efficacy was supported by evidences from the analyzed colon carcinogenesis and inflammation biomarkers. Limonin treated mice showed tubular adenoma, low-grade dysplasia and moderate inflammation in the colon mucosa.

The colorectal-cancer was confirmed from adenocarcinoma structures on the control non-treated CRC-induced mice. The limonin mitigated the carcinogen/inflammation-induced CRC and alleviated the colonic mucosal dysplasia, adenoma and adenocarcinoma formation (Figure 2). The limonin reduced the histological scores towards normal values (Figure 2d-e). The control non-treated CRC mice showed abnormal colon length and spleen weight, which were mitigated by the limonin treatment (Figure 2f-g).

The limonin enhanced serum GSH and suppressed serum PGE2 levels under the carcinogen/inflammation induced conditions (Figure 3a). Reduced glutathione (GSH) is an endogenous antioxidant response constituent that protects cells from oxidative damage and free radicals, a biomarker for anti-oxidant against oxidative stress. Prostaglandin E2 (PGE2) is a pro-inflammatory lipid mediator that cause inflammation, pain and fever in response to infection and injury.

The pro-inflammatory mRNA expressions for TNF- α were significantly ($p < 0.05$) upregulated in the CRC-induced mice. The treatment with limonin significantly ($p < 0.05$) down-regulated the expressions by about

75% (Fig. 3b), indicating that anti-inflammatory pathways are involved. The TGF- β 1 and iNOS expression was insignificantly ($p < 0.05$) affected by limonin as compared to non-treated CRC-induced mice (Fig. 3b and 3c).

Limonin upregulated Nrf2 and SOD2 mRNA expressions

The Nrf2 expression was significantly ($p < 0.05$) upregulated in all the CRC-induced mice (Figure 3c). Limonin significantly upregulated Nrf2 mRNA expressions further as compared to the non-treated CRC-induced mice (Figure 3c), to fight oxidative stress. All CRC-induced mice had significantly ($p < 0.05$) upregulated SOD2 mRNA expressions, and limonin administration significantly ($p < 0.05$) increased these SOD2 expressions further by 82% compared to non-treated CRC-induced group (Figure 3c).

Limonin modulated the immunophenotyping of lymphocytes CD4+, CD8+ and CD19+ and CD335

The limonin enhanced immunophenotyping of lymphocytes CD4+, CD8+ and CD19+ in the spleen of the CRC-induced mice, demonstrating its good immune-stimulating effects (Figure 3d). The percentage of immune markers (CD4, CD8 and CD19) were significantly ($p < 0.05$) decreased in the CRC-induced mice, and administration of limonin significantly ($p < 0.05$) increased the CD4 biomarker by about 6%; co-receptors for the T cell receptor CD8 biomarker by 0.5% and B cells (CD19 biomarker) by about 20% (Figure 3d). The CD335 which are expressed on natural killer cells were significantly increased ($p < 0.05$) in the CRC-induced mice and limonin treatment restored it to normal levels (Figure 3e), indicating attenuation of the CRC developments.

Discussion

Dextran sulfate sodium injures the intestinal epithelium, to expose the lamina propria (LP) and submucosal compartment to luminal antigens and bacteria, triggering inflammation, disrupting colonic crypts, macrophages and CD4+ T cells around the colitis areas (Laroui et al. 2012). In this study, limonin suppressed CRC-induced colitis without any observable adverse effects / toxicity in the mice. The significant anti-inflammatory effect of limonin was demonstrated by the decreases in the TNF- α , PGE2 and iNOS expression levels in the limonin treated CRC-induced colitis mice. Inflammation encourages tumor development for progression and metastasis. Inflammatory mediators such as TNF- α and iNOS contribute to IBD development and colitis by activating oncogenic signaling pathways such as Wnt and NF- κ B. The TNF- α is intricately linked to chronic inflammation and has pleiotropic functions for cell proliferation, differentiation, death and survival. The TNF- α is produced by invading immune and stromal cells and during mucosal injury.

Antioxidant defenses play crucial roles against the negative effects of reactive oxygen species. Among them, Nrf2 and SOD2 help suppress the activities of tumor promoters and pro-carcinogen activators. The Nrf2 (transcription factor NF-E2-related factor 2) is a key regulator for a subset of genes, to produce detoxication proteins for removing electrophiles, reactive oxygen species and repair damage, for cell survival and maintaining homeostasis (Sadeghi et al. 2017). Under oxidative stress, Nrf2 dissociates

from its repressor (Keap 1) and translocates to the nucleus, to mediate the transcriptional activity of antioxidant and cytoprotective genes (e.g. SOD1, HMOX1, NQO1 and GSTs) (Chikara et al. 2018). Superoxide dismutase (SOD) is the first antioxidant defense against oxidative stress /damage. The mitochondrial SOD2 isoform has an additional unique protective function as cancer suppressor (Kinnula and Crapo 2004). Excessive O_2^- production in rodents' colonic crypt epithelium, aggravated bile acids induced intestinal cell proliferation (Dossa et al. 2016) and SOD2 scavenges harmful and mutagenic mitochondrial ROS. In the present investigation, limonin probably helped counteract oxidative stress via increasing SOD, Nrf2 and other antioxidant defense mRNA expressions. The MDA is a lipid peroxidation product and is a biomarker for oxidative stress. The MDA can react with deoxyguanosine to form endogenous mutagenic DNA adducts that can hasten aging and metabolic disorders. Excessive reactive oxygen species (ROS) or free radicals generation accelerate lipid peroxidation, nucleic acid and protein damage.

Imbalance between antioxidants and ROS lead to the oxidative damage of biomolecules and disruption of redox homeostasis. The GSH suppress PGE2 synthesis via interaction with COX enzymes and inhibit the production of prostaglandin in inflammatory states (Hartl et al. 2005). PGE2 elicits various biological effects and plays prominent role in tumor development to not only sporadic cancer but also inflammation-associated intestinal cancer by targeting multiple signaling pathways (Nakanishi and Rosenberg 2013). In this study, limonin enhanced the serum GSH levels and reduced the PGE2 levels in the CRC-induced mice.

The limonin enhanced immunophenotyping of lymphocytes CD4+, CD8+ and CD19+ in the spleens of the AOM/DSS-induced mice, demonstrating its immune-stimulating effects. Cytotoxic CD8+ T cells produced IFN- γ as an important effector for antitumor immunity. A favorable overall survival was reported for CRC patients with high CD8A and low VEGFA (Zhang et al. 2018). An inverse correlation exist between CD8A and VEGFA expressions (Zhang et al. 2018). Here the limonin showed no significant effects on IFN- γ or CD8A mRNA expressions under the CRC induction.

CD4+ T cells help to produce pro- and anti-inflammatory cytokines especially IFN- γ and TNF- α . The interaction between TNF receptor (Fas), Fas ligand (FasL) activated apoptotic signaling pathways and activated caspases, triggered DNA fragmentation and apoptosis. The CD4+ T cells could induce tumour dormancy that prevents tumour escape (Müller-Hermelink et al. 2008). The CD8+ T cells are essential for immune defense against cytotoxic molecules, and CRC patients with high CD8+ T cell infiltration showed a better prognosis (Deschoolmeester et al. 2011). Limonin may help to increase T cells proliferation capacity by releasing perforin, granzymes and granulysin. The CD19+ is a type 1 transmembrane protein and co-receptor of the B cell. B cells are necessary to attain an optimal CD4+ and CD8+ T cell tumor immunity. The B cell-mediated antitumor immunity can suppress cancer metastasis, while depletion of B cells can promote tumor growth (Yuen et al. 2016). In animal models, B cell-based cancer immunotherapy produced promising results and the B cells can induce T-cell mediated antitumor immunity (Sorenmo et al. 2011). In this study, limonin enhanced the CD19+ in CRC-induced mice suggested improved therapeutic efficacy.

The limitations of this study include the small sample size (due to high mice mortality after the AOM/DSS induction and before starting the limonin treatment or experiment) and the use of only a single dose of limonin. The tumor incidence and latency period would be modulated by the dose of AOM or DSS used for the CRC-induction. Higher dose caused a higher incidence of cancer with a shortened latent period, but very high mortality rate. Too low a dose caused extremely long CRC-development duration, if the CRC develop at all. The extent of the lesion depended on the mice strain, AOM/DSS dose, induction duration, sample handling and other stress factors such as housing conditions (caging, feeding, microbiota, etc.). Several trials were conducted to get the right AOM/DSS dose for this particular mice strain, to obtain the acceptable CRC development rate, appropriate for the research resources available.

Conclusions

This study demonstrates that limonin help mitigate colorectal adenocarcinoma development, partly by enhancing adaptive immune responses (CD4, CD8, and CD19), suppressing inflammation (innate immune responses), and oxidative stress in the carcinogen/inflammation-induced colorectal adenocarcinoma mice. The CD335 (natural killer cells) were increased in the CRC-induced mice and limonin treatment restored it to normal levels suggesting reinstatement of near normal colon conditions. This was evidenced from the disease activity index, serum MDA, PGE2, GSH, tumor incidence, histopathological observation scores, mRNA expressions and immunophenotyping analysis. These results may be further corroborated by other animal models or in human complementary intervention studies to confirm or better understand the effects of limonin on CRC development.

Declarations

Ethical Approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The animal study protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (UPM/IACUC/AUP-R069/2017).

Consent to Participate: Not applicable.

Consent to Publish: Not applicable

Authors' Contributions: NIMI executed the experiments, planning, data collection, and manuscript preparation, NMM is the veterinary clinician for animal studies, and SM is the grant recipient, main supervisor, project planning coordinator and manuscript writing/editing, principal researcher. IFM and NME assisted in planning the animal study. All researchers approved the final manuscript. All authors read and approved the manuscript and all data were generated in-house and that no paper mill was used.

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Competing Interests: All authors (Nur Iliyani Mohd Ishak, Suhaila Mohamed, Noordin Mohamed Mustapha, Iffah Nadhira Madzuki. Norhaizan Mohd Esa) declare that we have no conflict of interest.

Availability of data and material: Provided as supplementary material.

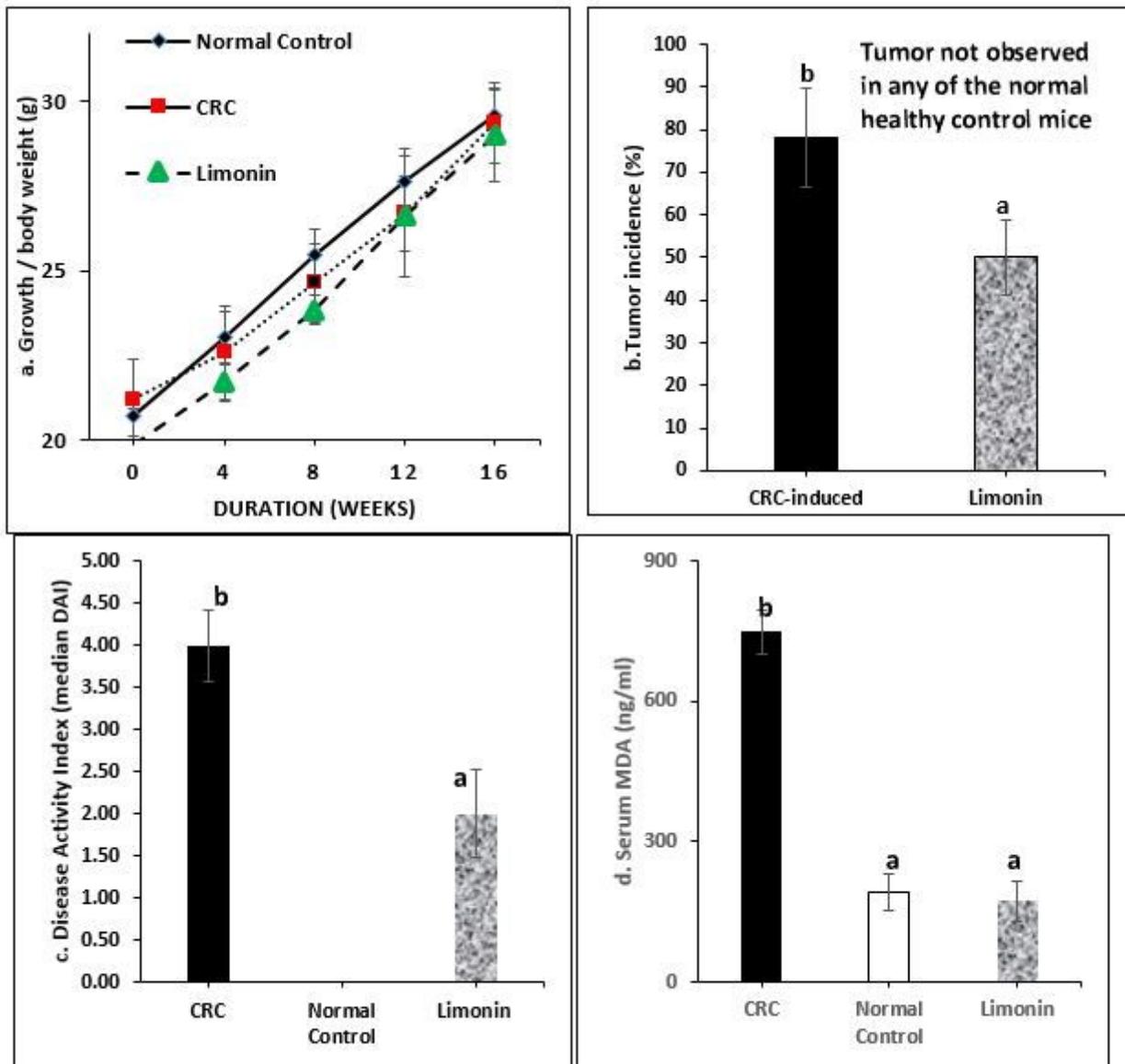
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Figures

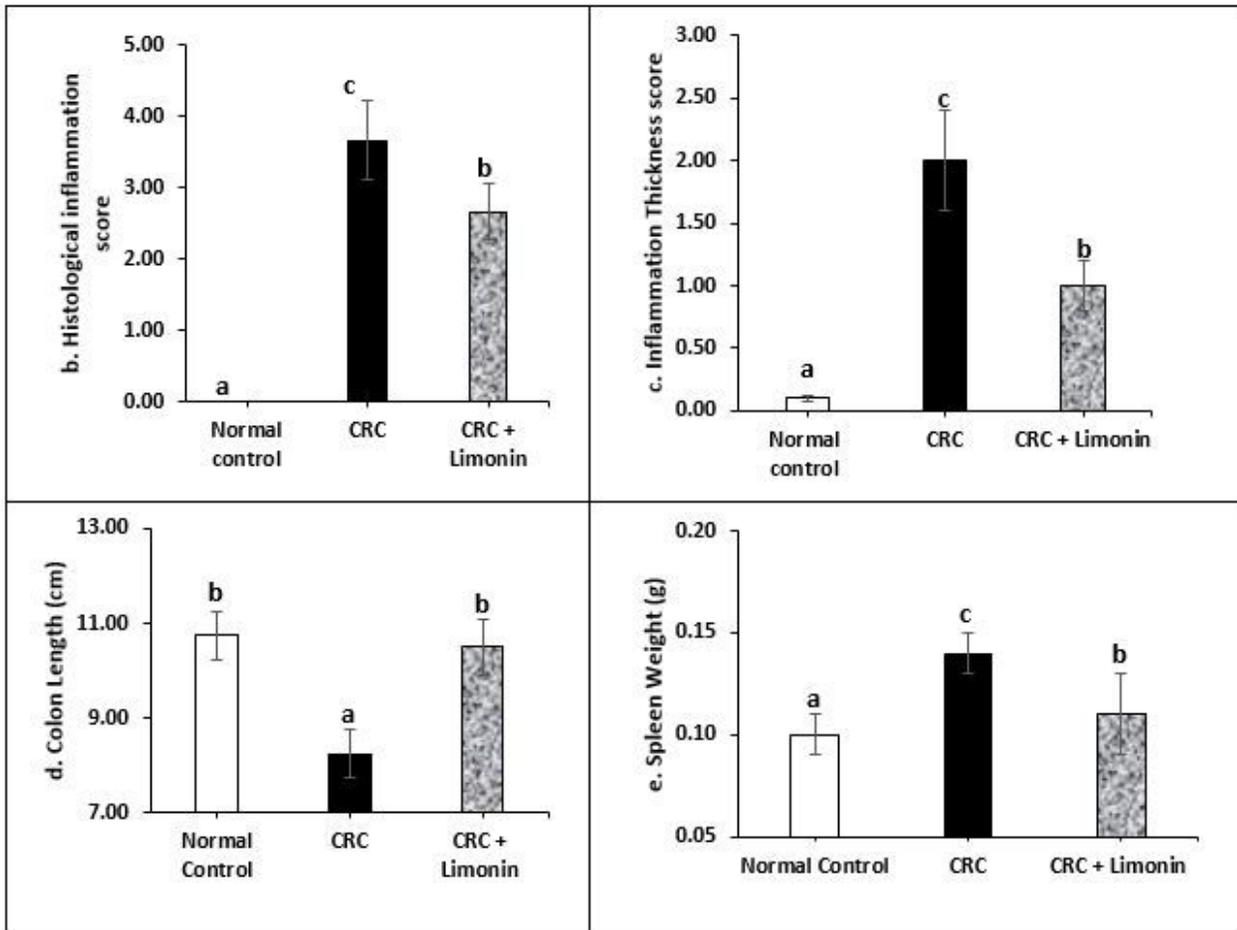
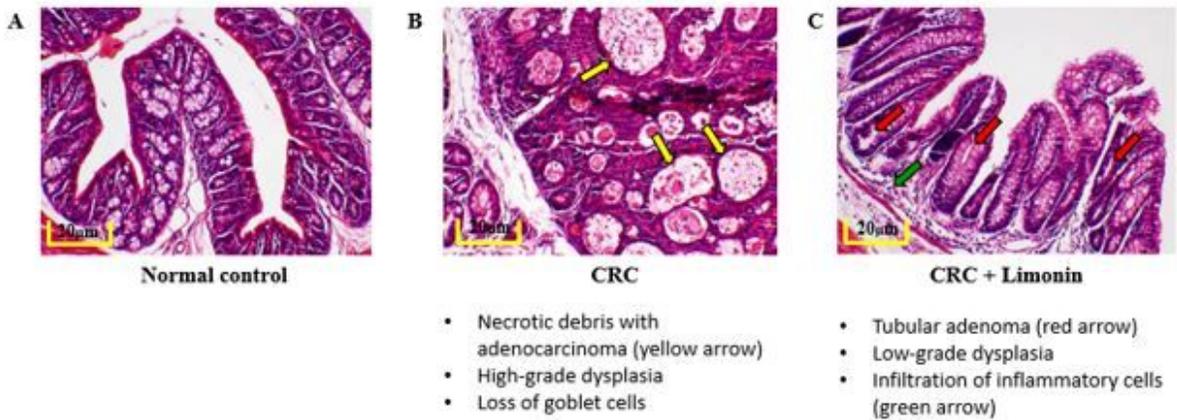


[Values are mean \pm SD of 6 mice. Values with similar letters/symbols are insignificantly different at $p < 0.05$. Colorectal cancer (CRC)].

Figure 1

The effects of limonin on (a) body weight, (b) tumor incidence (TI), (c) disease activity index (DAI), (d) serum malondialdehyde (MDA) (n=6).

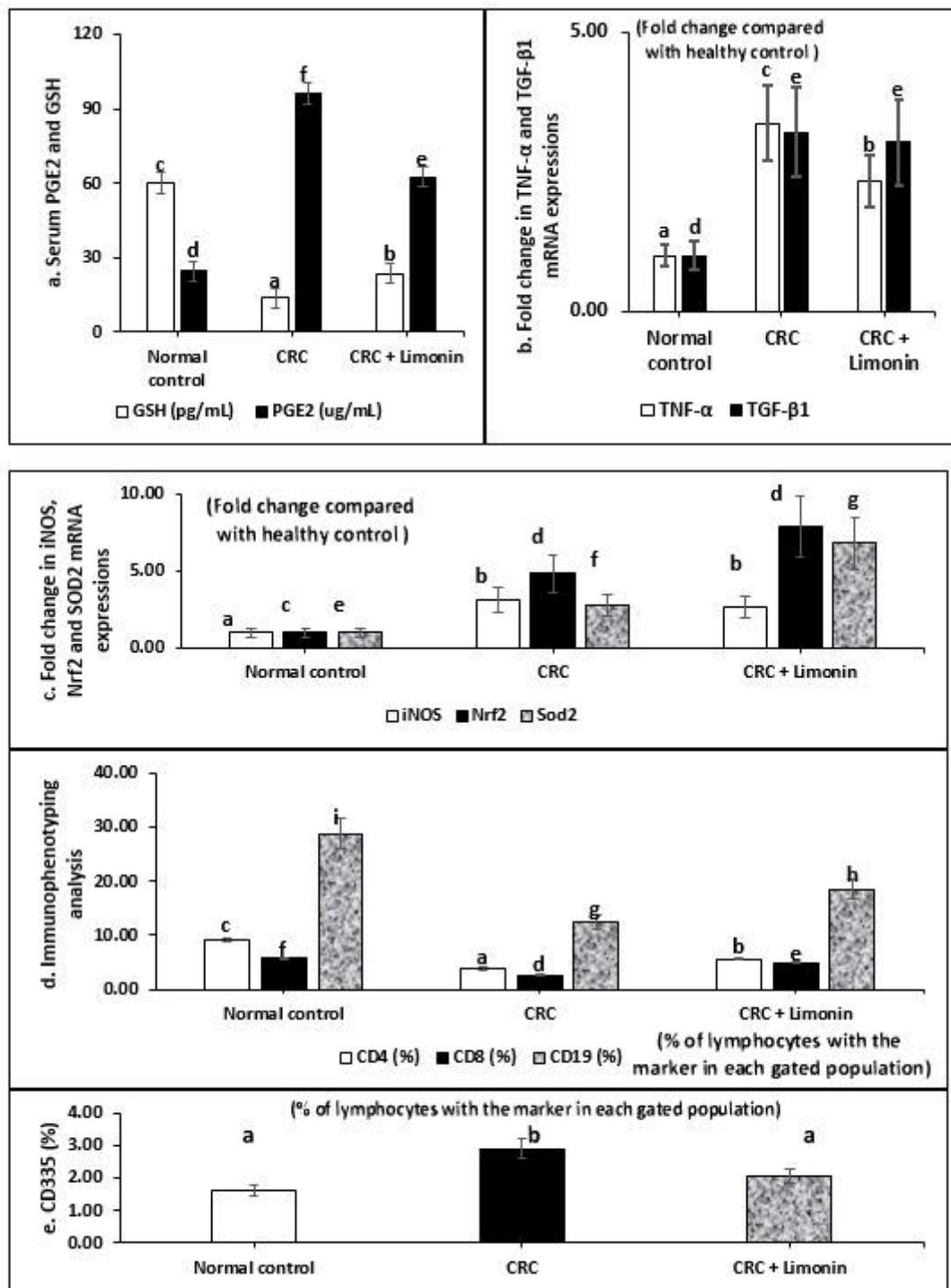
a. Histological alterations



The results are presented as mean ± SD of 6 mice. Values with similar letters are insignificantly different at $p < 0.05$. Colorectal cancer (CRC).

Figure 2

(a) Photomicrograph of the effects of limonin on the colon of CRC-induced mice (H&E, X20), (b-c) Histological inflammation score and inflammation thickness score from the histology of the mice colon, (d) colon length, (e) spleen weight (n=6).



[Values are mean ± SD of 3 or 4 mice. Values with similar letters/symbols are insignificantly different at $p < 0.05$. Colorectal cancer (CRC)].

Figure 3

The effects of limonin on (a) serum prostaglandin E2 (PGE2) and reduced glutathione (GSH) levels (n=4), (b) the fold change in mRNA expressions in the colon tissues for TNF-α and TGF-β1 (c) iNOS, Nrf2 and SOD2 (d) immunophenotyping analysis of CD4, CD19 and CD8, and CD335 in the spleen (single-cell suspensions stained with monoclonal antibodies and analysed by flow cytometry) (n=3).

Supplementary Files

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