Limonin modulated immune and inflammatory responses to suppress colorectal adenocarcinoma in mice model

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Abstract
Inflammation and compromised immune responses often increase colorectal cancer (CRC) risk. The immune-modulating effects of limonin on carcinogen/inflammation-induced colorectal cancer (CRC) were studied in mice. 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 developments were monitored via macroscopic, histopathological, ELISA, and mRNA expression analyses. 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 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 them to normal levels suggesting reinstatement to normal colon conditions. Limonin apparently mitigated CRC development, by ameliorating adaptive immune responses (CD8, CD4, and CD19), reducing inflammation (serum prostaglandin E2; TNF-α, innate immune responses) and oxidative stress, and enhancing the endogenous anti-oxidation defense reactions (GSH) in CRC-induced mice.

Keywords Limonin · Colorectal cancer · Inflammation · Immune responses · Flavonoid

Introduction
Colorectal cancer (CRC) is among the top three killer cancer worldwide, the 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) complication which can progress from the chronic inflamed mucosa to dysplasia and, ultimately, colorectal cancer. Surgery, neoadjuvant chemotherapy, and radiotherapy are the most common therapies used with many painful and undesirable side effects. The FDA-approved chemotherapeutical drug for CRC includes capecitabine, fluorouracil (5-FU), irinotecan, oxaliplatin, and trifluridine/tipiracil. Although chemotherapy is the foremost selected therapy, it usually causes nausea, vomiting, diarrhea, neuropathy, mouth sores, fatigue, hair loss, and increased infection risks, and often extends 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 cell proliferation (Chidambaram 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 Balb/c mouse model of colorectal adenocarcinoma induced by azoxymethane (AOM)/dextran sulfate sodium (DSS) model. This study provides indications that dietary limonin suppressed colon adenocarcinoma, at least partly by enhancing immune, anti-inflammatory, and endogenous anti-oxidant responses in colorectal adenocarcinoma–induced mice.

Materials and method

Chemicals and drugs

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

Animals

Five-week-old male Balb/c mice (25–30 g) were purchased from the Animal Resource Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia (Serendang, 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 to avoid any effects due to female 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). 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 was completely homogenized in distilled water (0.25 mg/ml) and given as drinking water to G3. The average daily Balb/c mouse 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-week experimental duration after 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: bloody; 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 blood collected 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 and cleaned, colon length measured, and tumor incidence/number was counted. The colon length was measured using a ruler, and recorded to the nearest centimeter. 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 an automated programmed tissue processing machine. The tissue was embedded in paraffin and tissue blocks sectioned at a 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 was measured throughout the layers of the colon wall, such as the mucosa, sub-mucosa, or transmural tissues and ranged from 0 to 3 (0, no inflammation; 1, mucosa; 2, mucosa plus submucosa; and 3, transmural).

The inflammation score (different from inflammation thickness) 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 assay

The serum malondialdehyde (MDA), reduced glutathione (GSH), and prostaglandin E2 (PGE2) levels were determined by using commercial enzyme-linked immunosorbent assay (ELISA) kits (Elabscience Biotechnology, Wuhan, China) following the manufacturer’s protocol.

RNA extraction and RT-qPCR analysis

Total RNA from large intestine of mice was extracted with RNaseasy Mini Kit (Qiagen, Hilden, Germany). Briefly, ~20 mg of large intestine was disrupted in liquid nitrogen utilizing a 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 RNaseasy 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 to 280 nm using the Nano Drop TM 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 $2^{\Delta \Delta C_T}$ normalized to β-actin since β-actin was the most stable gene compared to the other housekeeping genes analyzed in this study. Fold change ($2^{\Delta C_T}$) was calculated by dividing the average normalized gene expression ($2^{\Delta C_T}$) in the test group with the average normalized gene expression ($2^{\Delta C_T}$) 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 (10% FBS and 1% penicillin/streptomycin). The cell suspension was centrifuged at 500 g, 4 °C for 5 min. Then, the pellet was washed with 3 ml of PBS, followed by incubation with 3 ml of ACK lysis buffer (0.15 M NH4Cl, 1.0 mM KHCO3, 0.1 mM EDTA) for 3 min and centrifuged at 500 g for 5 min. The single-cell suspension was washed with BSA solution twice and centrifuged at 300 g, 4 °C for 5 min. 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 min. The antibodies were from BD Pharmingen™ and they were (i) PerCP-Cy5.5 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-Cy5.5  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 min. The cell pellets were suspended in BSA solution and analyzed 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 analyses are presented as mean ± SD, unless otherwise stated. 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 amounts of food and water. 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 (Fig. 1b and c). 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 \((p < 0.05)\) (Fig. 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 significantly reduced. The histopathology results showed that limonin suppressed CRC development (Fig. 2a–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. Limonin mitigated the carcinogen/inflammation-induced CRC and alleviated the colonic mucosal dysplasia, adenoma, and adenocarcinoma formation (Fig. 2). Limonin reduced the histological scores towards normal values (Fig. 2d, e). CRC mice showed abnormal colon length and spleen weight, which were mitigated by limonin treatment (Fig. 2f, g).

Limonin enhanced serum GSH and suppressed serum PGE2 levels under carcinogen/inflammation-induced conditions (Fig. 3a). Reduced glutathione (GSH) is an endogenous antioxidant response constituent that protects cells from oxidative damage and free radicals, a biomarker for anti-oxidants against oxidative stress. Prostaglandin E2 (PGE2) is a pro-inflammatory lipid mediator that causes 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 CRC-induced mice. The treatment with limonin significantly \((p < 0.05)\) downregulated the expressions by about 75% (Fig. 3b), indicating that anti-inflammatory pathways are involved. The TGF-β1 and iNOS expression was insignificantly \((p < 0.05)\) affected by limonin as compared to non-treated CRC-induced mice (Fig. 3b and c).
Limonin upregulated Nrf2 and SOD2 mRNA expressions

The Nrf2 expression was significantly ($p < 0.05$) upregulated in all the CRC-induced mice (Fig. 3c). Limonin significantly upregulated Nrf2 mRNA expressions further as compared to the non-treated CRC-induced mice (Fig. 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 (Fig. 3c).
Limonin modulated the immunophenotyping of lymphocytes CD4+, CD8+, and CD19+ and CD335

Limonin enhanced immunophenotyping of lymphocytes CD4+, CD8+, and CD19+ in the spleen of the CRC-induced mice, demonstrating its good immune-stimulating effects (Fig. 3d). The percentages 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% (Fig. 3d). The CD335 expressed on natural killer cells were significantly increased (p < 0.05) in the CRC-induced mice and limonin treatment restored them to normal levels (Fig. 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 and disrupting colonic crypts, macrophages, and
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 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-κB. TNF-α is intricately linked to chronic inflammation and has pleiotropic functions for cell proliferation, differentiation, death, and survival. 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. Nrf2 (transcription factor NF-E2-related factor 2) is a key regulator for a subset of genes to produce detoxication proteins for removing electrophiles and reactive oxygen species, and repairing 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 acid–induced intestinal cell proliferation (Dossa et al. 2016) and SOD2 scavenges harmful and mutagenic mitochondrial reactive oxygen species (ROS). In the present investigation, limonin probably helped counteract oxidative stress via increasing SOD, Nrf2, and other antioxidant defense mRNA expressions. MDA is a lipid peroxidation product that is a biomarker for oxidative stress. MDA can react with deoxyguanosine to form endogenous mutagenic DNA adducts that can hasten aging and metabolic disorders. Excessive ROS or free radical generation accelerates lipid peroxidation, nucleic acid, and protein damage.

Imbalance between antioxidants and ROS leads to the oxidative damage of biomolecules and disruption of redox homeostasis. GSH suppresses PGE2 synthesis via interaction with COX enzymes and inhibits the production of prostaglandin in inflammatory states (Hartl et al. 2005). PGE2 elicits various biological effects and plays a 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 increased the activity levels of reduced serum glutathione (GSH) and reduced PGE2 levels in the CRC-induced mice.

Limonin enhanced immunophenotyping of lymphocytes CD4+ (PerCP-Cy5.5), CD8+ (APC), and CD19+ (PE) 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, with an inverse correlation confirmed between CD8A and VEGFA expression (Zhang et al. 2018). Here, the limonin showed no significant effects on IFN-γ or CD8A mRNA expressions under CRC induction.

CD4+ T cells help in producing pro- and anti-inflammatory cytokines especially IFN-γ and TNF-α. The interactions between TNF receptor (Fas) and Fas ligand (FasL) activate apoptotic signalling pathway by caspase activation which contributes to DNA fragmentation and formation of apoptotic cells. The CD4+ T cells could induce tumor dormancy that prevents tumor escape (Müller-Hermelink et al. 2008). CD8+ T cells are essential for immune defense against cytotoxic molecules, and CRC patients with high CD8+ T cell infiltration showed better prognosis (Deschoolmeester et al. 2011). Limonin may help in increasing T cell proliferation capacity by releasing perforin, granzymes, and granulysin. CD19+ is a type 1 transmembrane protein and coreceptor of the B cell. B cells are necessary to attain optimal CD4+ and CD8+ T cell tumor immunity. B cell-mediated antitumor immunity can suppress cancer metastasis and depletion of B cells can promote tumor growth (Yuen et al. 2016). In animal models, B cell-based cancer immunotherapy achieved promising results and demonstrated that B cells can induce T-cell mediated antitumor immunity (Soreno et al. 2011).

In this study, limonin enhanced CD19+ in CRC-induced mice which could improve therapeutic efficacy.

The limitations of this study include the small sample size (due to high mouse mortality after 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 CRC induction. Higher doses caused a higher incidence of cancer with a shortened latency period, but a very high mortality rate. Too low a dose caused extremely long CRC development duration, if the CRC develops at all. The extent of the lesion in the mice depended on the mouse 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 mouse strain to obtain the acceptable CRC development, appropriate for the research resources available.
Conclusions

This study demonstrates that limonin helps 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. CD335 (natural killer cells) were increased in the CRC-induced mice and limonin treatment restored them 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.

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Author contribution 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 the researchers approved the final manuscript. All the authors read and approved the manuscript and all data were generated in-house and that no paper mill was used.

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Data availability Provided as supplementary material.

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.

Competing interests The authors declare no competing interests.
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