Food & Function

PAPER

Cite this: *Food Funct.*, 2016, **7**, 3233

Rosmarinus officinalis L. extract ameliorates intestinal inflammation through MAPKs/NF-κB signaling in a murine model of acute experimental colitis†

Kanakaraju Medicherla,[a] Avanee Ketkar,[a] Bidya Dhar Sahu,[b] Godi Sudhakar*[a] and Ramakrishna Sistla*[b]

Received 24th February 2016,
Accepted 14th June 2016

DOI: 10.1039/c6fo00244g

www.rsc.org/foodfunction

We investigated the anti-inflammatory and anti-colitis effects of *Rosmarinus officinalis* L. extract (RE) by using both *in vitro* LPS-activated mouse RAW 264.7 macrophages and *in vivo* dextran sulfate sodium (DSS)-induced experimental murine colitis and suggested the underlying possible mechanisms. Liquid Chromatography-Mass Spectrometry (LC-MS) analysis was performed to identify the major components present in the RE. The clinical signs, biochemistry, immunoblot, ELISA and histology in colon tissues were assessed in order to elucidate the beneficial effect of RE. RE suppressed the LPS-induced pro-inflammatory cytokine production and the expressions of inflammatory proteins in macrophages. Administration of RE (50 and 100 mg kg^{-1}) also significantly reduced the severity of DSS-induced murine colitis, as assessed by the clinical symptoms, colon length and histology. RE administration prevented the DSS-induced activation of p38, ERK and JNK MAPKs, attenuated IκBα phosphorylation and subsequent nuclear translocation and DNA binding of NF-κB (p65). RE also suppressed the COX-2 and iNOS expressions, decreased the levels of TNF-α and IL-6 cytokines and the myeloperoxidase activity in the colon tissue. Histological observation revealed that RE administration alleviated mucosal damage and inflammatory cell infiltration induced by DSS in the colon tissue. Hence, RE could be used as a new preventive and therapeutic food ingredient or as a dietary supplement for inflammatory bowel disease.

Introduction

Ulcerative colitis is a relapsing and chronic inflammatory bowel disease (IBD) that occurs selectively at the rectal colon and expands continuously into the large intestine.[1] It is characterized by ulceration, bleeding of the colonic mucosa with an imbalance in the intestinal mucosal immune system which shifts toward the pro-inflammatory side.[2] It has been well established that inflammation plays a crucial role in the pathogenesis of ulcerative colitis.[3] An increase in pro-inflammatory cytokine production, infiltration of inflammatory cells, nitric oxide production derived from inducible nitric oxide synthase (iNOS), and activation of NF-κB and MAPK signaling in colonic tissue are apparently associated with the pathogenesis of ulcerative colitis.[1,4,5] Apart from this, oxygen-derived free radicals and activated neutrophils releasing injurious molecules including reactive oxygen metabolites have also played an imperative role as triggers of intestinal inflammation and subsequent tissue injury.[3] Thus, suppressing the inflammatory pathways will be a reasonable strategy to alleviate IBD including ulcerative colitis.

Rosemary (*Rosmarinus officinalis*) is a common household aromatic perennial herb from the family Lamiaceae.[6] The aerial parts of rosemary are often used as a food preservative and also as a flavoring agent in foods, beverages and in cosmetics.[7] Recently, rosemary extracts have been adopted into European Union legislation and used as a safe, effective and natural alternative to synthetic antioxidants in many food preparations.[8] Carnosic acid, carnosol and rosmarinic acid are the most widely studied constituents of rosemary and are believed to be responsible for the wide pharmacological activities of rosemary.[9–11] The accumulated evidence supports the strong anti-inflammatory properties of rosemary. It has been demonstrated that rosemary extract inhibits NF-κB activation

[a]*Department of Human Genetics, College of Science and Technology, Andhra University, Visakhapatnam 530003, India. E-mail: gsudhakar2002@yahoo.com; Tel: +91-891-2844725*

[b]*Medicinal Chemistry and Pharmacology Division, CSIR-Indian Institute of Chemical Technology (IICT), Hyderabad 500 007, India. E-mail: sistla@iict.res.in; Fax: +91-40-27193189; Tel: +91-40-2719375*

†Electronic supplementary information (ESI) available: (1) MS spectra of major constituents present in Rosemary extract (RE). (2) Cell culture and *in vitro* evaluation of the anti-inflammatory effect of RE. See DOI: 10.1039/c6fo00244g

and blocks the tumor necrosis factor-alpha (TNF-α), prostaglandin E2 (PGE2) and nitrite secretion in LPS-stimulated RAW 264.7 cells.[12] Moreover, rosemary extract also suppressed the cyclooxygenase-2 (COX-2), TNF-α and interleukin-1β (IL-1β) gene expression and the infiltration of inflammatory cells in a phorbol 12-myristate 13-acetate (PMA)-induced ear edema model in mice.[13] Recently, it has also been demonstrated that oral treatment with rosemary extract inhibits neutrophil migration and the TNF-α, IL-6 and PGE2 secretion in the carrageenan-induced inflamed exudates.[14] Although the potent anti-inflammatory properties of rosemary extract have been well characterized, the effect on inflammatory disorders like ulcerative colitis is not yet explored. Hence, the present study was undertaken to investigate the possible protective effect of rosemary extract (RE) on intestinal inflammation by using a dextran sulphate sodium (DSS)-induced ulcerative colitis model in mice and explored the underlying possible mechanisms.

Materials and methods

Chemicals and reagents

Dextran Sulphate Sodium (DSS) (molecular weight: 36 000–50 000) was purchased from MP Biomedicals, Solon, OH, USA. Lipopolysaccharide (*Escherichia coli* 055:B5), Griess reagent, hexadecyltrimethylammonium bromide (HTAB) and *o*-dianisidine were obtained from Sigma-Aldrich Co., St Louis, MO, USA. Mouse specific TNF-α and IL-6 ELISA kits were purchased from BD Bioscience, San Diego, CA, USA. A NF-κB (p65) transcription factor assay kit was purchased from Cayman Chemical Company, Ann Arbor, MI. A bicinchoninic acid (BCA) protein assay kit, Halt protease inhibitor cocktail, radioimmunoprecipitation (RIPA) buffer, an NE-PER nuclear and cytoplasmic extraction kit were purchased from Pierce Biotechnology, Rockford, IL, USA. All other reagents were of analytical grade.

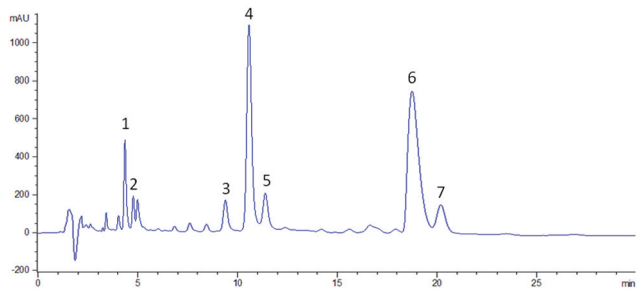

Fig. 1 Chromatogram (210 nm) corresponding to the LC-MS analysis of the rosemary extract (RE). Peak numbers corresponding to each of the compounds identified in the extract are listed in Table 1.

Rosemary extract and identification of major components by LC-MS

Rosmarinus officinalis L. (rosemary) leave methanolic extract (RE) containing carnosic acid as a major component was a kind gift from Exotic Naturals Pvt. Ltd, Mumbai, India. Liquid Chromatography-Mass Spectrometry (LC-MS) analysis was performed to identify the major components present in RE. The detailed procedures and the MS spectra of major ingredients are mentioned in the ESI-1.† Fig. 1 presents the chromatographic profile (210 nm) of the RE. A complete list of all the compounds identified in the RE is shown in Table 1. The compounds were characterized by their retention times, MS spectra and molecular ion identification, and identified by a comparison with published data. LC-MS analysis revealed that the major components present in the RE were carnosic acid (42.57%), carnosol (28.54%), rosmanol (5.68%), methylcarnosate (5.25%) and rosmanol methyl ether (4.87%).

Cell culture and *in vitro* evaluation of the anti-inflammatory effect of RE

The detailed procedures related to the culture of murine macrophage RAW 264.7 cells, the cytotoxicity of RE (concentrations ranging from 0 to 100 μg ml^{-1}) using the MTT reduction assay and the *in vitro* evaluation of the anti-inflammatory effect of RE are mentioned in the ESI-2.†

Animals

Male Balb/C mice (8 to 10 week-old, weighing between 22 and 25 g) were used to examine the effect of RE against

Table 1 List of major compounds identified in the rosemary leaf extract (RE) by LC-MS

Peak number	R_t (min)	Molecular weight	Major fragments (*m*/*z*)	Ionization mode	Proposed compound	g per 100 g	Ref.
1	4.3	346	345, 301, 283	N	Rosmanol	5.68	Ozarowski *et al.* (2013);[9]
2	4.6	346	345, 283	N	Epirosmanol	1.45	Borrás-Linares *et al.* (2014)[20]
3	9.3	360	359, 345, 301, 283	N	Rosmanol methyl ether	4.87	
4	10.5	330	329, 299, 285	N	Carnosol	28.54	
5	11.3	330	329, 317, 299, 285	N	Carnosol isomer	4.49	
6	18.7	332	331, 287	N	Carnosic acid	42.57	
7	20.1	346	345, 331, 301, 286	N	Methylcarnosate	5.25	

N indicates fragmentation of molecules obtained in negative ionization mode. Peak number correlates with numbers of major peaks on chromatogram UV in Fig. 1. R_t, retention time; g per 100 g, indicate gram of proposed compounds present in 100 g of rosemary leave extract powder.

DSS-induced ulcerative colitis. The study protocol was submitted to and approved by the Institutional Animal Ethics Committee (IAEC) of the CSIR-Indian Institute of Chemical Technology (IICT), Hyderabad, India (Permit no. IICT/PHARM/SRK/281/14/31). The study was performed in strict compliance with the guidelines for the safe use and care of experimental animals by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Animals were housed at a constant-temperature (22–24 °C) with a 12-hour dark/12-hour light cycle and fed a standard diet and water *ad libitum*.

Experimental design

As described in the earlier literature, mice were fed 4% DSS (molecular weight: 36 000–50 000; MP Biomedicals, Solon, OH, USA) in drinking water for 5 days to induce experimental acute ulcerative colitis.[4,15] To evaluate the effect of RE against DSS-induced colitis, mice were randomly divided into five groups each consisting of 8 animals.

1. Vehicle control (VC, n = 8): animals were administered orally with gum acacia suspension (2%) once a day and fed normal drinking water from day 1 to day 10.

2. Test drug control (RE, n = 8): animals were administered orally with RE (100 mg per kg body weight) in gum acacia suspension (2%) once a day and fed normal drinking water from day 1 to day 10.

3. DSS control (DSS, n = 8): animals were administered orally with gum acacia suspension (2%) once a day and fed 4% DSS in drinking water from day 6 to day 10.

4. DSS and RE at 50 mg kg^{-1} pretreatment (DSS + RE-50, n = 8): animals were administered orally with RE (50 mg per kg body weight) in gum acacia suspension (2%) once a day from day 1 to day 10 and fed 4% DSS in drinking water from day 6 to day 10.

5. DSS and RE at 100 mg kg^{-1} pretreatment (DSS + RE-100, n = 8): animals were administered orally with RE (100 mg per kg body weight) in gum acacia suspension (2%) once a day from day 1 to day 10 and fed 4% DSS in drinking water from day 6 to day 10.

The doses of RE were selected based on a previous study.[10] Animals were sacrificed through CO_2 asphyxiation on day 11 of the study (*i.e.* on the 6th day of DSS administration). The colon tissue was removed; the length was measured, and stored at −80 °C for further analysis. All the measurements presented in this study were from the distal portion of the colon tissues.

Assessment of disease activity index (DAI)

The assessment of the disease severity was evaluated as previously described.[16] Stool consistency, body weight loss and presence of blood in faeces were assessed to evaluate the disease severity and a scoring system was assigned to each disease symptom to calculate the disease activity index (DAI). Briefly, the DAI was calculated as the sum of the stool consistency (scored as: 0, well-formed pellets; 2, loose stools; 4, diarrhea), the body weight loss (scored as: 0, none; 1, 1–5%; 2, 5–10%; 3, 10–20%; 4, over 20%) and the presence or absence of blood in faeces (scored as: 0, negative hemoccult test; 2, positive hemoccult test; 4, gross bleeding).

Histopathology of colon tissue

For the microscopic histological examination, colon tissues (distal part) were collected and immediately fixed in 10% phosphate-buffered formalin. Then the specimens were processed, embedded in paraffin, sectioned at 5 μm thicknesses, and stained with hematoxylin and eosin (H and E). The tissue sections were then examined for histological changes under light microscopy (Axiovision software, Axioplan 2 Imaging, Zeiss microscope).

Extraction of whole, nuclear and cytoplasmic proteins

Colonic tissues were weighed, minced and homogenized in ice cold RIPA lysis buffer (Pierce Biotechnology, Rockford, IL, USA) containing 1% Halt protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA) to prepare whole protein extracts. The nuclear and cytoplasmic extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL, USA) as per instructions of the kit manuals. Protein concentration in the tissue extracts was estimated using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA) and bovine serum albumin (BSA) as standard.

Western blot analysis

Aliquots of tissue extracts containing equal amounts of protein (40 μg per lane) were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane, blocked in 3% bovine serum albumin (BSA) and incubated overnight at 4 °C with the appropriate antibody. All the primary and secondary antibodies were obtained from Cell Signaling Technology, Baverly, USA. The nuclear extracts were used for the quantification of NF-κB (p65) (rabbit monoclonal, 1 : 500), the cytoplasmic extracts were used for the quantification of IκBα (mouse monoclonal, 1 : 1000) and phospho-IκBα (rabbit monoclonal, 1 : 1000), and the whole tissue extracts were used for the quantification of COX-2 (rabbit monoclonal, 1 : 1000), iNOS (rabbit monoclonal, 1 : 1000), p38 (rabbit monoclonal, 1 : 1000), p-p38 (rabbit monoclonal, 1 : 1000), ERK (rabbit monoclonal, 1 : 1000), p-ERK (rabbit monoclonal, 1 : 1000), JNK (rabbit monoclonal, 1 : 1000) and p-JNK (rabbit monoclonal, 1 : 1000) expression. β-Actin (rabbit monoclonal, 1 : 1000) was used for whole and cytoplasmic extracts loading control, lamin B (rabbit monoclonal, 1 : 1000) was used for nuclear extract loading control and horseradish-peroxidase (HRP)-conjugated anti-mouse or anti-rabbit antibodies (1 : 5000) were used as secondary antibodies. The membranes were washed and signals were detected using the SuperSignal West Femto Maximum Sensitivity Substrate kit (Pierce Biotechnology, Rockford, IL, USA) and digitally analyzed using the ImageJ program (NIH).

Determination of TNF-α and IL-6 in colon tissue

The concentrations of TNF-α and IL-6 in the colon tissue homogenates were determined by using mouse specific TNF-α and IL-6 ELISA kits (BD Bioscience, San Diego, CA, USA), respectively. The concentrations were expressed as pg mg^{-1} protein.

Myeloperoxidase activity assay

Myeloperoxidase (MPO) activity, as a marker for neutrophil infiltration into the colon was determined as described in the previous literature and was expressed as U g^{-1} tissue.[16]

NF-κB (p65)-DNA binding assay

The DNA-binding activity of NF-κB (p65) in 2 μg of nuclear extract of colon tissues from each experimental animal was evaluated using NF-κB (p65) transcription ELISA kits as per manufacturer instructions (Cayman Chemical Company, Ann Arbor, MI).

Determination of nitrite levels in colon tissue

The contents of nitrite in the colon tissue, as an indicator of nitric oxide production was determined using Griess reagent (Sigma-Aldrich Co., St Louis, MO, USA) and sodium nitrite as standard.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Data were evaluated using Microsoft Excel 2007 and GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison tests. $p < 0.05$ was considered statistical significant.

Results

Effect of RE on cell viability, pro-inflammatory cytokines and nitrite release in LPS-induced RAW 264.7 cells

We analyzed the anti-inflammatory potential of RE in RAW 264.7 cells, following stimulation with LPS (Fig. 2). To determine the concentration of RE to use in *in vitro* studies, the cytotoxicity of RE was assessed by MTT reduction assay after cells were treated with various concentrations of RE. The result revealed that incubation of cells (24 h) with RE up to 12.5 μg ml^{-1} was not cytotoxic on RAW 264.7 cells. Thus we have selected up to 10 μg ml^{-1} of RE for further studies to evaluate the anti-inflammatory potential without affecting the cell viability (Fig. 2A and B). LPS treatment induced a significant ($p < 0.001$) elevation of TNF-α, IL-6 and nitrite levels in RAW 264.7 cells when compared with the control (no treatment) cells. Pretreatment with RE (5 and 10 μg ml^{-1}) followed by LPS (100 ng ml^{-1}) stimulation significantly suppressed the LPS-induced pro-inflammatory cytokines, TNF-α (Fig. 2C, $p < 0.001$ at both 5 and 10 μg ml^{-1}) and IL-6 (Fig. 2D, $p < 0.05$ at 5 μg ml^{-1} and $p < 0.001$ at 10 μg ml^{-1}) production and also inhibited the nitrite (Fig. 2E, $p < 0.001$ at both 5 and 10 μg ml^{-1}) production when compared with the only LPS-induced cells.

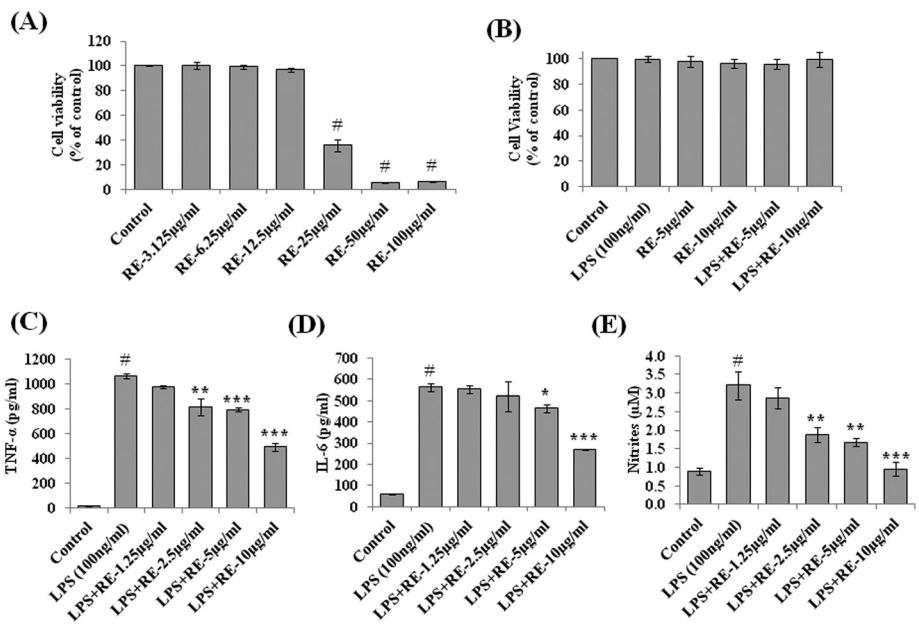

Fig. 2 Effect of rosemary extract (RE) on the cell viability and the pro-inflammatory cytokines (TNF-α and IL-6) and nitrites release in LPS-activated mouse RAW 264.7 cells. (A) Effect of RE on the viability of RAW 264.7 cells. (B) Combined effect of LPS and RE on the viability of RAW 264.7 cells. Effect of RE on (C) tumor necrosis factor-α (TNF-α), (D) interleukin-6 (IL-6) and (E) nitrites release in LPS-activated mouse RAW 264.7 cells. Data are expressed as mean ± S.D. $^{\#}p < 0.001$ *vs.* control cells (no treatment); $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* LPS alone treated cells.

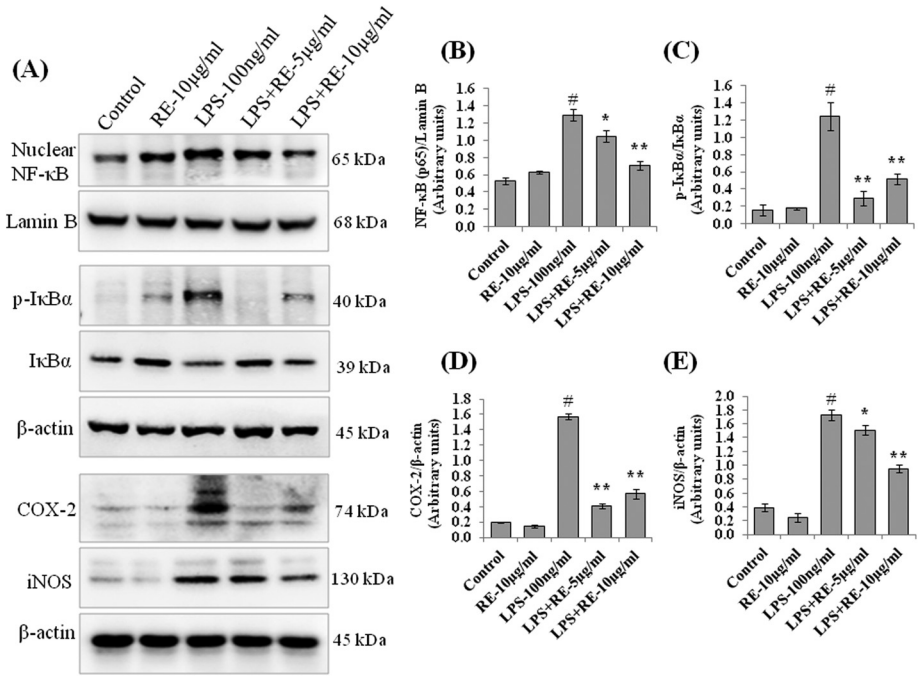

Fig. 3 Effect of rosemary extract (RE) on proteins of NF-κB signaling and the COX-2 and iNOS expressions in LPS-activated mouse RAW 264.7 cells. (A) Representative western blots and bar diagrams showing densitometric analyses of (B) NF-κB (p65), (C) p-IκBα/IκBα ratio, (D) COX-2, and (E) iNOS proteins. β-Actin and lamin B were used as loading control for whole and nuclear extracts, respectively. The data are presented as mean ± S.D. of three different experiments. $^{\#}p < 0.01$ *vs.* control cells (no treatment); $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* LPS alone treated cells.

Effect of RE on LPS-induced inflammation related proteins in RAW 264.7 cell lysate

To elucidate the mechanism underlying the anti-inflammatory effect of RE *in vitro*, we determined the effect on LPS-induced NF-κB signaling and its downstream, COX-2 and iNOS proteins through western blot analyses (Fig. 3). LPS at 100 ng ml^{-1} significantly ($p < 0.01$) induced nuclear translocation of NF-κB with subsequent phosphorylation of IκBα (p-IκBα/IκBα ratio) when compared with the control (no treatment) cells. Similarly, the amounts of COX-2 and iNOS proteins were also significantly ($p < 0.001$) increased in the LPS alone induced cells. Treatment with RE significantly suppressed the LPS-induced nuclear accumulation of NF-κB (p65) ($p < 0.05$ at 5 μg ml^{-1} and $p < 0.01$ at 10 μg ml^{-1}) and also prevented the phosphorylation of IκBα ($p < 0.01$) when compared with the only LPS-stimulated cells. Moreover, RE treatment significantly reduced the COX-2 ($p < 0.01$ at both 5 and 10 μg ml^{-1}) and iNOS ($p < 0.05$ at 5 μg ml^{-1} and $p < 0.01$ at 10 μg ml^{-1}) expressions in LPS-induced RAW 264.7 cells.

Effect of RE on DSS-induced weight loss and clinical symptoms

No mortality was observed in animals treated with DSS and/or rosemary extract during the study period. The clinical symptoms of mice were assessed by disease activity index (DAI) based on the daily scoring of the stool consistency, the body weight loss and the presence of blood in faeces. As shown in Fig. 4A, the DAI markedly ($p < 0.001$) increased in the DSS-model group when compared with the normal control group obviously from day 5. On day 6 of DSS admin-

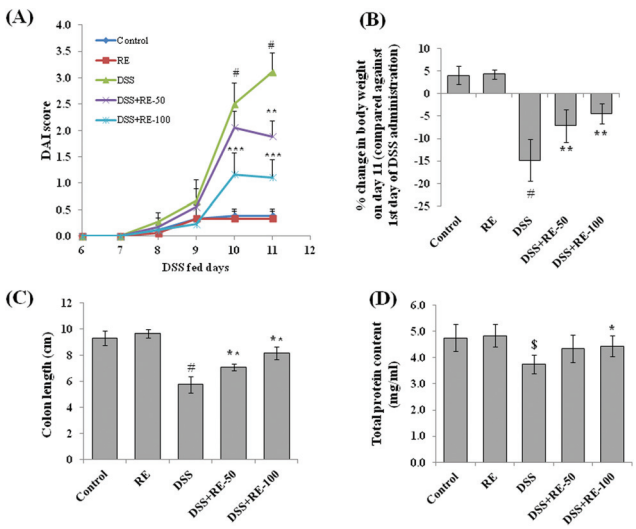

Fig. 4 Effect of rosemary extract (RE) on clinical symptoms of DSS-induced ulcerative colitis in mice. (A) Disease activity index (DAI) score, comprising the scores assigned to the body weight loss, the stool consistency and the presence of blood in feces, (B) changes in body weight (%) on end day, (C) changes in colon length, and (D) total protein content in colon tissue. Data are expressed as mean ± S.D. (n = 6). $^{\$}p < 0.05$ and $^{\#}p < 0.001$ *vs.* vehicle control group; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* DSS alone treated group.

istration (*i.e.* on the 11th day of the study), mice were sacrificed with CO_2 asphyxiation. The results showed a significant ($p < 0.001$) loss in the body weight (Fig. 4B) and a significant ($p < 0.001$) shortening in the length of colon (Fig. 4C) in the DSS model group when compared with that of the vehicle control group. Treatment with RE (both 50 and 100 mg kg^{-1}) significantly ($p < 0.01$ at 50 mg kg^{-1} and $p < 0.001$ at 100 mg kg^{-1}) decreased DAI scores and also prevented body weight loss ($p < 0.01$ at both 50 and 100 mg kg^{-1}) and the shortening in the length of the colons ($p < 0.01$ at both 50 and 100 mg kg^{-1}) in colitis mice. Moreover, a significant ($p < 0.05$) decrease in the amount of total protein content was observed in the colon tissues of DSS administered mice when compared with the vehicle control group (Fig. 4D). Though treatment with RE at 50 mg kg^{-1} did not produce any significant ($p > 0.05$) change in the total protein content, RE at 100 mg kg^{-1} significantly ($p < 0.05$) restored the total protein content when compared with that of the DSS model group.

Effect of RE on histological changes in colon tissue

The Hematoxylin and Eosin (H and E) staining and light microscopic examination of colon tissue from vehicle control (Fig. 5A) and RE alone (Fig. 5B) administered mice showed an intact colon structure without any histological change. Sections from DSS-induced mice (Fig. 5C) showed evidence of mucosal damage, ulceration, crypt distortion, loss of goblet cells and disruption of the epithelial layer with the infiltration of inflammatory cells in the colon tissues. The colon tissue sections from RE treated groups, 50 mg kg^{-1} (Fig. 5D) and 100 mg kg^{-1} (Fig. 5E) revealed a reduction in histological signs of tissue injury with evidence of less cryptic damage with preserved goblet cells and mild infiltration of inflammatory cells into the colon tissue.

Effect of RE on colonic MPO activity, pro-inflammatory cytokines and nitrites

The results showed that MPO activity in the DSS-administered group, an index of neutrophil infiltration, was significantly ($p < 0.001$) increased when compared with the vehicle control (no DSS) group (Fig. 6A). Similarly, a significant ($p < 0.001$) increase in the concentration of pro-inflammatory cytokines such as TNF-α (Fig. 6B) and IL-6 (Fig. 6C) was observed in DSS-induced colitic mice when compared with the vehicle control group. Following RE (50 and 100 mg kg^{-1}) treatment, the MPO activity ($p < 0.05$ at 50 mg kg^{-1} and $p < 0.01$ at 100 mg kg^{-1} RE) and the concentration of pro-inflammatory cytokines were significantly (TNF-α: $p < 0.01$ at both 50 and 100 mg kg^{-1} RE; IL-6: $p < 0.01$ at 50 mg kg^{-1} and $p < 0.001$ at 100 mg kg^{-1} RE) decreased when compared with the only DSS-induced group. Moreover, DSS significantly ($p < 0.001$) increased the colonic nitrite levels, a nitrosative stress marker (Fig. 6D) when compared with that of the vehicle control group. The colonic nitrite levels of DSS-colitis mice were significantly ($p < 0.001$ at both 50 and 100 mg kg^{-1} RE) decreased following treatment with RE.

Effect of RE on colonic NF-κB (p65) activation and inflammatory COX-2 and iNOS expressions

Western blot analyses revealed that the amount of nuclear NF-κB (p65) ($p < 0.01$), the ratio of p-IκBα/IκBα proteins ($p < 0.05$) and the expressions of COX-2 ($p < 0.01$) and iNOS ($p < 0.01$)

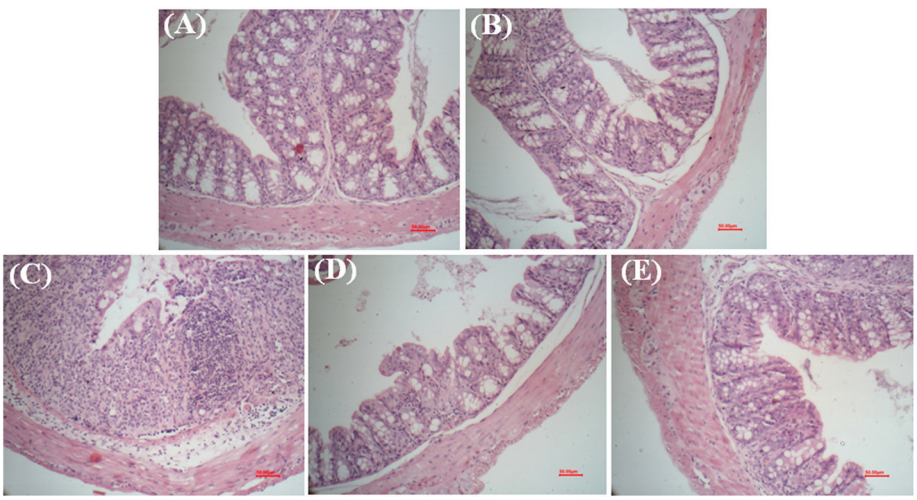

Fig. 5 Hematoxylin and Eosin (H & E) staining and light microscopic examination of colon tissues treated with DSS and/or RE. Representative photographs of colon tissue sections from vehicle control (A) and rosemary alone (B) treated groups of mice showing colon with intact histo-architecture. The tissue sections from DSS alone treated mice (C) showing destruction of crypt structure with loss of goblet cells, ulceration, erosion of the epithelial layer, massive influx of inflammatory cells. The tissue sections from RE at 50 mg kg^{-1} plus DSS treated mice (D) showing evidence of less cryptic damage and inflammatory cells with moderate ulceration in the colon tissue compared with that of DSS alone treated mice. The tissue sections from RE at 100 mg kg^{-1} plus DSS treated mice (E) showing mild cryptic damage with preserved goblet cells and mild inflammatory cells infiltrate in the colon tissue compared with that of DSS alone treated mice.

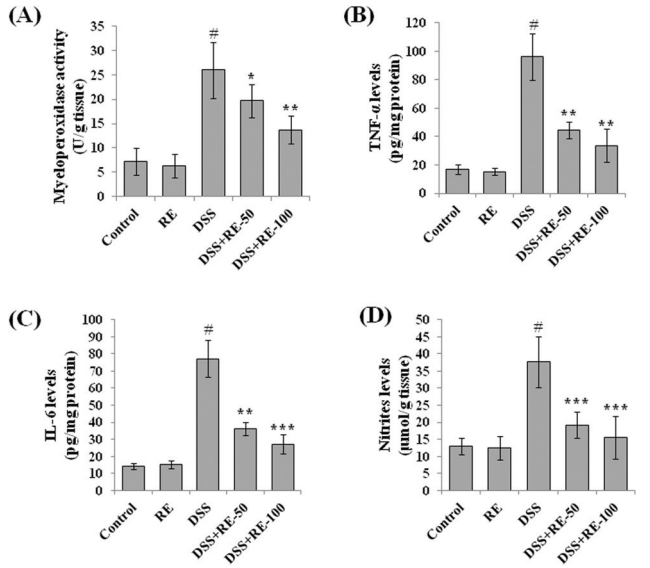

Fig. 6 Effect of rosemary extract (RE) on DSS-induced inflammatory parameters in colon tissue. (A) Myeloperoxidase (MPO) activity, (B) tumor necrosis factor-α (TNF-α), (C) interleukin-6 and (D) nitrites levels. Data are expressed as mean ± S.D. (n = 6). $^{\#}p < 0.001$ *vs.* vehicle control group; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* DSS alone treated group.

were significantly increased in the colon tissues of DSS alone treated mice (Fig. 7A). Similarly, NF-κB (p65) transcription ELISA revealed that there was a significant ($p < 0.01$) increase in the NF-κB (p65)-DNA binding activity in nuclear extracts of colon tissue from DSS-induced mice compared to that of vehicle control mice (Fig. 7B). Treatment with RE at both doses (50 and 100 mg kg^{-1}) significantly attenuated the nuclear translocation of NF-κB (p65) ($p < 0.01$ at both 50 and 100 mg kg^{-1}), prevented the phosphorylation and degradation of IκBα (p-IκBα/IκBα ratio) ($p < 0.05$ at both 50 and 100 mg kg^{-1}) and suppressed the inflammatory proteins, COX-2 ($p < 0.01$ at both 50 and 100 mg kg^{-1} RE) and iNOS ($p < 0.05$ at both 50 and 100 mg kg^{-1} RE) in colon tissues when compared with that of DSS alone administered mice. Treatment with RE at both doses (50 and 100 mg kg^{-1}) also significantly ($p < 0.01$) reduced the NF-κB (p65)-DNA binding activity in the DSS-induced colitic mice.

Effect of RE on colonic MAPK activation

As shown in Fig. 8, DSS administration significantly ($p < 0.01$ for p-p38/p38 and p-ERK/ERK; $p < 0.05$ for p-JNK/JNK) induced the phosphorylation of p38, ERK and JNK proteins in colon tissues. Treatment with RE at both doses effectively attenuated the DSS-induced increase in phosphorylation of p38 ($p < 0.01$ at both 50 and 100 mg kg^{-1}), ERK ($p < 0.01$ at both 50 and

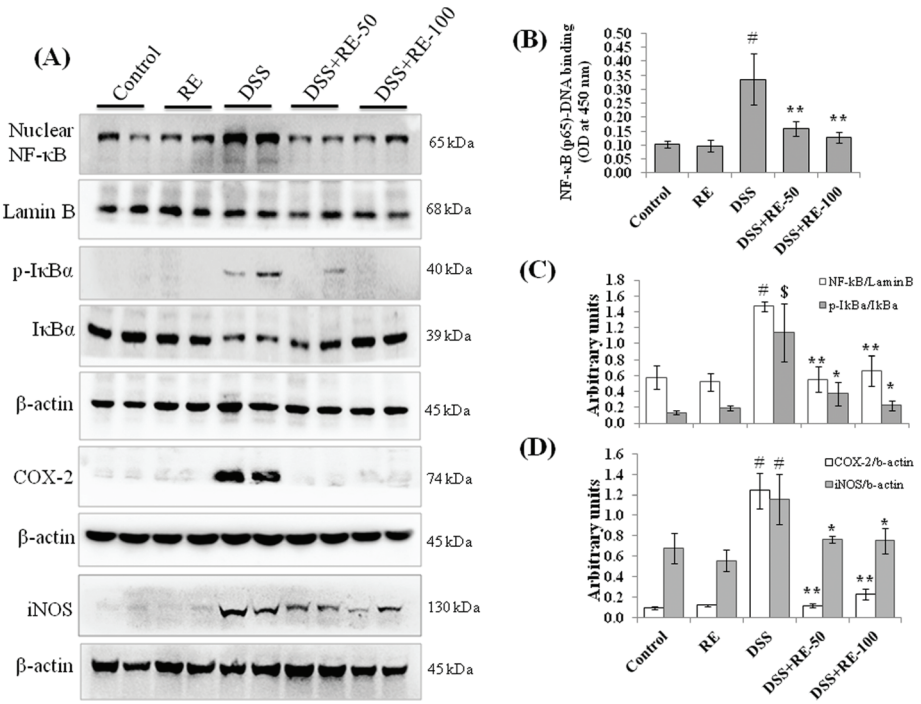

Fig. 7 Effect of rosemary extract (RE) on DSS-induced colonic NF-κB activation and pro-inflammatory COX-2 and iNOS expressions. (A) Representative western blots of NF-κB (p65), p-IκBα, IκBα, COX-2 and iNOS in colon tissues. (B) NF-κB (p65)-DNA binding activity of nuclear extracts from the colon tissues was determined by using the NF-κB transcription ELISA assay kit and data are expressed as mean ± S.D. (n = 6). Densitometric analysis of (C) nuclear-NF-κB (p65) and p-IκBα/IκBα ratio and (D) COX-2 and iNOS expressions. Lamin B was used as loading control for nuclear extracts and the β-actin was used for whole extracts. The results are representative of three independent experiments and expressed as mean ± S.D. $^{\$}p < 0.05$ and $^{\#}p < 0.01$ *vs.* vehicle control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* DSS alone treated group.

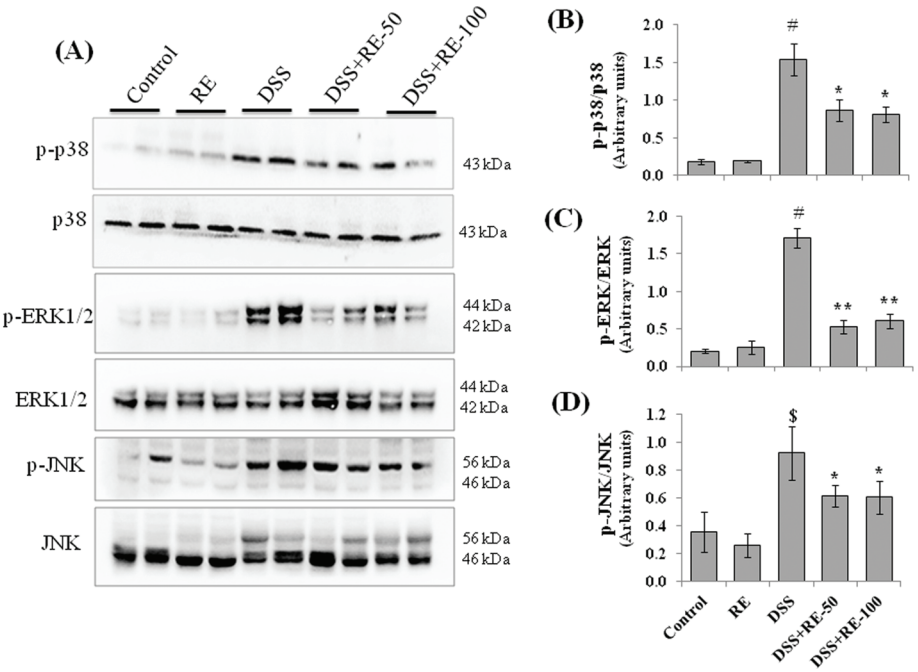

Fig. 8 Effect of rosemary extract (RE) on DSS-induced MAPK activation in colon tissue. Representative western blots (A) and bar diagrams showing densitometric analyses of (B) p-p38/p38, (C) p-ERK/ERK and (D) p-JNK/JNK proteins. Densitometric analyses were performed following normalization to the control (p38, ERK and JNK housekeeping genes, respectively). The results are representative of three independent experiments and expressed as mean ± S.D. $^{\$}p < 0.05$ and $^{\#}p < 0.01$ *vs.* vehicle control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* DSS alone treated group.

100 mg kg^{-1}) and JNK ($p < 0.05$ at both 50 and 100 mg kg^{-1}) in the colon tissue of mice with DSS-induced colitis.

Discussion

The increasing interest in naturally occurring plant products and herbal supplements has gained considerable attention in the primary health care system.[17] With regard to low toxicity and minimal side effects, recent investigations on management of inflammation have focused on the use of phytomedicine to offer alternative and effective anti-inflammatory therapies.[18] Rosemary is a common household aromatic herb much utilized in the food industry for its functional properties as well as for its beneficial health properties.[19] Our LC-MS analysis revealed that carnosic acid (42.57%), carnosol (28.54%), rosmanol (5.68%), methylcarnosate (5.25%) and rosmanol methyl ether (4.87%) are the major components present in rosemary extract (RE) and the findings are in good agreement with the previously published data of RE extracts.[9,20] Studies on rosemary have revealed that it has potent antioxidative and anti-inflammatory properties.[12,14] However, the beneficial effect of RE on inflammatory disorders like ulcerative colitis is not known. In the present study, an ulcerative colitis model was successfully established by administering mice with 4% DSS in drinking water for 5 days, and then the protective effect of RE was investigated. Consistent with the previous reports, our data showed that DSS administration produced a loss in body weight, bloody diarrhea, neutrophilic infiltration as evidenced by MPO activity, loss of crypts and desquamation in the colon with reduced colon length. Treatment with RE (both 50 and 100 mg kg^{-1}) effectively ameliorated the clinical features of colitis and histological changes and also prevented body weight loss and the shortening of colon length in colitic mice.

The etiology of IBD remains ambiguous and it is believed that a complex interaction between environmental, genetic and immune systems in the gut has a profound role during the initiation, progression and pathogenesis of IBD.[21] Studies in human and animal models have indicated that the imbalance of inflammatory cytokines in the gastrointestinal tract has a key role in the pathogenesis of IBD.[22] The release of pro-inflammatory cytokines causes the activation and perpetuation of the inflammatory response in intestinal mucosa.[23] Out of many pro-inflammatory cytokines, TNF-α is known to have a crucial role in the pathogenesis of colitis and the clinical inhibition of TNF-α (infliximab and adalimumab) has been shown to reduce disease pathology and morbidity in IBD patients.[24] Accumulating literature suggests that both activated macrophages and the transcription factor NF-κB are the major contributors in the progression of IBD by releasing pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and inducible pro-inflammatory enzymes (COX-2 and iNOS).[21,25] Moreover, it has also been reported that there is an increase in the production of proinflammatory cytokines and the expression of NF-κB signaling in the mucus of IBD patients and in the mouse DSS-induced acute colitis model.[26,27] Under resting conditions, the transcription factor NF-κB remains in

dimerization with the IκB protein in the cytosol. The activation of NF-κB due to the presence of external stimuli, the IκB kinase (IKK) phosphorylates the IκB protein, leading to its subsequent ubiquitination and degradation and the dissociation of NF-κB. As a consequence, the freed NF-κB translocates into the nucleus where it binds to the specific site of the target genes to regulate their transcription.[16,25] Our findings revealed that treatment with RE prevented the phosphorylation and degradation of IκBα and also markedly suppressed the nuclear translocation and DNA binding activity of NF-κB (p65) in the colon tissue of DSS-induced colitic mice. Consistent with the *in vivo* result, RE also interfered with NF-κB signaling and subsequent inflammatory response in LPS-activated mouse RAW 264.7 cells. Our findings are in line with the previously published data in which RE exerted its anti-inflammatory effect *via* inhibition of NF-κB pathways.[12,13] Hence, these results suggest that the anti-inflammatory effects of RE could be mediated, at least in part, by inhibition of NF-κB pathways.

MAPKs are serine/threonine kinases and are believed to be involved in the regulation of pro-inflammatory cytokine production and the upregulation of pro-inflammatory enzymes, COX-2 and iNOS in intestinal epithelial cells.[28] Recent studies have demonstrated that p38, ERK1/2, JNK MAPKs, as well as NF-κB are dramatically activated during the development of colitis and attenuation of these pathways ameliorates experimental colitis.[4,27] Studies also suggest that the upregulation of COX-2 and iNOS enzymes has a detrimental role in the colonic damage and precipitates the inflammatory response. Moreover, MAPKs are the upstream enzymes and signaling molecules for NF-κB.[4] In this study, we found that RE inhibited both NF-κB signaling and also the phosphorylation of MAPK signaling molecules, including p38, ERK and JNK in colon tissue of colitic mice. Treatment with RE also markedly reduced the expression of COX-2 and iNOS in colitic mice. Hence, our findings suggest that RE exhibits anti-inflammatory properties *via* the modulation of MAPKs and NF-κB signaling.

Infiltration of inflammatory cells especially neutrophils has been suggested to be involved in the development of colitis.[23,25] Myeloperoxidase is an enzyme most abundantly found in azurophilic granules of neutrophils and monocytes.[26] The estimation of MPO activity is thus used as a marker for neutrophil infiltration and also widely used to quantify tissue inflammation. Moreover, it has also been reported that the MPO is an important enzyme involved in the free radical generation and the increase in MPO activity is believed to aggravate tissue injury.[26,29] In the present study, MPO activity increased significantly following DSS administration, suggesting the recruitment of neutrophils. Treatment with RE reduced the activity of the MPO enzyme and can be interpreted as a sign of the anti-inflammatory activity of the RE.

In conclusion, the results of the present study support the possible beneficial effects of RE consumption in the prevention of DSS-induced ulcerative colitis by alleviating the disease severity, the infiltration of inflammatory cells and the histological changes in colon tissue. The inhibition of MAPKs and NF-κB signaling and subsequent suppression of proinflammatory cytokines and mediator release in the colon tissue may account for the anti-inflammatory and anti-colitis properties of RE. Hence, our study encourages the use of *Rosmarinus officinalis* L. extract (RE) as a complementary and alternative supplement for the prevention of ulcerative colitis.

Conflict of interest

The authors declare that there are no conflicts of interest associated with this study.

Abbreviations

RE	*Rosmarinus officinalis* L. extract
LC-MS	Liquid chromatography-mass spectrometry
DSS	Dextran sulfate sodium
IBD	Inflammatory bowel disease
DAI	Disease activity index
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TNF-α	Tumor necrosis factor-alpha
IL-6	Interleukin-6
BSA	Bovine serum albumin
H&E	Hematoxylin and eosin
NF-κB	Nuclear factor-kappa B
IκBα	NF-κB inhibitor alpha
MAPKs	Mitogen activated protein kinases
COX-2	Cyclooxygenase-2
iNOS	Inducible nitric oxide synthase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MPO	Myeloperoxidase

Acknowledgements

This work was partially supported by the CSIR 12th 5-year plan project 'SMiLE' (CSC 0111). The authors thank Director-IICT, Hyderabad for constant support and encouragement.

References

1 X. Tun, K. Yasukawa and K. Yamada, Involvement of nitric oxide with activation of toll-like receptor 4 signaling in mice with dextran sodium sulfate-induced colitis, *Free Radicals Biol. Med.*, 2014, **74**, 108–117.

2 F. Wei, Y. Zhang, J. Jian, J. J. Mundra, Q. Tian, J. Lin, *et al.*, PGRN protects against colitis progression in mice in an IL-10 and TNFR2 dependent manner, *Sci. Rep.*, 2014, **4**, 7023.

3 S. Matsuo, W. L. Yang, M. Aziz, S. Kameoka and P. Wang, Fatty acid synthase inhibitor C75 ameliorates experimental colitis, *Mol. Med.*, 2014, **20**, 1–9.

4 S. Sanchez-Fidalgo, I. Villegas, M. A. Rosillo, M. A. Soto and C. A. D. L. Lastra, Dietary squalene supplementation improves DSS-induced acute colitis by down regulating p38 MAPK and NF-κB signaling pathways, *Mol. Nutr. Food Res.*, 2015, **59**, 284–292.

5 S. J. Koh, J. W. Kim, B. G. Kim, K. L. Lee, J. Chun and J. S. Kim, Fexofenadine regulates NF-κB signaling and ER stress in intestinal epithelial cells, and ameliorates acute and chronic colitis in mice, *J. Pharmacol. Exp. Ther.*, 2015, **352**, 455–461.

6 I. Takaki, L. E. Bersani-Amado, A. Vendruscolo, S. M. Sartoretto, S. P. Diniz, C. A. Bersani-Amado, *et al.*, Anti-Inflammatory and Antinociceptive Effects of *Rosmarinus officinalis* L. Essential Oil in Experimental Animal Models, *J. Med. Food*, 2008, **11**, 741–746.

7 C. Ulbricht, T. R. Abrams, A. Brigham, J. Ceurvels, J. Clubb, W. Curtiss, *et al.*, An evidence-based systematic review of rosemary (Rosmarinus officinalis) by the Natural Standard Research Collaboration, *J. Diet. Suppl.*, 2010, **7**, 351–413.

8 A. Lopez-Jimenez, M. Garcia-Caballero, M. A. Medina and A. R. Quesada, Anti-angiogenic properties of carnosol and carnosic acid, two major dietary compounds from rosemary, *Eur. J. Nutr.*, 2013, **52**, 85–95.

9 M. Ozarowski, P. L. Mikolajczak, A. Bogacz, A. Gryszczynska, A. Kujawska, J. Jodynis-Liebert, *et al.*, Rosmarinus officinalis L. leaf extract improves memory impairment and affects acetylcholinesterase and butyrylcholinesterase activities in rat brain, *Fitoterapia*, 2013, **91**, 261–271.

10 B. D. Sahu, U. K. Putcha, M. Kuncha, S. S. Rachamalla and R. Sistla, Carnosic acid promotes myocardial antioxidant response and prevents isoproterenol-induced myocardial oxidative stress and apoptosis in mice, *Mol. Cell. Biochem.*, 2014, **394**, 163–176.

11 M. Romo-Vaquero, M. Larrosa, M. J. Yanez-Gascon, N. Issaly, J. Flanagan, M. Roller, *et al.*, A rosemary extract enriched in carnosic acid improves circulating adipocytokines and modulates key metabolic sensors in lean Zucker rats: Critical and contrasting differences in the obese genotype, *Mol. Nutr. Food Res.*, 2014, **58**, 942–953.

12 M. H. Yu, J. H. Choi, I. G. Chae, H. G. Im, S. A. Yang, K. More, *et al.*, Suppression of LPS-induced inflammatory activities by Rosmarinus officinalis L., *Food Chem.*, 2013, **136**, 1047–1054.

13 E. S. Mengoni, G. Vichera, L. A. Rigano, M. L. Rodriguez-Puebla, S. R. Galliano, E. E. Cafferata, *et al.*, Suppression of COX-2, IL-1β and TNF-α expression and leukocyte infiltration in inflamed skin by bioactive compounds from Rosmarinus officinalis L., *Fitoterapia*, 2011, **82**, 414–421.

14 A. M. Silva, I. D. Machado, J. R. Santin, I. L. P. Melo, G. V. Pedrosa, M. I. Genovese, *et al.*, Aqueous Extract of Rosmarinus officinalis L. Inhibits Neutrophil Influx and Cytokine Secretion, *Phytother. Res.*, 2015, **29**, 125–133.

15 Y. Zhang, M. Brenner, W. L. Yang and P. Wang, Recombinant human MFG-E8 ameliorates colon damage in DSS- and TNBS-induced colitis in mice, *Lab. Invest.*, 2015a, **95**, 480–490.

16 B. D. Sahu, J. Mahesh Kumar and R. Sistla, Fisetin, a dietary flavonoid, ameliorates experimental colitis in mice: Relevance of NF-κB signaling, *J. Nutr. Biochem.*, 2016, **28**, 171–182.

17 R. Sreedhar, S. Arumugam, V. Karuppagounder, R. A. Thandavarayan, V. V. Giridharan, V. Pitchaimani, *et al.*, Jumihaidokuto effectively inhibits colon inflammation and apoptosis in mice with acute colitis, *Int. Immunopharmacol.*, 2015, **29**, 957–963.

18 E. Giner, M. C. Recio, J. L. Rios, J. M. Cerda-Nicolas and R. M. Giner, Chemopreventive effect of oleuropein in colitis-associated colorectal cancer in c57bl/6 mice, *Mol. Nutr. Food Res.*, 2016, **60**, 242–255.

19 M. Romo-Vaquero, M.-J. Yanez-Gascon, R. Garcıa Villalba, M. Larrosa, E. Fromentin, I. Alvin, *et al.*, Inhibition of Gastric Lipase as a Mechanism for Body Weight and Plasma Lipids Reduction in Zucker Rats Fed a Rosemary Extract Rich in Carnosic Acid, *PLoS One*, 2012, **7**, e39773.

20 I. Borrás-Linares, Z. Stojanović, R. Quirantes-Piné, D. Arraez-Roman, J. Svarc-Gajic, A. Fernandez-Gutierrez, *et al.*, Rosmarinus Officinalis Leaves as a Natural Source of Bioactive Compounds, *Int. J. Mol. Sci.*, 2014, **15**, 20585–20606.

21 T. Liu, J. Ren, W. Wang, X. W. Wei, G. B. Shen, Y. T. Liu, *et al.*, Treatment of dextran sodium sulfate-induced experimental colitis by adoptive transfer of peritoneal cells, *Sci. Rep.*, 2015, **5**, 16760.

22 J. Yin, M. Wu, J. Duan, G. Liu, Z. Cui, J. Zheng, *et al.*, Pyrrolidine Dithiocarbamate Inhibits NF-KappaB Activation and Upregulates the Expression of Gpx1, Gpx4, Occludin, and ZO-1 in DSS-Induced Colitis, *Appl. Biochem. Biotechnol.*, 2015, **177**, 1716–1728.

23 M. Zhou, Z. Wang, J. Chen, Y. Zhan, T. Wang, L. Xia, *et al.*, Supplementation of the diet with Salecan attenuates the symptoms of colitis induced by dextran sulphate sodium in mice, *Br. J. Nutr.*, 2014, **111**, 1822–1829.

24 H. Zhang, J. Kovacs-Nolan, T. Kodera, Y. Eto and Y. Mine, γ-Glutamyl cysteine and γ-glutamyl valine inhibit TNF-α signaling in intestinal epithelial cells and reduce inflammation in a mouse model of colitis via allosteric activation of the calcium-sensing receptor, *Biochim. Biophys. Acta*, 2015, **1852**, 792–804.

25 T. Ren, T. Tian, X. Feng, S. Ye, H. Wang, W. Wu, *et al.*, An adenosine A3 receptor agonist inhibits DSS-induced colitis in mice through modulation of the NF-κB signaling pathway, *Sci. Rep.*, 2015, **5**, 9047.

26 M. Pervin, M. A. Hasnat, J. H. Lim, Y. M. Lee, E. O. Kim, B. H. Um, *et al.*, Preventive and therapeutic effects of Blueberry (Vaccinium corymbosum) extract against DSS-induced ulcerative colitis by regulation of antioxidant and inflammatory mediators, *J. Nutr. Biochem.*, 2016, **28**, 103–113.

27 W. Dou, J. Zhang, G. Ren, L. Ding, A. Sun, C. Deng, *et al.*, Mangiferin attenuates the symptoms of dextran sulfate sodium-induced colitis in mice via NF-κB and MAPK signaling inactivation, *Int. Immunopharmacol.*, 2014, **23**, 170–178.

28 M. A. Rosillo, M. Sanchez-Hidalgo, A. Cardeno and C. A. de la Lastra, Protective effect of ellagic acid, a natural polyphenolic compound, in a murine model of Crohn's disease, *Biochem. Pharmacol.*, 2011, **82**, 737–745.

29 X. Li, Y. Xu, C. Zhang, L. Deng, M. Chang, Z. Yu and D. Liu, Protective Effect of Calculus Bovis Sativus on Dextran Sulphate Sodium-Induced Ulcerative Colitis in Mice, *J. Evidence-Based Complementary Altern. Med.*, 2015, **2015**, 469506, DOI: 10.1155/2015/469506.