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Kelley M.K. Haarberg
Iowa State University

Meghan J. Wymore Brand
Iowa State University

Anne-Marie C. Overstreet
Iowa State University

Catherine C. Hauck
Iowa State University

Patricia A. Murphy
Iowa State University

See next page for additional authors

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ORIGINAL ARTICLE

Basic Study

Orally administered extract from *Prunella vulgaris* attenuates spontaneous colitis in mdr1a$^{-/-}$ mice

Kelley MK Haarberg, Meghan J Wymore Brand, Anne-Marie C Overstreet, Catherine C Hauck, Patricia A Murphy, Jesse M Hostetter, Amanda E Ramer-Tait, Michael J Wannemuehler

Kelley MK Haarberg, Meghan J Wymore Brand, Anne-Marie C Overstreet, Michael J Wannemuehler, Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, United States

Catherine C Hauck, Patricia A Murphy, Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011, United States

Jesse M Hostetter, Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, United States

Amanda E Ramer-Tait, Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, NE 68583, United States

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Correspondence to: Dr. Michael J Wannemuehler, Professor, Chair, Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, 2178 Vet Med, Ames, IA 50011, United States. mjwannem@iastate.edu
Telephone: +1-515-2943534
Fax: +1-515-2943839

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Abstract

AIM: To investigate the ability of a *Prunella vulgaris* (*P. vulgaris*) ethanolic extract to attenuate spontaneous typhlocolitis in mdr1a$^{-/-}$ mice.

METHODS: Vehicle (5% ethanol) or *P. vulgaris* ethanolic extract (2.4 mg/d) were administered daily by oral gavage to mdr1a$^{-/-}$ or wild type FVB^{WT} mice from 6 wk of age up to 20 wk of age. Clinical signs of disease were noted by monitoring weight loss. Mice experiencing

weight loss in excess of 15% were removed from the study. At the time mice were removed from the study, blood and colon tissue were collected for analyses that included histological evaluation of lesions, inflammatory cytokine levels, and myeloperoxidase activity.

RESULTS: Administration of *P. vulgaris* extracts to mdr1a$^{-/-}$ mice delayed onset of colitis and reduced severity of mucosal inflammation when compared to vehicle-treated mdr1a$^{-/-}$ mice. Oral administration of the *P. vulgaris* extract resulted in reduced ($P < 0.05$) serum levels of IL-10 (4.6 ± 2 *vs* 19.4 ± 4), CXCL9 (1319.0 ± 277 *vs* 3901.0 ± 858), and TNFα (9.9 ± 3 *vs* 14.8 ± 1) as well as reduced gene expression by more than two-fold for *Ccl2, Ccl20, Cxcl1, Cxcl9, IL-1α, Mmp10, VCAM-1, ICAM, IL-2,* and *TNFα* in the colonic mucosa of mdr1a$^{-/-}$ mice compared to vehicle-treated mdr1a$^{-/-}$ mice. Histologically, several microscopic parameters were reduced ($P < 0.05$) in *P. vulgaris*-treated mdr1a$^{-/-}$ mice, as was myeloperoxidase activity in the colon (2.49 ± 0.16 *vs* 3.36 ± 0.06, $P < 0.05$). The numbers of CD4^{+} T cells (2031.9 ± 412.1 *vs* 5054.5 ± 809.5) and germinal center B cells (2749.6 ± 473.7 *vs* 4934.0 ± 645.9) observed in the cecal tonsils of *P. vulgaris*-treated mdr1a$^{-/-}$ were significantly reduced ($P < 0.05$) from vehicle-treated mdr1a$^{-/-}$ mice. Vehicle-treated mdr1a$^{-/-}$ mice were found to produce serum antibodies to antigens derived from members of the intestinal microbiota, indicative of severe colitis and a loss of adaptive tolerance to the members of the microbiota. These serum antibodies were greatly reduced or absent in *P. vulgaris*-treated mdr1a$^{-/-}$ mice.

CONCLUSION: The anti-inflammatory activity of *P. vulgaris* ethanolic extract effectively attenuated the severity of intestinal inflammation in mdr1a$^{-/-}$ mice.

Key words: *Prunella vulgaris*; Spontaneous colitis; Inflammatory bowel disease; Mdr1a; Botanical extract; Mucosal inflammation; Nutraceutical

Core tip: Extracts of *Prunella vulgaris* (*P. vulgaris*) contain multiple anti-inflammatory phenolics and flavonoids and we report that oral administration of an ethanolic extract of *P. vulgaris* ameliorated the severity of spontaneous colitis in 20 wk old mdr1a$^{-/-}$ mice. Because these mice are genetically prone to develop colitis by 10 wk of age, daily oral treatments were initiated at 6 wk of age. This treatment regimen resulted in the inhibition of multiple parameters of inflammation that collectively contributed to ameliorate the severity of mucosal inflammation suggesting that botanical extracts may be used as effective complementary intervention strategies for the treatment of colitis.

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INTRODUCTION

The intestinal epithelium is the interface between the host and the lumen of the gastrointestinal tract and cooperates with other innate immune mechanisms to protect the host from microbial-induced inflammation as well as to hinder colonization and invasion by intestinal microorganisms. The ability to maintain low levels of mucosal inflammation in the gut is believed to be important for mucosal homeostasis. However, in the context of inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), regulation of intestinal inflammation often fails resulting in mucosal damage and chronic disease[1]. While idiopathic in nature, current hypotheses regarding the etiology of IBD point to complex multifactorial causalities, which include disruption of the intestinal epithelial barrier, dysbiosis of the microbiota, genetic predispositions, chronically activated inflammatory immune cells, and failed adaptive immune regulatory responses[2,3].

Much IBD research has focused on aberrant adaptive immune responses to antigens derived from the microbiota. More emphasis is now being placed on elucidating the role innate immune cells (*e.g.*, neutrophils), cytokines, chemokines, and their related transcription factors play in the initiation and/or maintenance of epithelial damage as the initial step in the onset of IBD[4]. In the absence of effective epithelial barrier function, compartmentalization that is meant to separate immune cells in the lamina propria from the numerous bacterial and food antigens normally sequestered in the lumen is lost[5]. A loss of epithelial barrier integrity is characteristic of UC and CD, and the consequential loss of immunologic tolerance to the microbiota initiates a cascade of signaling pathways that activate both innate and adaptive immune mechanisms[6].

The most common therapies used for the treatment of IBD are immune suppressive and anti-inflammatory drugs and biologicals such as monoclonal antibodies (*e.g.*, anti-TNF)[7,8]. Metronidazole and ciprofloxacin have also been utilized in several clinical trials related to the treatment of UC, CD, and pouchitis with underwhelming results[9]. Immunosuppressive therapies include monoclonal antibodies against TNF-α, 5-aminosalicylates (5-ASA), and steroids[10-12]. For many of these treatments, there is the potential for adverse effects that may include increased susceptibility to bacterial and viral infections and increased risk of cancer. One study showed that IBD related hospitalizations at high volume IBD treatment centers around the United States increased 6-fold

from 1998 to 2004[13]. This data illustrates that despite advances in IBD research, current therapies have not decreased the frequency of IBD related hospitalizations and surgical interventions are still common for severe forms of IBD. These facts and the financial burdens associated with expensive therapeutic regimens have lead patients to explore unconventional means of coping with IBD.

A 1998 study showed that up to 51% of surveyed IBD patients had used alternative or complementary therapies and, in particular, 16% of patients used the alternative therapies specifically for their IBD[14]. For most complementary therapies (*e.g.*, nutraceuticals), many anecdotal claims of health benefits exist with very little scientific data to support or negate those claims.

Prunella vulgaris (*P. vulgaris*is) commonly used in traditional Chinese medicine for wound healing, indigestion, burns and anti-inflammatory therapy. *P. vulgaris* contains several bioactive phenolics, triterpenoids and flavonoids[15]. Dietary phenolics such as rosmarinic acid, ursolic acid, and caffeic acid are all found in extracts of *P. vulgaris*, and have been shown to possess antioxidant, anti-inflammatory and anti-cancer activities[16-21]. Caffeic acid has also been shown to ef)fectively attenuate chemically induced experimental colitis through upregulation of cytochrome P450 (CYP4B1)[22]. Flavonoids, like those found in *P. vulgaris*, have been implicated as potential therapeutics for IBD as well[23]. In contrast to its ability to attenuate DSS-induced colitis, the flavonoid luteolin was found to attenuate spontaneous colitis by inhibiting the activation of NF-κB. Despite this promising evidence, there are no published reports evaluating the use of *P. vulgaris* extracts as a treatment for IBD. In this context, we have designed this study to test the hypothesis that an ethanolic extract of *P. vulgaris* will decrease gastrointestinal mucosal inflammation and thereby ameliorate the severity of spontaneous colitis in mdr1a$^{-/-}$ mice.

MATERIALS AND METHODS

Prunella vulgaris extract preparation

Information about the specific provenance of *P. vulgaris* accession Ames 27664, obtained from Dr. Mark Wiederlichner at the USDA-ARS North Central Regional Plant Introduction Station (Ames, IA), is available on the Germplasm Resources Information Network database at http://www.ars-grin.gov/npgs/acc/acc_queries.html. Above ground portions of plants from *P. vulgaris* (Ames 27664), harvested in 2008 were prepared for storage by drying for 8 d at 38 ℃ in a forced-air dryer with constant humidity. The dried material was ground with a 40-mesh screen and stored at -20 ℃ under N_2 until extraction. Weighed plant material was extracted with 95 ethanol with Soxhlet extractors for 6 h. The extract was concentrated by rotary evaporation at < 30 ℃ and lyophilized. The residue weight was recorded and the residues stored at -20 ℃ until solubilized in a final working solution of 5% ethanol in sterile distilled water at a final plant extract concentration of 12 mg/mL. The working *P. vulgaris* extract was divided into 2 mL aliquots and stored at -20 ℃ until use. *P. vulgaris* extracts from North Central Regional Plant Introduction Station were screened for endotoxin by using the Limulus Amebocyte Lysate Test (BioWhittaker, Inc., Walkersville, MD) according to manufacturers' specifications, and there was no detectable endotoxin present in the extract (data not shown). Extracts were tested for antimicrobial activity *in vitro* with no activity demonstrated.

Animals

Prior to the initiation of any work being performed, all animal related experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University. Incumbent with IACUC approval, methods and procedures were used tomimimize pain and/or distress of all animals used in this study. Four to five week old male mdr1a$^{-/-}$ FVB.129P2-Abcb1a tm1BorN7 and wild type (WT) FVB.129P2 mice were obtained from Taconic Farms, Inc. (Germantown, NY). Animals were housed and maintained in the Laboratory Animal Resource facility at the College of Veterinary Medicine, Iowa State University. Established specific pathogen-free husbandry practices were followed, and twelve-hour light/dark cycles were applied. Upon arrival and throughout the study, mice were fed a defined Harlan Teklad AIN93 (M) rodent chow (Madison, WI) to control the amount of phytochemicals in their diet.

Experimental design

Three treatment groups of mice where utilized: Mdr1a$^{-/-}$ mice that were orally gavaged with 2.4 mg/d *P. vulgaris* extract in a 200 μL volume (prepared as described above) and mdr1a$^{-/-}$ and FVB^{WT} mice were orally gavaged with 5% ethanol vehicle alone; there were 4 to 10 mice/group per experiment. Gavage was performed using a 20 gauge feeding needle once daily beginning at 6 wk of age until the mice reached 20 wk of age or were removed from the study because of severe clinical wasting and/or weight loss exceeding 15% of their peak body weight in order to minimize pain and discomfort. At necropsy, mice were euthanized by CO_2 asphyxiation. Following euthanasia, blood was collected by cardiac puncture and separate sections of each cecum and proximal colon were excised, washed, and stored for further histological, myeloperoxidase (MPO) enzymatic and real-time PCR analysis. Serum was analyzed by multiplex assay to measure cytokine and chemokine levels as well as western blot analysis for antibody reactivity to antigens derived from selected members of the microbiota. Cecal tonsils were also collected for flow cytometric analysis of T and B cell populations. All results are representative of two independent experiments.

Macroscopic typhlocolitis assessment

Following euthanization, the colon and cecum were excised, photographed, measured and scored for

severity of macroscopic lesions. Gross typhlocolitic lesions were scored using a 9-point additive scale: A score of zero being a healthy animal and a score of 9 being a maximally diseased animal. Score parameters evaluated included: (1) cecal atrophy; (2) enlarged cecal tonsil or other enlarged lymphoid aggregates; (3) emptying of cecal contents; (4) abnormally watery or mucoid intraluminal cecal and/or colonic contents; (5) bloody cecal contents; (6) bloody colonic contents; (7) visible thickening and rigidity of the cecum; (8) presence of visible thickening and rigidity of the colon; and (9) absence of formed fecal pellets in the colon. In accordance with approved IACUC protocol, mice that developed severe colitis prior to 20 wk of age were removed from study when they lost ≥ 15% of their maximal body weight. Mice were also removed from the study within 5 d of the onset of persistent clinical signs of disease as characterized by bloody stools, diarrhea, ruffled fur, and hunched gate.

Histopathological assessment

Sections of excised cecum and proximal colon were placed in 10% buffered formalin overnight, paraffin embedded, sectioned, and routinely stained with hematoxylin and eosin. Stained colonic and cecal sections were scored by a board-certified veterinary pathologist, Dr. Jesse Hostetter of Iowa State University (Ames, IA), blinded to the treatments as previously described[24,25]. Microscopic mucosal lesion scores were assessed by five parameters, with each parameter scored on a scale of 0-5 (5 = maximum severity). Score parameters include: (1) ulceration of the mucosa; extent of inflammatory cell infiltrate; (2) mucosal edema characterized by the extent of lymphatic and vascular distortion and expansion of the mucosa/submucosa by clear space; (3) stromal collapse and necrosis of the glands; and (4) glandular hyperplasia characterized by the crowding and immaturity of enterocytes along the gland and gland dilation. In addition to score, mucosal height was determined and recorded as a ratio of gland height to gland width, and the specific inflammatory cell populations, if present, were recorded. Score parameters were considered individually and as an additive histopathological score with mucosal height included in the additive score.

Myeloperoxidase assay

MPO activity was assessed as a measure of neutrophil/granulocyte accumulation in proximal colonic tissues. The MPO assay was performed as previously described with several modifications[22]. Proximal colon sections collected at necropsy were gently flushed with PBS to remove luminal contents and stored in 1 mL of freshly prepared PBS supplemented with the protease inhibitor phenylmethanesulfonyl fluoride (PMSF) at 0.1 mmol/L and 15% dimethylsulphoxide (DMSO) at -20 ℃ for no more than 7 d prior to assay. Samples used as positive controls for MPO activity were prepared fresh the day the assay from peripheral blood. One FVB^{WT} mouse, not on study, was euthanized by CO_2 asphyxiation, and 500 μL to 1 mL of blood was immediately collected by cardiac puncture with a heparinized needle (heparin at 5000 USP heparin units/0.5 mL is drawn into the needle and syringe and then expelled to coat the inside of the needle with heparin). The heparinized blood was centrifuged at 250 x g for 10 min, the supernatant discarded and the red blood cells (RBC) lysed. In brief, 1 mL of ACK lysis buffer (8042 mg/L ammonium chloride, 1001 mg/L potassium bicarbonate, 3.722 mg/L ethylene diamine tetraacetic acid disodium, pH 7.2) was added to the pellet, vortexed gently for 1 min, 1 mL of PBS was added, and the mixture was centrifuged for 10 min at 250 x g. The lysis was repeated until the pellet was white and the supernatant was clear. Following RBC lysis, the pellet was resuspended in 1 mL of PBS/PMSF (0.1 mmol/L), cell numbers were enumerated using a cell counter (average yield of 3 x 10^6 cells/mL) and the cells were sonicated at an amplitude of 5, pulse on for 4 s, pulse off for 1 s, for 20 s total. The sonicated tissue samples were then centrifuged at 250 x g for 15 min and the supernatant stored at 4 ℃ until the tissue samples were prepared. Frozen proximal colonic sections were thawed, blotted to remove as much excess fluid as possible, trimmed to roughly 35 mg and their weights recorded. Tissues were then homogenized for 1 min at maximum power in 1 mL PBS/PMSF (0.1 mmol/L) and the homogenizer probe was washed 5 times with PBS between tissue samples. Homogenate cell counts were recorded, and each sample was then sonicated as described above. The tissue sonicates were then centrifuged at 250 x g for 15 min, the supernatant collected and the pellet discarded. Each lysate prepared from tissue or peripheral blood monocytes (PBMC) was analyzed for total protein using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Individual lysates were pipetted into 96-well, flat bottom microtiter plates. The PBMC lysates (150 μL/well) were serially diluted (10, two-fold dilutions) and analyzed in triplicate wells. For each tissue lysate, 150 μL was pipetted into separate wells and analyzed in triplicate. To each well, 50 μL of 0.78 mg/mL 3,3,5,5-tetramethylbenzidine dihydrochloride hydrate was added, followed immediately by the addition of 50 μL hydrogen peroxide (H_2O_2) (5 mmol/L). The reaction was allowed to proceed for 2 min (wells turned bright blue), followed by the addition of 50 μL of sulfuric acid (1 mol/L) to stop the reaction. The optical density (OD) was measured at 405 nm spectrophotometerically (V-Max, Molecular Devices, United States) using SOFTmax PRO 4.0 software. The MPO content was determined by comparison to the standard curve and MPO activity was expressed as the relative units of enzyme activity per gram of wet weight of tissue.

Serum cytokine/chemokine quantification

Following euthanasia of mdr1a$^{-/-}$ and FVB^{WT} mice, blood was collected *via* cardiac puncture. The blood was allowed to clot for 24 h at 4 ℃ after which samples were

centrifuged at 10000 x g for 10 min. Serum was then removed and stored at -20 ℃ until use. The day of assay, serum samples were thawed to room temperature. Concentrations of cytokines and chemokines of interest were measured using the Millipore (Billerica, MA) mouse cytokine-chemokine multiplexed assay kit. Analytes screened include: Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC, LIF, LIX, M-CSF, MCP-1, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, and VEGF. The assay was performed according to the manufacturer's instructions. In brief, supplied analyte standards (range: 10000 to 3.2 pg/mL), quality control standard, and buffer only control samples were analyzed in duplicate wells of the supplied 96 well plate. Mouse serum samples were diluted 1:1 in supplied assay buffer plated for each mouse. Supplied serum matrix and supplied assay buffer were added to all wells. Supplied pre-conjugated multiplex analyte beads were added to each well and the samples were incubated at 4 ℃ overnight on a plate shaker (Barnstead International Titer Plate Shaker, setting 5, Model No. 4625). Supplied detection antibody was added to all wells and allowed to incubate at room temperature while shaking for 2 h. Supplied streptavidin-phycoerythrin was incubated for 30 min at room temperature while shaking. The mean fluorescence intensity (MFI) was measured using Luminex platform technology (The FlowMetric System, Luminex, Austin, TX). MFIs were subsequently converted to concentrations using a 5-parameter logistic or line curve-fitting method in MasterPlex QT Software (MiraiBio Group, San Francisco, CA).

Flow cytometric analysis of cecal tonsil cell populations

Cecal tonsils from mdr1a$^{-/-}$ and FVB^{WT} mice were excised, placed in complete cell culture medium (10 mL heat-inactivated FBS, 1 mL penicillin/streptomycin, 1 mL glutamine, 0.1 mL 50 mmol/L β-mercaptoethanol, 2.5 mL 1M Hepes buffer in 85.4 mL DMEM containing 4.5 g/L glucose and sodium pyruvate), and homogenized mechanically on ice. Stainless steel wire strainers (60 mesh) were used to prepare single cell suspensions and remove particulate matter. Cells (5 × 10^5 cells/tube) were washed in FACS buffer, centrifuged at 250 x g and incubated in FACS buffer containing 1:100 rat IgG and fluorochrome labeled reagents for 15 min on ice. Following labeling, cells were washed with FACS buffer, centrifuged and fixed in 200 μL of BD stabilizing fixative. Cellular preparations from individual mice were labeled with the following fluorochrome-labeled reagents: Germinal center B cells (PNA$^+$B220$^+$)[26] identified using FITC-conjugated PNA and Alexa 700-conjugated anti-B220 mAb, CD4$^+$ T cells were identified using PE-Cy7-conjugated anti-CD4 mAb and CD8$^+$ T cells were identified using APC-conjugated anti-CD8β mAb. The following isotype controls were utilized: Alexa 700-conjugated anti-rat IgG2aκ, PE-Cy7-conjugated anti-rat IgG2a$^+$, APC-conjugated anti-rat IgG2b$^+$ and PE-conjugated anti-rat IgG2a$^+$ (eBioscience, San Diego, CA). PNA has no isotype control. Analysis was performed using a BD FACSCanto flow cytometer (BD, San Jose, CA) made available through the Flow Cytometry Core Facility at Iowa State University (Ames, IA). Data analysis was performed using FlowJo software (TreeStar Inc., Ashland, OR).

Western blot analysis

Sera from mdr1a$^{-/-}$ and FVB^{WT} mice were used to evaluate the presence of serum antibody against select members of the intestinal microbiota. Whole cell sonicates (WCS) of three members of the clostridial cluster group XIVa (ASF356, ASF500, and ASF502) were cultivated anaerobically, cells were harvested by centrifugation, washed in PBS, lyophilized, and stored at -20 ℃ until use[27,28]. Cells were then weighed and suspended in PBS to 2 mg/mL. The resulting suspension was sonicated on ice for 3 min at the following settings: Amplitude of 50 for 2, 30 s pulses with 5 s between each pulse; amplitude 75 for 2, 30 s pulses with 5 s between each pulse; amplitude 100 for 2, 30 s pulses with 5 s between each pulse. The sonicate was sterilized by UV light (six-minute exposure) and sterility was confirmed bacteriologically. For each preparation, protein content was determined by bicinchoninic acid (BCA) analysis (Pierce Laboratories, New Haven, Connecticut, United States), aliquoted and stored at -20 ℃. Whole cell sonicates of ASF356, ASF500, and ASF502 (8 μg of total protein content) were subjected to SDS-PAGE using 12% tris-glycine gels (BioRad, Hercules, CA) and transferred to PVDF membranes. Each individual antigen was analyzed using pooled anti-sera (1:250) from separate treatment groups as described above The membranes were then reacted with alkaline phosphatase (AP) conjugated anti-mouse IgG (H+L) (1:1000, Southern Biotech, Birmingham, AL) in a solution containing tris buffered saline (pH 7.6), 1% Tween 20 (TBST) and 2.5% non-fat, skim milk. Immunoreactive proteins were visualized using Sigma fast red tablets (Sigma, St. Louis, MO) according to manufacturers' instructions.

Pathway finder R2 profiler PCR array analysis

To evaluate the activation of signal transduction pathways modulated by treatment with the *P. vulgaris* extract, cecal gene expression was analyzed using the RT^2 profiler signal transduction pathway finder PCR array from QIAGEN (Germantown, MD) as per the manufacturer's instructions. In brief, total RNA was isolated from cecal tissue collected that had been stored at -20 ℃ in RNAlater using the TRIzol method[29]. RNA was further purified using the RT^2 qPCR-grade RNA isolation kit from QIAGEN (Germantown, MD) according to manufacturer's instructions. RNA quality (8.2 to 9.4) was assessed using an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA). Prior to preparation of cDNA, RNA samples were tested by PCR using oligonucleotide primers for GAPDH to

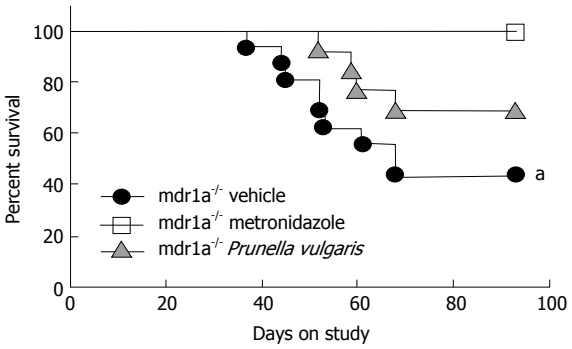

Figure 1 Effect of treatment with a *Prunella vulgaris* ethanolic extract on the onset of colitis in mdr1a$^{-/-}$ mice. Mdr1a$^{-/-}$ mice were removed from the study as they developed severe clinical disease (*e.g.*, > 15% weight loss) before the termination of the experiment as described in Materials and Methods. $^{a}P < 0.05$, as compared to FVB^{WT} control mice. Vehicle-treated mdr1a$^{-/-}$ mice $n = 16$, metronidazole-treated mdr1a$^{-/-}$ mice $n = 10$, *Prunella vulgaris*-treated mdr1a$^{-/-}$ mice $n = 13$. This survival (*i.e.*, mice remaining on study) curve is representative of two independent experiments.

confirm the absence of genomic DNA contamination. Invitrogen SYBR Green/ROX, primers and 1 μg of isolated RNA from each mouse were subjected to the following PCR conditions and were run on an ABI 5700 (Applied Biosystems Inc., Carlsbad, CA): 95 ℃ for 10 min, followed by 40 cycles of amplification (95 ℃ for 10 s, 60 ℃ for 15 s). All cycle threshold (CT) values were greater than 30, and were acceptable for further use (data not shown). GAPDH oligonucleotide primers used were: 5'-TGTGTCCGTCGTGGATCTGA-3' and 5'-CCTGCTTCACCACCTTCTTGA-3'. RNA (1 μg) from each mouse was then converted to cDNA using QIAGEN RT^2 First Strand kit according to manufacturers' instructions. Resulting cDNA from individual mice was pooled into diseased and healthy groups of mice for each treatment group and each experiment, mixed with the kit's array master mix experimental cocktail preparation, and subjected to the same PCR conditions and equipment noted above. PCR array data was analyzed using QIAGEN RT^2 PCR array analysis software and fold changes were calculated relative to house-keeping genes by the software. Only 2-fold changes or greater were considered.

Statistical analysis

Following review by a biostatistician for appropriateness of the statistical methods used, all data, except survival curves, were evaluated by the Kruskal-Wallis test with Dunn's multiple comparisons test. Because the Kruskal-Wallis test has no analog of the ANOVA linear contrast that focuses attention on a specific pre-specified comparison of groups, differences in the mdr1a$^{-/-}$ groups were further evaluated by the Mann-Whitney test for ordinal data and unpaired *t*-test with Welch's correction for continuous data. Survival curves were evaluated by the Log-rank (Mantel-Cox) test. A *P*-value of < 0.05 was considered statistically significant. Prism 6 software was used for all statistical calculations.

RESULTS

The ethanolic extract of P. vulgaris decreases severity of macroscopic disease parameters and delays onset of severe colitis in mdr1a$^{-/-}$ mice

To determine the efficacy of *P. vulgaris* extract in the treatment of spontaneous colitis, mdr1a$^{-/-}$ and FVB^{WT} mice were gavaged daily with vehicle (5% ethanol) or 2.4 mg *P. vulgaris* extract. Previously published data shows that mdr1a$^{-/-}$ mice develop disease between 8 and 36 wk of age, with the average age of disease onset occurring at 20 wk[30]. As expected, FVB^{WT} mice treated with *P. vulgaris* were not adversely affected by the administration of the extract despite the long course (14 wk) of treatment (data not shown). As anticipated, many vehicle-treated mdr1a$^{-/-}$ mice developed severe colitis and weight loss and were removed from study prior to 20 wk of age. Out of 16 mdr1a$^{-/-}$ mice treated with vehicle, 7 required removal from study prior to 20 wk of age, compared to only 4 out of 13 *P. vulgaris*-treated mdr1a$^{-/-}$ mice. Treatment with the *P. vulgaris* extract was able to delay onset of severe colitis and reduce the number of mdr1a$^{-/-}$ mice that had to be removed from study, the difference was not significant when compared to vehicle-treated mdr1a$^{-/-}$ mice (Figure 1). In addition, the phlogistic nature of the resident microbiota contributes to the mucosal inflammation in mdr1a$^{-/-}$ mice as evidenced by the ability of metronidazole treatment to prevent the onset of clinical disease (Figure 1).

Representative photographs (Figure 2) show the extent of macroscopic and microscopic tissue damage in vehicle-treated mdr1a$^{-/-}$ mice. In these mice, ceca were atrophied with visibly enlarged cecal tonsils suggesting immune activation. The ceca of the vehicle-treated mdr1a$^{-/-}$ mice were almost devoid of contents, and both cecal and colonic tissues are notably thickened and rigid. Occasional blood was noted in cecal and colonic contents while no formed fecal pellets were noted in the vehicle-treated mdr1a$^{-/-}$ mice. Conversely, the ceca and colons of *P. vulgaris*-treated mdr1a$^{-/-}$ mice were markedly improved and more closely resembled the tissue appearance of healthy FVB^{WT} as well as metronidazole-treated mdr1a$^{-/-}$ mice with regard to all parameters assessed.

Macroscopically, mild to severe typhlocolitis (a score of 2 to 9, respectively) was observed in 100% of vehicle-treated mdr1a$^{-/-}$ mice while all of the *P. vulgaris*-treated mice presented with macroscopic scores below the average score of the vehicle-treated mdr1a$^{-/-}$ mice (Figure 2C). *P. vulgaris* prophylaxis significantly ($P < 0.05$) improved macroscopic parameters of disease when compared to vehicle treatment in mdr1a$^{-/-}$ mice. In addition, the median colon length for *P. vulgaris*-treated mdr1a$^{-/-}$ mice was longer than that for the vehicle-treated in mdr1a$^{-/-}$ mice indicating less severe epithelial injury (Figure 2). Regardless of the treatment, FVB^{WT} mice did not exhibit any signs of clinical disease or tissue damage (Figure 2). These results indicate that treatment with the *P. vulgaris* ethanolic extract

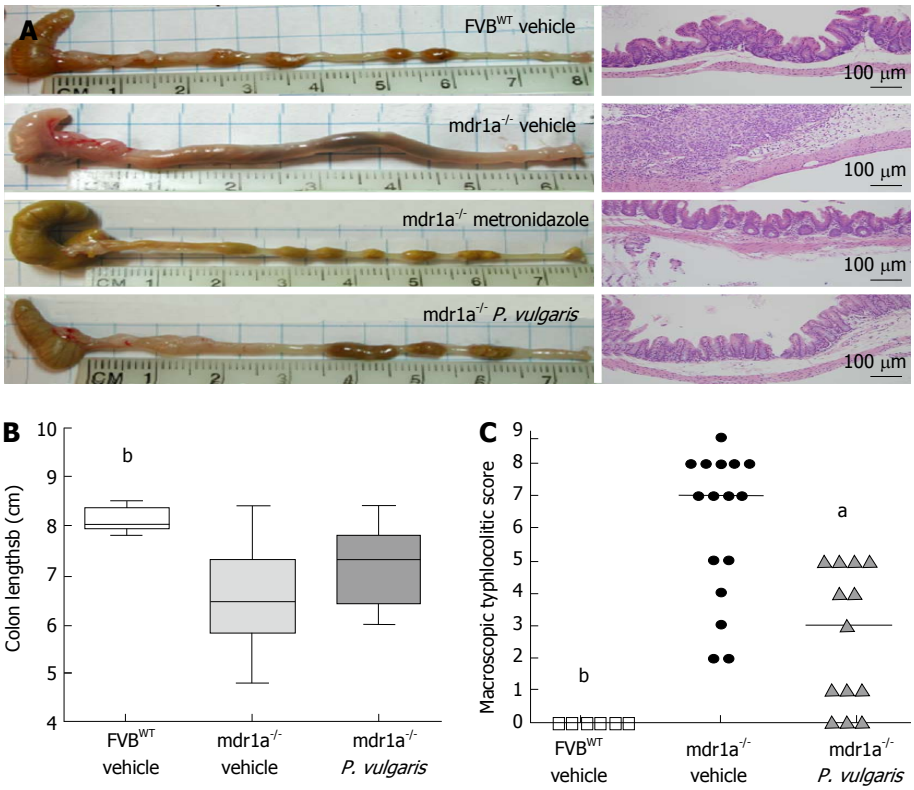

Figure 2 Oral administration of a *Prunella vulgaris* extract attenuated both microscopic and macroscopic cecal lesions in mdr1a$^{-/-}$ mice. A: Representative photographs of ceca and colons (left) and representative photomicrographs (200 ×) of histological sections of ceca (right) collected at necropsy from FVB^{WT} or mdr1a$^{-/-}$ mice treated with either vehicle or *Prunella vulgaris* (*P. vulgaris*) extract; B: Colon lengths were measured at necropsy and the group range is represented. Whiskers indicate minimum and maximum values, while the horizontal line represents the group median; C: Macroscopic typhlocolitic scores were assigned at necropsy as described in the Materials and Methods (Max/Severe = 9, Min/Healthy = 0). $^{a}P < 0.05$, $^{b}P < 0.01$ compared to mdr1a$^{-/-}$ vehicle as calculated by Kruskal-Wallis test. Vehicle-treated FVB^{WT} mice $n = 6$, vehicle-treated mdr1a$^{-/-}$ mice $n = 16$, *P. vulgaris*-treated mdr1a$^{-/-}$ mice $n = 13$.

Table 1 Histopathological scores of cecal tissue

Microscopic parameter	FVB^{WT} vehicle (n = 6)	mdr1a$^{-/-}$ vehicle (n = 10)	mdr1a$^{-/-}$ *P. vulgaris* (n = 7)
Mucosal height (μm)	3.5 ± 0.2^{a}	4.6 ± 0.2	3.9 ± 0.2
Ulceration	0.2 ± 0.2	1.9 ± 0.3	1.2 ± 0.4
Inflammation	1.3 ± 0.2^{b}	3.6 ± 0.2	2.5 ± 0.4^{c}
Edema	0.7 ± 0.5^{a}	2.4 ± 0.3	0.9 ± 0.3^{c}
Stromal collapse (necrosis)	0.0 ± 0.0^{a}	1.7 ± 0.4	0.5 ± 0.4
Gland hyperplasia	1.2 ± 0.2^{b}	2.8 ± 0.2	2.1 ± 0.3^{c}
Additive cecal score	6.9 ± 0.9^{b}	17.0 ± 1.2	11.1 ±1.5
Mice exhibiting cecal neutrophil infiltrate	17%^{b}	100%	38%^{c}

Average values are shown here ± standard error of the mean except where noted. $^{a}P < 0.05$, $^{b}P < 0.01$ compared to mdr1a$^{-/-}$ vehicle with Kruskal-Wallis test with Dunn's multiple comparisons test. $^{c}P < 0.05$ compared to mdr1a$^{-/-}$ vehicle with Mann-Whitney test.

attenuated macroscopic disease and delayed the onset of spontaneous colitis in mdr1a$^{-/-}$ mice.

Impact of P. vulgaris treatment on the severity of histopathological lesions

Histological inflammation of the cecum (Table 1) and colon (data not shown) was evaluated in the context of mucosal height, ulceration, extent and character of inflammatory cell infiltrate, edema, stromal collapse and glandular necrosis, and glandular hyperplasia. The ceca of vehicle-treated mdr1a$^{-/-}$ mice were characterized by crypt hyperplasia, extensive transmural ulceration and inflammatory cell infiltration, as well as submucosal edema and occasional stromal collapse (Figure 2 and Table 1). *P. vulgaris*-treated mdr1a$^{-/-}$ mice exhibited statistically significant ($P < 0.05$) improvement in inflammation, edema, gland hyperplasia, and neutrophil infiltration (Table 1). As expected, FVB^{WT} mice presented with no evidence of mucosal inflammation. While 100% of vehicle-treated mdr1a$^{-/-}$ mice exhibited extensive neutrophilic infiltration into the cecal lamina propria, neutrophils were only noted in cecal mucosa of 38% of *P. vulgaris*-treated mdr1a$^{-/-}$ mice (Figure 2 and Table 1). As a measure of the infiltration of granulocytes

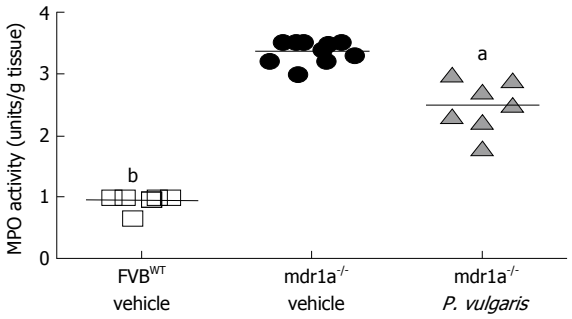

Figure 3 Administration of a *Prunella vulgaris* ethanolic extract reduced local myeloperoxidase activity in the colon of mdr1a$^{-/-}$ mice. Homogenates of colonic tissue were subjected to an assay for MPO activity. $^{a}P < 0.05$, $^{b}P < 0.01$ compared to mdr1a$^{-/-}$ vehicle as calculated by Kruskal-Wallis test. Vehicle-treated FVB^{WT} mice $n = 6$, vehicle-treated mdr1a$^{-/-}$ mice $n = 10$, *P. vulgaris*-treated mdr1a$^{-/-}$ mice $n = 7$. MPO: Myeloperoxidase; *P. vulgaris: Prunella vulgaris.*

into the mucosal tissue, MPO activity was assessed in tissue homogenates. In comparison to tissue samples from vehicle-treated mdr1a$^{-/-}$ mice, the associated MPO activity was significantly diminished in *P. vulgaris*-treated mdr1a$^{-/-}$ mice ($P < 0.05$) (Figure 3). In contrast to colon length, there was no histological evidence that the *P. vulgaris* treatment attenuated microscopic lesions when compared to vehicle-treated mdr1a$^{-/-}$ mice (data not shown), suggesting that the bioactive benefit of *P. vulgaris* localized in the cecum. Together, these data indicated that the benefits provided by the oral administration of *P. vulgaris* were to attenuate the severity of inflammation and injury in the cecal mucosa in association with a reduction of the presence or recruitment of inflammatory granulocytes.

Impact of the ethanolic extract of P. vulgaris on the induction of innate chemotactic and pro-inflammatory cytokines

To further investigate the mechanism(s) related to improved mucosal homeostasis and the associated reduction in neutrophils and MPO activity in the colons of *P. vulgaris*-treated mdr1a$^{-/-}$ mice, serum samples collected at necropsy were examined for the presence of chemokines and cytokines. Of those present in the kit, multiple cytokines/chemokines (Eotaxin, IL-13, IL-15, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, LIF, LIX, M-CSF, MCP-1, MIP-1α, MIP-2, and RANTES) were not detectable in the serum of any treatment group (data not shown). However, several analytes were significantly elevated in mdr1a$^{-/-}$ mice compared to FVB^{WT} mice including G-CSF, IL-10, CXCL10, KC, CXCL9, and TNF-α ($P < 0.01$), and IL-9 ($P < 0.05$) (Table 2). When comparing *P. vulgaris* extract-treated to vehicle-treated mdr1a$^{-/-}$ mice, the levels of IL-10 ($P < 0.01$) and CXCL9 ($P < 0.05$) and TNF-α ($P < 0.05$) were significantly lower in the *P. vulgaris*-treated mdr1a$^{-/-}$ mice (Table 2). For the remainder of the cytokines/chemokines listed in Table 2, there was a trend for lower amounts in the serum of *P. vulgaris*-treated mdr1a$^{-/-}$ mice when compared to vehicle-treated mdr1a$^{-/-}$ mice. This data indicates that oral administration of the ethanolic extract of *P. vulgaris* is able to attenuate production of several innate chemokines and cytokines induced by the inflammatory response in mdr1a$^{-/-}$ mice.

Differential regulation of gene expression pathways by in vivo treatment with the ethanolic extract of P. vulgaris

To further characterize the attenuation of mucosal inflammation provided by *P. vulgaris* treatment, a microarray analysis for inflammatory gene expression was performed in order to identify differential gene regulation between disease phenotypes of botanical extract-treated mdr1a$^{-/-}$ mice (*e.g.*, healthy = macroscopic score < 2 ; colitic = macroscopic score ≥ 2) and between FVB^{WT} mice and mdr1a$^{-/-}$ mice treated with vehicle. At the extremes of microscopic and macroscopic lesion scores, it was observed that no vehicle treated mdr1a$^{-/-}$ mice were characterized as "healthy" and no FVB^{WT} mice were characterized as "colitic" (data not shown). Genes encoding *CCL2, CXCL1, CXCL9, IL-1α, MMP10, TNF-α, VCAM-1, CCL20,* and *IL-2* were all downregulated more than 2-fold by *P. vulgaris* treatment in mdr1a$^{-/-}$ mice that did not develop colitis (Table 3). *P. vulgaris* treatment appears to modulate the NF-κB pathway in the preservation of mucosal homeostasis in mdr1a$^{-/-}$ mice.

Influence of P. vulgaris on local T cell and B cell populations

Because T and B cells are activated as a consequence of inflammation, T cell and B cell populations in the cecal tonsils of mdr1a$^{-/-}$ and FVB^{WT} mice were analyzed to evaluate the effects of *P. vulgaris* treatment on local lymphocyte populations (Figure 4). Severe colitis in vehicle-treated mdr1a$^{-/-}$ mice resulted in 3-fold more CD4^{+} T cells (Figure 4A) and 6-fold more CD8^{+} T cells (Figure 4B) in the cecal tonsil as compared to vehicle gavaged FVB^{WT} mice. In *P. vulgaris*-treated mdr1a$^{-/-}$ mice, the numbers of CD4^{+} T cells in the cecal tonsils were significantly lower ($P < 0.05$) when compared to vehicle-treated mdr1a$^{-/-}$ mice (Figure 4A), and there was a trend indicating fewer CD8^{+} T cells in the *P. vulgaris*-treated mdr1a$^{-/-}$ mice (Figure 4B).

Vehicle-treated mdr1a$^{-/-}$ mice exhibited a 2.5-fold increase in the number of PNA^{+}B220^{+} germinal center B cells (Figure 4C) in the cecal tonsil as compared to vehicle gavaged FVB^{WT} mice. Remarkably, *P. vulgaris* treatment significantly ($P < 0.05$) decreased PNA^{+}B220^{+} germinal center B cells in mdr1a$^{-/-}$ mice (Figure 4C). Together, these data indicate that the expansion CD4^{+} T cell and PNA^{+}B220^{+} germinal center B cell populations were significantly lower in the cecal tonsils of *P. vulgaris*-treated mdr1a$^{-/-}$ mice.

P. vulgaris prevents antigenic responses to some members of the intestinal microbiota

Antibody responses to antigens derived from the gut

Table 2 Assessment of selected cytokines in the serum of mice

Cytokine/chemokine	FVB^{WT} vehicle (n = 6)	mdr1a$^{-/-}$ vehicle (n = 16)	mdr1a$^{-/-}$ *P. vulgaris* (n = 13)
G-CSF	140.1 ± 15[b]	9694.0 ± 2563	4597.0 ± 1931
GM-CSF	ND	21.1 ± 11	1.5 ± 1
IL-9	77.9 ± 27[a]	200.1 ± 38	117.8 ± 14
IL-10	ND[b]	19.4 ± 4	4.6 ± 2[a,d]
IL-17	4 ± 2	16 ± 4	1 ± 1
CXCL10	8.4 ± 5[b]	724.3 ± 136	397.2 ± 85
KC	69.5 ± 24[b]	527.1 ± 119	253.4 ± 58
CXCL9	108.7 ± 37[b]	3901.0 ± 858	1319.0 ± 277[c]
TNFα	9.3 ± 0.1[b]	14.8 ± 1	9.9 ± 3[c]

Serum samples were collected at the time mice were euthanized and analyzed as described in Materials and Methods. Average values (pg/mL serum) are shown here ± standard error of the mean except where noted. [a]P < 0.05, [b]P < 0.01 compared to mdr1a$^{-/-}$ vehicle with Kruskal-Wallis test with Dunn's multiple comparisons test. [c]P < 0.05, [d]P < 0.01 compared to mdr1a-/- vehicle with unpaired t-test with Welch's correction. ND: Not detectable; *P. vulgaris*: *Prunella vulgaris*.

Table 3 Attenuation of inflammatory gene expression in mdr1a$^{-/-}$ mice treated orally with an extract from *Prunella vulgaris*

Gene	Pathway affiliation	Fold change compared to vehicle-treated mdr1a$^{-/-}$ mice[a]		
		FVB^{WT} vehicle healthy	mdr1a$^{-/-}$ *P. vulgaris* healthy	mdr1a$^{-/-}$ *P. vulgaris* colitic
Ccl2	NF-κB, LDL	-7.3	-3.4	-1.1
Cxcl1	NF-κB	-11.3	-7.2	-1.0
Cxcl9	NF-κB, Jak/Stat	-23.9	-4.2	-1.1
Icam1	NF-κB, Phospholipase C	-3.2	-1.2	1.8
Il1a	NF-κB	-13.4	-8.0	1.3
Mmp10	NF-κB, Jak/Stat	-6.6	-8.5	1.2
TNFα	NF-κB	-10.2	-2.6	1.2
Vcam1	NF-κB, Phospholipase C, LDL	-3.8	-2.0	1.2
Ccl20	NF-κB	-3.6	-2.0	1.4
Il2	NF-κB, NFAT, Calcium, PKC	-3.7	-2.2	-1.5

[a]A negative value indicates that there was a lower level of gene expression when compared to the level of gene expression in vehicle-treated mdr1a$^{-/-}$ mice that developed severe colitis. *P. vulgaris*: *Prunella vulgaris*.

microbiota (*e.g.*, clostridial cluster group XIVa) have been noted in IBD patients and in murine models of IBD[31-33]. These antibody responses are indicative of a loss of epithelial integrity and immune tolerance to the microbiota and do not occur in healthy humans or mice. Pooled serum samples from mdr1a$^{-/-}$ mice treated with *P. vulgaris* were evaluated by immunoblot analysis against antigens (*i.e.*, whole cell sonicate) derived from select members of the clostridial cluster group XIVa within the microbiota (Figure 5). As anticipated, sera from FVB^{WT} mice did not display antibody reactivity against these bacterial antigens, suggesting that these mice maintained immunological tolerance to their gut microbiota. Conversely, sera from vehicle-treated mdr1a$^{-/-}$ mice did contain antibodies reactive to these bacterial antigens, indicating a loss of immunologic tolerance to these members of the microbiota. Sera from *P. vulgaris*-treated mdr1a$^{-/-}$ mice displayed little to no detectible antibody response to the three bacterial antigens (Figure 5).

DISCUSSION

As the long term safety and efficacy of current parenteral therapeutics for IBD are a concern and antibiotics are deemed unreliable for long-term use in IBD patients, there is a need for new therapies that may include complementary treatments[8,9,34]. Complementary and alternative therapy including nutraceuticals hold realistic potential in treating or supplementing treatment of inflammatory disorders, as the anti-inflammatory and antioxidant benefits of plant-derived components are becoming more extensively characterized[35-39]. *P. vulgaris*, already popular in Asian medicine, is a viable candidate for study as a therapeutic agent in the treatment of IBD as it contains several anti-inflammatory, immunomodulatory, and antioxidant flavonoids, polyphenols, and triterpenoids and has no documented toxic side-effects[37,40-44]. In this regard, rosmarinic acid, the most plentiful phenolic compound found in *P. vulgaris*, was found to protect mice against the deleterious effects associated with sepsis by downregulating inflammatory genes in the NF-κB pathway including the related pro-inflammatory cytokines TNF-α and IL-6[45].

The mdr1a$^{-/-}$ mouse model is ideal to use for studies of potential IBD therapeutics that are relevant to human medicine as mdr1a$^{-/-}$ mice are immunocompetent, develop spontaneous colitis in the context of a leaky intestinal epithelium, and exhibit cytokine profiles and

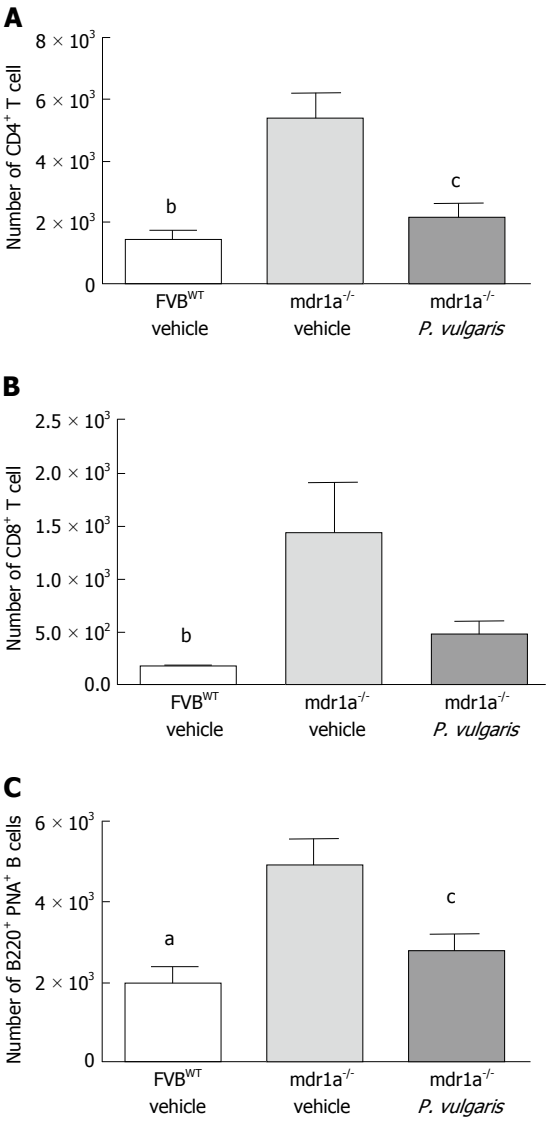

Figure 4 Evaluation of T cell and B cell subsets in the cecal tonsil of mdr1a$^{-/-}$ mice treated with *prunella vulgaris* ethanolic extract. Cecal tonsils were excised at necropsy, single cell suspensions prepared and labeled for flow cytometric analysis as described in Materials and Methods. Absolute numbers of (A) CD4+ T cells; (B) CD8ß+ T cells; and (C) B220+PNA+ germinal center B cells in the cecal tonsils of mice. $^{a}P < 0.05$, $^{b}P < 0.01$ compared to mdr1a$^{-/-}$ vehicle as calculated by Kruskal-Wallis test. $^{c}P < 0.05$ compared to mdr1a$^{-/-}$ vehicle as calculated by unpaired *t*-test. The *n* for each group is equal to that noted in Figure 1 and data are representative of two independent experiments. Vehicle-treated FVB^{WT} mice *n* = 5-6, vehicle-treated mdr1a$^{-/-}$ mice *n* = 8-10, *P. vulgaris*-treated mdr1a$^{-/-}$ mice *n* = 5-7.

immune responses similar to those documented in clinical IBD cases[30,46-51]. It was previously demonstrated that administration of curcumin attenuated mucosal inflammation in mdr1a$^{-/-}$ mice[52]. In these studies, similar to previously published studies, onset of clinical disease (*e.g.*, weight loss) in vehicle-treated mdr1a$^{-/-}$ mice was observed at roughly 10 wk of age (Figure 1)[30]. In contrast, onset of clinical disease was delayed by treatment with the *P. vulgaris* extract; in addition, markedly fewer of the mdr1a$^{-/-}$ mice treated developed severe clinical disease by 20 wk of age (Figure 1). *P. vulgaris* treatment of mdr1a$^{-/-}$ mice was also found

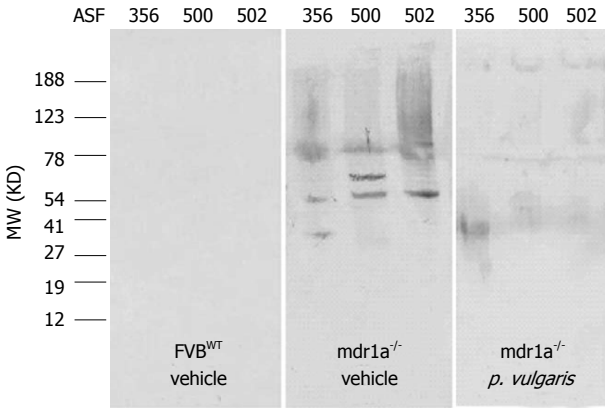

Figure 5 Vehicle-treated mdr1a$^{-/-}$ mice with severe colitic inflammation developed serum antibody to antigens derived from gut microbiota while those treated with *prunella vulgaris* extract do not. Whole cell sonicates of three separate clostridial species present as part of the intestinal microbiota (altered Schaedler flora members 356, 500 and 502) were subjected to SDS-PAGE. Western blot analysis was performed using sera collected at necropsy. Antigens in lanes represented in each panel are as follows from left to right: ASF502, ASF500 and ASF356.

to attenuate macroscopic lesions associated with the characteristic severe typhlocolitis observed in this murine model (Figure 2). In contrast to the vehicle-treated mdr1a$^{-/-}$ mice, ceca of mdr1a$^{-/-}$ mice treated with a *P. vulgaris* extract retained normal mucosal architecture, lacked enlarged lymphoid aggregates, and retained luminal contents devoid of blood or mucus. Macroscopically, the colons of botanical-treated mdr1a$^{-/-}$ mice were more similar in appearance to the colons of the FVB^{WT} control mice with regard to presence of formed feces, and lack of grossly visible tissue edema and rigidity (Figure 2). Mdr1a$^{-/-}$ mice treated with *P. vulgaris* extract also presented with normal colon lengths (Figure 2); microscopically, colonic lesions were less attenuated in the botanical extract-treated mdr1a$^{-/-}$ mice than those present in the cecum (data not shown). The microscopic lesions observed in the ceca of mdr1a$^{-/-}$ mice treated with *P. vulgaris* were markedly less severe when compared to those observed for vehicle-treated mdr1a$^{-/-}$ mice (Table 1). Together, these findings highlight the differences between colonic and cecal compartments in terms of the magnitude of the disease. Perhaps these differences arise from the more dense concentration of metabolically active microbes in the cecum as compared to the colon, which may lead to more efficient metabolism/degradation of the extract and greater health benefit at more proximal gastrointestinal sites.

Although the etiology of IBD is still ill defined, many recognize that the inductive phase of colitis involves a compromised intestinal epithelium and activation of innate immune responses, including neutrophil activation, transmigration across the mucosal epithelium, and enzymatic damage to host tissues[3,53-56]. Flavonoids from licorice have been shown to inhibit neutrophil infiltration into lung tissue after lipopolysaccharide-induced inflammation and reduce the severity of associated inflammatory damage to host pulmonary

tissues[57]. As a group, the mdr1a$^{-/-}$ mice treated with the *P. vulgaris* ethanolic extract presented with markedly less neutrophilic infiltration into the cecal mucosa when compared to vehicle-treated mdr1a$^{-/-}$ mice (Table 1). Reduction of the neutrophilic infiltrate in these mice was correlated with less severe microscopic scores and a significant decrease in tissue levels of MPO activity (Figures 2 and 3, Table 1). MPO enzymatic activity is a known correlate to intestinal damage and is often used as a marker of IBD severity in many animal models of colitis[58-62].

Since neutrophils are not resident in tissues, cytokine and chemokine signals produced by epithelial cells and local macrophages are responsible for the recruitment of neutrophils into the mucosal tissues[63,64]. Homeostatic production of these innate chemokines is central to mucosal health, while over-production contributes to the development of severe inflammation in colitis[55,56,65]. Debate regarding the role of NF-κB activation and the exacerbated recruitment innate immune cells in acute intestinal inflammatory models continues, with some research pointing to a protective effect by these components and others revealing a deleterious effect[23,66-68]. However, many agree that dysregulation of NF-κB signaling and the consequent innate cellular responses are causative factors in the inductive phase and maintenance of chronic inflammation associated with human CD and UC[69-71]. Pro-inflammatory mediators induced by activation of NF-κB are abnormally upregulated in CD and UC patients[72]. Moreover, it concludes nuclear translocation of NF-κB in epithelial cells and local monocytes upregulates production of pro-inflammatory cytokines and chemokines such as TNF-α, IL-1β, KC, and CXCL9. These cytokines increase expression of adhesion molecules (*i.e.*, VCAMs, ICAMs, and MadCAM) on endothelial cells, while chemokines create chemical gradients to attract neutrophils and other innate inflammatory cells to sites of injury[55,64,65,73]. Others have reported that the ability to regulate or attenuate cytokine production (*e.g.*, TNF-α, IL-1α, IL-1β, IFN-γ) decreases the expression of chemokines (*e.g.*, IL-8/KC and VEGF) and adhesion molecules on endothelial cells resulting in amelioration of inflammatory tissue damage in several disease models, including colitis[1,45,66,74]. The patterns of cytokine and chemokine production observed in aged-matched, vehicle-treated mdr1a$^{-/-}$ mice in the current study (Table 2) was consistent with that previously reported[51]. Serum samples from mdr1a$^{-/-}$ mice treated with the ethanolic extract of *P. vulgaris* had lower levels of cytokines that would contribute to the production of granulocytes and monocytes (G-CSF and GM-CSF) as well as the neutrophil chemotactic factor KC (Table 2). Importantly, treatment with the *P. vulgaris* extract reduced serum levels of TNF-α (Table 2), a cytokine known to be a key regulator of inflammatory responses in colitis[75]. These data indicate that *P. vulgaris* extract reduced the production of cytokines and chemokines central to the induction and maintenance of chronic inflammation.

Activation of the transcription factor NF-κB and the regulation of its target genes have well documented links to the chronicity of inflammation associated with IBD[29,69-71]. Recent studies have shown that flavonoids similar to those identified in *P. vulgaris* are capable of downregulating NF-κB and ultimately regulating the production of innate chemotactic factors and pro-inflammatory cytokines[76,77]. One such study showed that the flavonoid luteolin decreased NF-κB expression in the ceca and colons of IL-10$^{-/-}$ mice, and effectively ameliorated spontaneous colitis[23]. Similarly, the ethanolic extract of *P. vulgaris*, which is known to contain several flavonoids (data not shown)[15], downregulated expression of chemokine genes (*Ccl2, Cxcl1/KC, Cxcl9/CXCL9,* and *Ccl20*) and genes involved in the increased expression of adhesion molecules (*VCAM-1, ICAM, TNFα* and *IL-1α)* and tissue remodeling to allow for inflammatory cell transmigration (*MMP-10*) (Table. 3). All of these genes participate in the activation of or are regulated by NF-κB[78-83]. Based on our findings, the ethanolic extract of *P. vulgaris* likely attenuates neutrophil recruitment into the colonic tissues of mdr1a$^{-/-}$ mice by downregulating genes regulated by NF-κB signaling. The importance of regulating inflammation in mdr1a$^{-/-}$ mice prior to the onset of clinical disease is underscored by recent data showing that regulation of inflammatory gene expression is altered in mdr1a$^{-/-}$ mice and in mice treated with dextran sodium sulfate (DSS) prior to any histologic signs of inflammation[84,85]. Since defects in gene expression precede inflammation, prophylactic approaches to control mucosal inflammation rather than providing therapy at or after the onset of an inflammatory flare may prove advantageous. In the current study, therapeutic initiation of *P. vulgaris* treatment (*i.e.*, after colitic onset) was ineffective at reducing the severity of inflammation (data not shown). This observation supports the current hypothesis that the ethanolic extract of *P. vulgaris* modulates innate inflammatory gene expression, and that effective treatment should begin prior to the onset of clinical disease.

In addition to NF-κB signaling and innate immune activation, adaptive immune responses also play an integral role in mediating the chronicity and severity of colitic disease in experimental models and in humans with IBD. In particular, aberrant CD4$^+$ T cell responses to antigens derived from the resident microbiota have been implicated in the pathogenesis of IBD[31,86]. Pretreatment with the ethanolic extract of *P. vulgaris* decreased production of CXCL10 and CXCL9, two proteins that are induced by IFN-γ and are chemotactic for T cells (Table 2)[79,87]. These chemokines and other cytokines participate in inflammatory feedback loops that may be interrupted by treatment with the ethanolic extract of *P. vulgaris*. The observation of reduced numbers of CD4$^+$ and CD8$^+$ T cells in the cecal tonsils of mdr1a$^{-/-}$ mice (Figure 4) is consistent with the lower amounts of CXCL10 and CXCL9 in the serum. There were also lower levels of IL-9 in the serum of mdr1a$^{-/-}$ mice treated with

the ethanolic extract of *P. vulgaris* (Table 2), a cytokine known to enhance $CD4^+$ T cell proliferation and inhibit apoptosis[88]. Moreover, the decreased expression of *CXCL9, Ccl2, IL-1α, TNF-α,* and *Ccl20* genes in extract treated mdr1a$^{-/-}$ mice provides additional evidence that treatment with *P. vulgaris* extract impacted the robustness of the local T cell response (Table 3). CCL20 is strongly chemotactic for immature dendritic cells, which would mature upon collecting antigen in the tissues, present that antigen to T cells, and stimulate an adaptive immune response[89].

With respect to the induction of antibody specific to antigens derived from the resident microbiota, germinal centers will develop in lymphoid tissue upon B cell activation by T dependent antigens[90]. The results of this study demonstrated that the number of PNA^+B220^+ B cells present in *P. vulgaris*-treated mice was significantly less than that detected in the vehicle-treated mdr1a$^{-/-}$ mice (Figure 4). As a consequence of the attenuated germinal center B cell response, there was a lack of antibody production towards bacterial antigens derived from the resident microbiota in *P. vulgaris*-treated mdr1a$^{-/-}$ mice (Figure 5). Collectively, these data present evidence that the ethanolic extract of *P. vulgaris* acts to maintain mucosal homeostasis in mdr1a$^{-/-}$ mice by regulating gene expression associated with innate inflammatory responses and attenuating the activation of the adaptive immune response.

It has been recently reported that 40% to 50% of adults suffering with inflammatory bowel disease or irritable bowel disease utilize complementary and alternative medicine to treat their symptoms[34]. Because of the prevalence at which patients use complementary approaches to attenuate clinical symptoms, it is critical to evaluate the efficacy of nutraceuticals in pre-clinical controlled studies. The work highlighted in this study indicates that an ethanolic extract derived from *P. vulgaris* was safe when administered daily for 14 wk and markedly attenuated the severity of colitis in mice that are genetically prone to develop mucosal inflammation. The health benefits associated with consuming plant-derived nutraceuticals are likely associated with the richness and complexity of anti-inflammatory compounds present in botanical extracts. There is a need to further evaluate the underlying mechanism(s) that contributed to the anti-inflammatory activity of *P. vulgaris* extracts in order to provide a basis for their legitimate use as a prophylactic or supplementary option for the treatment of IBD and other chronic inflammatory disorders.

ACKNOWLEDGMENTS

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COMMENTS

Background

Extracts of *Prunella vulgaris* (*P. vulgaris*) have been shown to contain anti-inflammatory components but there is limited information regarding the ability of these extracts to attenuate or prevent inflammation *in vivo*. Mice that are deficient in the expression of the multiple drug resistance gene (*i.e.*, mdr1a$^{-/-}$) develop spontaneous colitis by 12 to 15 wk of age. As opposed to chemically-induced models of colitis, these mice offer a excellent model to assess the anti-inflammatory capabilities of botanical extracts administered as an oral formulation.

Research frontiers

Treatment of inflammatory bowel disease is dominated by the use of drugs and biologicals that systemically target inflammatory processes. The development of effective treatment modalities that can be delivered orally and target the inflammatory response in the gastrointestinal mucosa would reduce the systemic side-effects observed with other treatment regimen. The studies presented herein demonstrate that botanical extracts can effectively attenuate the severity of colitis on a murine model of spontaneous colitis.

Innovations and breakthroughs

This is the first study to demonstrate the oral administration of an ethanolic extract derived from *P. vulgaris* can be used to delay the onset of and ameliorate the severity of spontaneously occurring colitis in mdr1a-deficient mice.

Applications

It is estimated that 30% to 70% of patients suffering from inflammatory bowel diseases use some form of complementary and alternative therapy to treat their symptoms. Many of the parenteral therapies (*e.g.*, steroids, 5-aminosalicylates, monoclonal antibodies) used to control gastrointestinal inflammation are associated side-effects. The development of extracts derived from medicinal plants, such as *P. vulgaris,* that can be delivered orally and ameliorate gastrointestinal inflammation may be useful adjunct treatments for IBD patients.

Terminology

Mice lacking the multiple drug resistance (*mdr1a*) gene fail to produce an epithelial cell transporter protein (a 107 kDa P-glycoprotein) responsible for pumping various compounds across the cell membrane. The pathological lesions and cytokine profiles observed in the colon of mdr1a-deficient mice resembles that noted in human ulcerative colitis patients.

Peer-review

This is a well written manuscript, investigating the ability of an orally-supplemented *P. vulgaris* extract to attenuate the clinical symptoms of colitis in an animal model of spontaneous colitis (mdr1a$^{-/-}$ mice). The authors used valid methodological approaches to compare the histopathological, biochemical and immunological profile of the supplemented and control animals, the presentation of the results was clear and the discussion was thorough.

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