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

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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 mdrla<sup>−/−</sup> mice delayed onset of colitis and reduced severity of mucosal inflammation when compared to vehicle-treated mdrla<sup>−/−</sup> 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 TNF<sub>α</sub> (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 TNF<sub>α</sub> in the colonic mucosa of mdrla<sup>−/−</sup> mice compared to vehicle-treated mdrla<sup>−/−</sup> mice. Histologically, several microscopic parameters were reduced (P < 0.05) in *P. vulgaris*-treated mdrla<sup>−/−</sup> mice, as was myeloperoxidase activity in the colon (2.49 ± 0.16 vs 3.36 ± 0.06, P < 0.05). The numbers of CD4<sup>+</sup> 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 mdrla<sup>−/−</sup> were significantly reduced (P < 0.05) from vehicle-treated mdrla<sup>−/−</sup> mice. Vehicle-treated mdrla<sup>−/−</sup> mice 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 mdrla<sup>−/−</sup> mice.

CONCLUSION: The anti-inflammatory activity of *P. vulgaris* ethanolic extract effectively attenuated the severity of intestinal inflammation in mdrla<sup>−/−</sup> mice.

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

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**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 mdrla<sup>−/−</sup> 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](#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([5]).

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-TNFα<sup>−/−</sup>, 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\textsuperscript{[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\textsuperscript{[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.

\textit{Prunella vulgaris} (\textit{P. vulgaris}) commonly used in traditional Chinese medicine for wound healing, indigestion, burns and anti-inflammatory therapy. \textit{P. vulgaris} contains several bioactive phenolics, triterpenoids and flavonoids\textsuperscript{[15]}. Dietary phenolics such as rosmarinic acid, ursolic acid, and caffeic acid are all found in extracts of \textit{P. vulgaris}, and have been shown to possess antioxidant, anti-inflammatory and anti-cancer activities\textsuperscript{[16–21]}. Caffeic acid has also been shown to efffectively attenuate chemically induced experimental colitis through upregulation of cytochrome P450 (CYP4B1)\textsuperscript{[22]}. Flavonoids, like those found in \textit{P. vulgaris}, have been implicated as potential therapeutics for IBD as well\textsuperscript{[22]}. 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 \textit{P. vulgaris} extracts as a treatment for IBD. In this context, we have designed this study to test the hypothesis that an ethnic extract of \textit{P. vulgaris} will decrease gastrointestinal mucosal inflammation and thereby ameliorate the severity of spontaneous colitis in mdr1a\textsuperscript{-/-} mice.

**MATERIALS AND METHODS**

\textit{Prunella vulgaris} extract preparation

Information about the specific provenance of \textit{P. vulgaris} accession Ames 27664, obtained from Dr. Mark Wiederchinrner at the USDA-ARS North Central Regional Plant Introduction Station (Ames, IA), is available on the Germplasm Resources Information Network database that can be accessed [here](http://www.ars-grin.gov/npgs/acc/acc_queries.html). Above ground portions of plants from \textit{P. vulgaris} (Ames 27664), harvested in 2008 were prepared for storage by drying for 8 d at 38 °C in a forced-air dryer with constant humidity. The dried material was ground with a 40-mesh screen and stored at -20 °C 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 °C and lyophilized. The residue weight was recorded and the residues stored at -20 °C 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 \textit{P. vulgaris} extract was divided into 2 mL aliquots and stored at -20 °C until use. \textit{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 \textit{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 to minimize pain and/or distress of all animals used in this study. Four to five week old male mdr1a\textsuperscript{-/-} 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\textsuperscript{-/-} mice that were orally gavaged with 2.4 mg/d \textit{P. vulgaris} extract in a 200 μL volume (prepared as described above) and mdr1a\textsuperscript{-/-} and FVB\textsuperscript{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\textsubscript{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

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severity of macroscopic lesions. Gross typhlocolitotic
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. 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
caracterized 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. 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
phenylmethylsulfonyl fluoride (PMSF) at 0.1 mmol/L
and 15% dimethylsulphoxide (DMSO) at -20°C 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 FVBmice, not on
study, was euthanized by CO2 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 PBS/PMSF (0.1 mmol/L), cell
numbers were enumerated using a cell counter (average
yield of 3 x 106 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°C 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
content using a NanoDrop ND-1000 UV-Vis Spectrophotometer
(NanoDrop Technologies Inc., Wilmington, DE). Individual
lysatess were pipetted into 96-well, flat bottom microtiter
plates. The PBMC lyments (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 (H2O2) (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
spectrophotometrically (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 FVBmice, blood
was collected via cardiac puncture. The blood was
allowed to clot for 24 h at 4°C after which samples were
centrifuged at 10000 x g for 10 min. Serum was then removed and stored at -20 °C 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-α, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, 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 °C 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-phycocerythrin 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 mdr1α−/− and FVB wildtype 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 µm mesh) were used to prepare single cell suspensions and remove particulate matter. Cells (5 × 10^6 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 IgG2ak, 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 FACSAnalyze 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 mdr1α−/− and FVB wildtype 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 °C 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 °C. 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 RT2 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 °C in RNalater using the TRIzol method[29]. RNA was further purified using the RT2 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
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 °C for 10 min, followed by 40 cycles of amplification (95 °C for 10 s, 60 °C 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’-TGTGTCCGGTCGTGGATCTGA-3’ and 5’-CCTGCTTCACCACCTTCTTGA-3’. RNA (1 µg) from each mouse was then converted to cDNA using QIAGEN RT2 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 RT2 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
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, FVBWT 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

### Table 1 Histopathological scores of cecal tissue

<table>
<thead>
<tr>
<th>Microscopic parameter</th>
<th>FVBWT vehicle (n = 6)</th>
<th>mdr1a−/− vehicle (n = 10)</th>
<th>mdr1a−/− P. vulgaris (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal height (µm)</td>
<td>3.5 ± 0.2</td>
<td>4.6 ± 0.2</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>Ulceration</td>
<td>0.2 ± 0.2</td>
<td>1.9 ± 0.3</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>Inflammation</td>
<td>1.3 ± 0.2</td>
<td>3.6 ± 0.2</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>Edema</td>
<td>0.7 ± 0.5</td>
<td>2.4 ± 0.3</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Stromal collapse (necrosis)</td>
<td>0.0 ± 0.0</td>
<td>1.7 ± 0.4</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>Gland hyperplasia</td>
<td>1.2 ± 0.2</td>
<td>2.6 ± 0.2</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>Additive cecal score</td>
<td>6.9 ± 0.9</td>
<td>17.0 ± 1.2</td>
<td>11.1 ± 1.5</td>
</tr>
<tr>
<td>Mice exhibiting cecal neutrophil infiltrate</td>
<td>17%b</td>
<td>100%</td>
<td>38%c</td>
</tr>
</tbody>
</table>

Average values are shown here ± standard error of the mean except where noted. *P < 0.05, **P < 0.01 compared to mdr1a−/− vehicle as calculated by Kruskal-Wallis test. Vehicle-treated FVBWT mice n = 6, vehicle-treated mdr1a−/− mice n = 16, P. vulgaris-treated mdr1a−/− mice n = 13.
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 FVBWT 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 FVBWT 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 FVBWT 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 FVBWT 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 FVBWT 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 FVBWT 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
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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 vehicle 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 detectable 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* colitic, 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

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**Table 2** Assesment of selected cytokines in the serum of mice

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>FVB vehicle (n = 6)</th>
<th>mdr1a−/− vehicle (n = 16)</th>
<th>mdr1a−/− <em>P. vulgaris</em> (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>140.1 ± 15</td>
<td>9694.0 ± 2563</td>
<td>4597.0 ± 1931</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>ND</td>
<td>21.1 ± 11</td>
<td>1.5 ± 1</td>
</tr>
<tr>
<td>IL-9</td>
<td>77.9 ± 27</td>
<td>200.1 ± 38</td>
<td>117.8 ± 14</td>
</tr>
<tr>
<td>IL-10</td>
<td>ND</td>
<td>19.4 ± 4</td>
<td>4.6 ± 2.4</td>
</tr>
<tr>
<td>IL-17</td>
<td>4 ± 2</td>
<td>16 ± 4</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>CXCL10</td>
<td>8.4 ± 5</td>
<td>724.3 ± 136</td>
<td>397.2 ± 85</td>
</tr>
<tr>
<td>KC</td>
<td>69.5 ± 24</td>
<td>527.1 ± 119</td>
<td>253.4 ± 58</td>
</tr>
<tr>
<td>CXCL9</td>
<td>108.7 ± 37.6</td>
<td>3901.0 ± 858</td>
<td>1319.0 ± 277</td>
</tr>
<tr>
<td>TNFα</td>
<td>9.3 ± 0.3</td>
<td>14.8 ± 1</td>
<td>9.9 ± 3</td>
</tr>
</tbody>
</table>

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. *P < 0.05, *P < 0.01 compared to mdr1a−/− vehicle with Kruskal-Wallis test with Dunn’s multiple comparisons test. *P < 0.05, *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*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Pathway affiliation</th>
<th>Fold change compared to vehicle-treated mdr1a−/− mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FVB vehicle healthy</td>
<td>mdr1a−/− <em>P. vulgaris</em> healthy</td>
</tr>
<tr>
<td>Ccl2</td>
<td>NF-κB, LDL</td>
<td>-7.3</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>NF-κB</td>
<td>-11.3</td>
</tr>
<tr>
<td>Cxcl9</td>
<td>NF-κB, Jak/Stat</td>
<td>-23.9</td>
</tr>
<tr>
<td>Icam1</td>
<td>NF-κB, Phospholipase C</td>
<td>-3.2</td>
</tr>
<tr>
<td>Il1α</td>
<td>NF-κB</td>
<td>-13.4</td>
</tr>
<tr>
<td>Mmp10</td>
<td>NF-κB, Jak/Stat</td>
<td>-6.6</td>
</tr>
<tr>
<td>Tnfα</td>
<td>NF-κB</td>
<td>-10.2</td>
</tr>
<tr>
<td>Vcam1</td>
<td>NF-κB, Phospholipase C, LDL</td>
<td>-3.8</td>
</tr>
<tr>
<td>Ccl20</td>
<td>NF-κB</td>
<td>-3.6</td>
</tr>
<tr>
<td>Il2</td>
<td>NF-κB, NFAT, Calcium, PKC</td>
<td>-3.7</td>
</tr>
</tbody>
</table>

*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.*
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![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**Figure 4** Evaluation of T cell and B cell subsets in the cecal tonsil of mdr1a<sup>−/−</sup> 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<sup>+</sup> T cells, (B) CD8<sup>+</sup> T cells, and (C) B220<sup>+</sup>PNA<sup>+</sup> germinal center B cells in the cecal tonsils of mice. *P* < 0.05 or *P* < 0.01 compared to mdr1a<sup>−/−</sup> vehicle as calculated by Kruskal-Wallis test.

**Figure 5** Vehicle-treated mdr1a<sup>−/−</sup> 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: ASF356, ASF500 and ASF502.

Immune responses similar to those documented in clinical IBD cases<sup>30,46-51</sup>. It was previously demonstrated that administration of curcumin attenuated mucosal inflammation in mdr1a<sup>−/−</sup> mice<sup>52</sup>. In these studies, similar to previously published studies, onset of clinical disease (e.g., weight loss) in vehicle-treated mdr1a<sup>−/−</sup> mice was observed at roughly 10 wk of age (Figure 1)<sup>30</sup>. In contrast, onset of clinical disease was delayed by treatment with the *P. vulgaris* extract; in addition, markedly fewer of the mdr1a<sup>−/−</sup> mice treated developed severe clinical disease by 20 wk of age (Figure 1). *P. vulgaris* treatment of mdr1a<sup>−/−</sup> mice was also found to attenuate macroscopic lesions associated with the characteristic severe typhlocolitis observed in this murine model (Figure 2). In contrast to the vehicle-treated mdr1a<sup>−/−</sup> mice, ceaca of mdr1a<sup>−/−</sup> 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<sup>−/−</sup> mice were more similar in appearance to the colons of the FVB<sup>WT</sup> control mice with regard to presence of formed feces, and lack of grossly visible tissue edema and rigidity (Figure 2). Mdr1a<sup>−/−</sup> 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<sup>−/−</sup> mice than those present in the cecum (data not shown). The microscopic lesions observed in the ceca of mdr1a<sup>−/−</sup> mice treated with *P. vulgaris* were markedly less severe when compared to those observed for vehicle-treated mdr1a<sup>−/−</sup> 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<sup>23,33-50</sup>. 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...
Debate regarding the role of NF-κB mediators induced by activation of NF-κB levels of TNF-α induction and maintenance of chronic inflammation. Production of cytokines and chemokines central to the key regulator of inflammatory responses in colitis (Table 2). Importantly, extract of P. vulgaris is consistent with that previously reported (1). Other innate inflammatory cells to sites of injury and MadCAM-1 on endothelial cells, while chemokines expression of adhesion molecules (pro-inflammatory cytokines and chemokines such as IL-1α, 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. 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. 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. 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. 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. 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. 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. Similarly, the ethanolic extract of P. vulgaris, which is known to contain several flavonoids (data not shown), downregulated expression of chemokine genes (CCL2, CXCL1/KC, CXCL9/CXCL19, and CCL20) and genes involved in the increased expression of adhesion molecules (VCAM-1, ICAM, TNFα) 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. 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 (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. 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). 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[28]. 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[90].

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[36]. 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**

The authors thank Dr. Philip Dixon for helpful discussion and review of the statistics.

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**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.

**CONFIRMED**

Haarberg KMK et al. Prunella vulgaris attenuates colitis

**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.
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