 doi:10.20944/preprints201803.0146.v1

Article

Panaxynol, a Bioactive Component of American Ginseng, Targets Macrophages and Suppresses Colitis in Mice

Anusha Chaparala [1], Deepak Poudyal [2], Erin E. Witalison [3], Hossam Tashkandi [1], Alexander A. Chumanevich [1], Jenna L. Hofseth [1], Douglas L. Pittman [1], Michael D. Wyatt [1], Anthony Windust [4], Mitzi Nagarkatti [5], Prakash Nagarkatti [5] and Lorne J. Hofseth [1,*]

[1] College of Pharmacy, University of South Carolina, Columbia, SC 29208, USA; chaparal@email.sc.edu
[2] Laboratory of Human Retrovirology and Immunoinformatics, Leidos Biomedical Research Inc., Frederick National Laboratory for Cancer Research, Frederick, MD 21702, USA; Deepak.poudyal@nih.gov
[4] Department of Biological and Biomedical Sciences, Julius L. Chambers Biomedical/Biotechnology Research Institute (BBRI), North Carolina Central University, Kannapolis, NC 28081, USA.; ewitalis@nccu.edu
[4] Measurement Science and Standards, National Research Council, Ottawa, Canada; Anthony.Windust@nrc-cnrc.gc.ca
[5] Department of Pathology, Microbiology, and Immunology, School of Medicine, University of South Carolina, Columbia, SC 29208, USA; mitzi.nagarkatti@uscmed.sc.edu
* Correspondence: hofseth@cop.sc.edu; Tel.: +01-803-403-5588

Abstract: Ulcerative colitis has a significant impact on the quality of life for the patients, and can substantially increase the risk of colon cancer in patients suffering long-term. Conventional treatments provide only modest relief paired with a high risk of side effects, while complementary and alternative medicines can offer safe and effective options. Over the past decade, we have shown that American ginseng has anti-oxidant and anti-inflammatory properties that can suppress mouse colitis and prevent colitis associated colon cancer. With the goal of isolating a single active compound, we further fractionated the hexane fraction, and found the most abundant molecule in this fraction was the polyacetylene, Panaxynol. After isolating and characterizing Panaxynol, we tested the efficacy of Panaxynol in the treatment and prevention of colitis in mice and studied the mechanism of action. We demonstrate here that Panaxynol effectively treats colitis in a Dextran Sulfate Sodium mouse model by targeting macrophages for DNA damage and apoptosis. Positive outcomes from this study could take American ginseng one-step further towards becoming a conventional drug for the treatment of colitis, and possibility exploring other autoimmune diseases associated with macrophage dysfunction.

Keywords: Inflammatory Bowel Diseases; ulcerative colitis; American ginseng; Panaxynol; macrophages

1. Introduction

Inflammatory bowel diseases (IBDs), including ulcerative colitis (UC) and Crohn's disease (CD), are debilitating, significantly affect lifestyle, and carry a high colon cancer risk. IBD prevalence is particularly high in North America and Europe (affecting 3.8 million people), with an economic burden of $30 - $45 billion [1–4]. Of note, incidence has been increasing for both males and females over the past 20 years [5], making this a health problem that needs to be addressed for both sexes. As a frustration to patients with IBD, conventional treatment outcomes are modest, e.g., 20% do not respond to anti-TNFα antagonists [6], and toxicity leads to dangerous side effects. As such, about half of all IBD patients (millions) turn to complementary and alternative medicines (CAMs). Although CAMs have been used for thousands of years, there is a gap in our knowledge of the mechanisms

 doi:10.20944/preprints201803.0146.v1

that support their effectiveness. Understanding these mechanisms will not only lead to standardized and more efficient treatment for IBD outside of toxic FDA-approved drugs but will also better our understanding of the potential applications of CAMs for other diseases with similar mechanisms.

The natural herb, American ginseng (*Panax quinquefolius*; AG), improves mental performance and detrimental end points associated with diseases, such as cardiovascular disease, diabetes, and influenza [7,8]. Over the past decade, we have shown that AG has anti-oxidant and anti-inflammatory properties and is able to suppress mouse colitis and prevent colon cancer associated with colitis [9–11]. Using bioassay-guided fractionation, more recently, we have shown that a hexane fraction of AG (HAG) was particularly potent in this capacity [12–14].

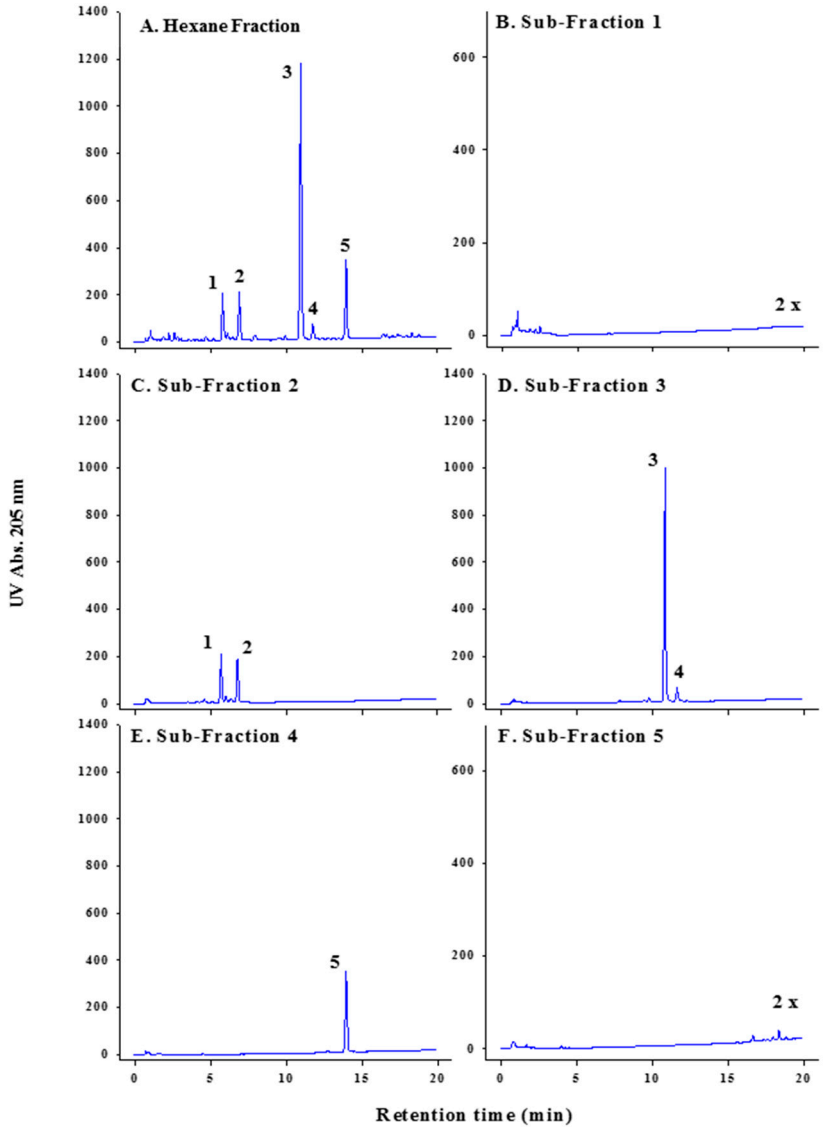
With a goal of isolating a single, bioactive compound from AG and HAG, we further fractionated HAG and found that the most abundant molecule in this fraction was the polyacetylene, Panaxynol. Polyacetylenes are a distinct group of naturally occurring products, whose numerous pharmacological properties have been recognized [15]. Panaxynol ([3(R)-(9Z)-heptadeca-1, 9-dien-4, 6-diyn-3-ol]; falcarinol) is a bioactive member of this family. It has been identified in both traditional herbal medicines (such as AG), and in common dietary plants, e.g., carrots, celery, fennel, parsley, and parsnip [16]. Interestingly, Panaxynol has been shown to have anti-cancer properties [16–19] and neuroprotective effects [20–22]. However, there remains an unanswered question regarding Panaxynol's potential as an anti-inflammatory molecule and, therefore, its capacity to suppress chronic inflammatory diseases, such as UC. Intriguingly, Panaxynol (as compared to the hundreds of other potential CAMs currently used with success in animals) not only comes from a natural source, but s a single ingredient, allowing the potential to be standardized on its own, or in a cocktail. What makes this molecule innovative is the putative mechanism we explore here by addressing the hypothesis that Panaxynol targets macrophages for apoptosis resulting in the suppression of colitis in mice.

2. Results

2.1. Panaxynol is the most abundant and a potent anti-inflammatory molecule in AG

We have previously shown that AG and HAG are effective in the treatment of colitis and prevention of colon cancer [9–14]. We have also demonstrated that fatty acids and polyacetylenes are both components in AG and HAG [12]. In moving forward, to better understand the active components of HAG, we have sub-fractionated this fraction of AG (**Fig 1A)**. Sub-fraction 1 (F1; <10% of the whole HAG) contains multiple minor components including two minor polyacetylenes tentatively identified based on UV spectra (**Fig 1B**). F2 (30% of HAG) contains two major polyacetylenes, Panaxydiol (peak-1) and Panaxydol (peak-2), and four minor polyacetylenes tentatively identified based on UV spectra (**Fig 1C**). F3 (24% of HAG) contains a major polyacetylene, Panaxynol (peak-3), and a fatty acid, linolenic acid (peak-4) (18:3n3) (**Fig 1D**). F4 (27% of HAG) contains linoleic acid (peak-5) and no detectable polyacetylenes (**Fig 1E**). F5 (10%) contains minor fatty acids including saturates, and no polyacetylenes (**Fig 1F**). F2 and F3, the only fractions containing major polyacetylenes, suppress iNOS induction in ANA-1 macrophages polarized to the M1 type with IFNγ (**Fig 1G**), which is predictive of colitis suppression [10,12]. Of the three major polyacetylenes in F2 and F3 sub-fractions of HAG, Panaxynol was the most abundant (10.2%) molecule [12].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 March 2018 doi:10.20944/preprints201803.0146.v1



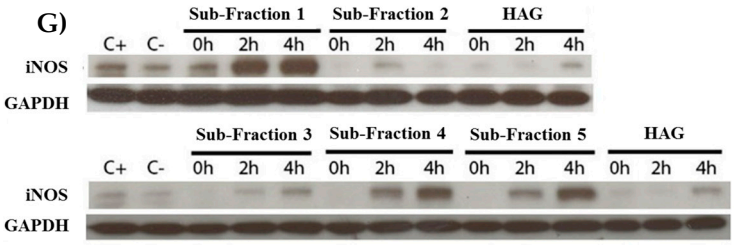


Figure 1. Isolation and characterization of various sub-fractions of HAG. A-F) LC-UV/DAD analysis of Hexane fraction and each sub-fraction. F1 to F5 represent the collected fractions, 4 minutes each. Peak identities: **1.** Panaxydiol, **2.** Panaxydol, **3.** Panaxynol, **4.** linolenic acid, **5.** linoleic acid Column C-18 2.1 x 100 mm, 1 µl injection of a 5 mg mL^{-1} (whole) or equivalent fraction, gradient 55% to 90% acetonitrile/water in 15 minutes; hold 5 minutes; re-equilibration 10 minutes. Note: The scale magnification for sub-fractions 1 and 5 is 2X. **G)** Effect of HAG and different sub-fractions of HAG on IFNγ-induced iNOS expression. ANA-1 mouse macrophages were incubated for 12 hours with HAG or the indicated sub-fractions (10 µg/ml), washed, then exposed to IFNγ (10 ng/ml) for 0, 2, and 4 hours. C+ indicates the positive control, which is ANA-1 cells induced by IFNγ, and then incubated with media.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 March 2018 doi:10.20944/preprints201803.0146.v1

2.2. Panaxynol is effective as a treatment for colitis in Dextran Sulfate Sodium (DSS) mouse model

Following the isolation of Panaxynol from HAG, and an initial screening (iNOS suppression *in vitro* [23]), we tested the efficacy of this compound in the prevention and treatment of DSS-induced mouse colitis. In the prevention model, where mice were treated with Panaxynol for a week before the induction of colitis using DSS (**Fig S1A**), treatment with Panaxynol did not inhibit colitis in mice when compared to the control group. Moreover, there was a marginal increase in the inflammation score with the highest dose of Panaxynol (**Fig S2A, B**) when compared to the vehicle group, indicating that pre-treatment with Panaxynol slightly exacerbated DSS-induced colitis. Although we are currently exploring the possible mechanism of this finding, it appears caution has to be made when considering Panaxynol for any chemoprevention purposes.

Excitingly, Panaxynol was very effective in the treatment model of colitis (**Fig S1B**), where colitis was induced with DSS for a week followed by Panaxynol treatment. Panaxynol (PA) significantly decreased the Clinical Disease Index (CDI) (**Fig 2E**) and the inflammation score (**Fig 2A, 2B**) in a dose-dependent manner. Colonic inflammation from Panaxynol-treated mice was limited to the distal end of the colon, while in the vehicle group, inflammation involved a larger area. To examine a biomarker of inflammation, we tested each colon section for cyclooxygenase-2 (COX-2) immunoreactivity. There was decreased expression of COX-2 with Panaxynol treatment (**Fig 2C, 2D**). Taken together, the results are consistent with the hypothesis that Panaxynol can be used to treat mouse colitis. To note, we monitored the weights of the mice over the course of the experiment and did not observe any unexpected weight loss even with the highest dose of Panaxynol, indicating the non-toxic nature of Panaxynol.

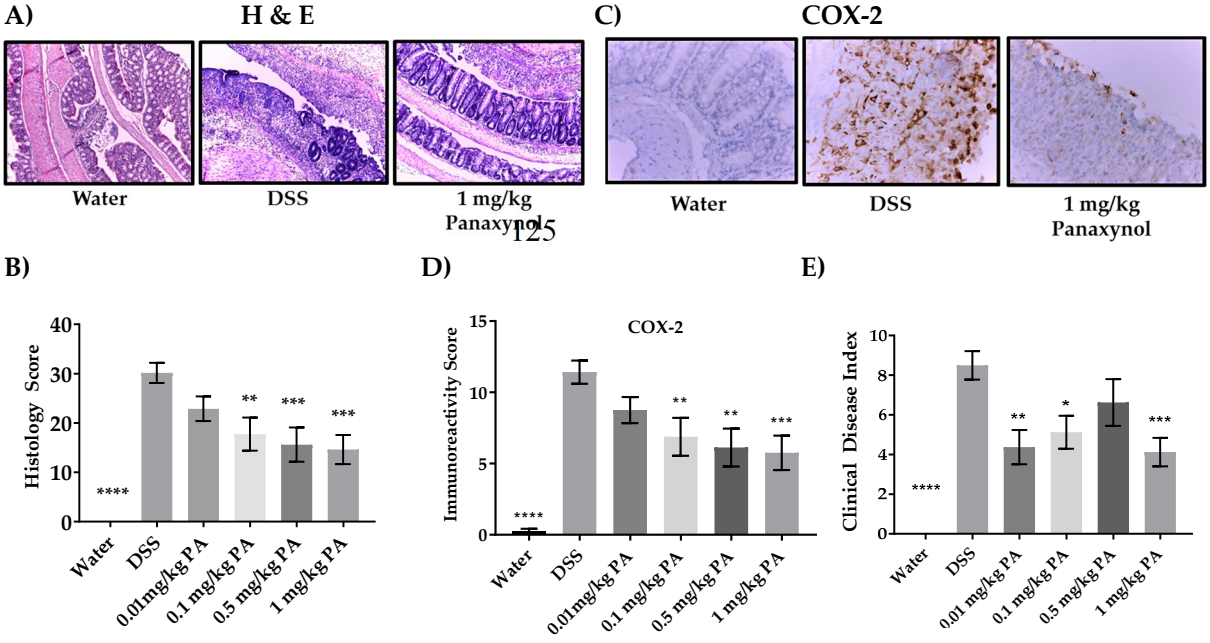


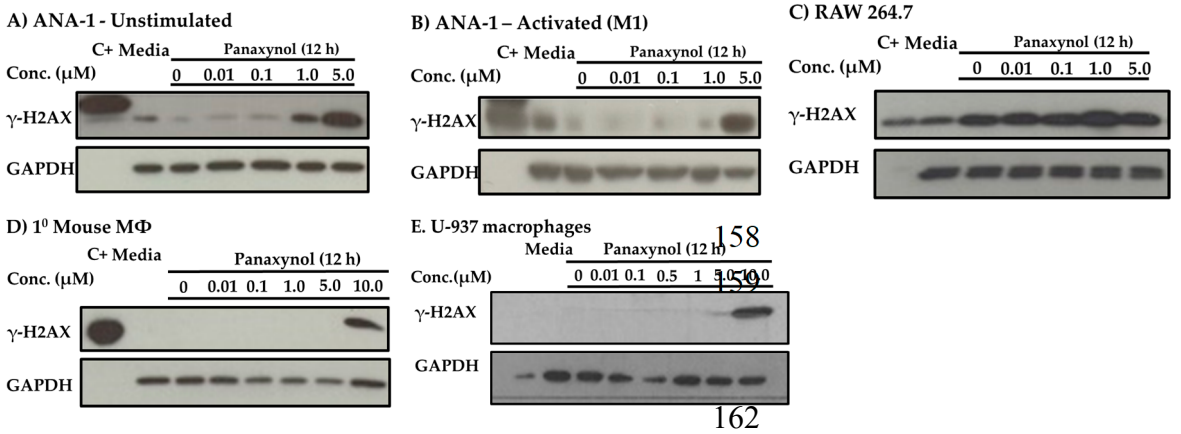
Figure 2. Panaxynol suppresses DSS-induced colitis in mice. A) Representative images (magnification – 100X) of histological sections from 3 groups; water, DSS only and highest dose of Panaxynol (1 mg/kg/day). **B)** Inflammation scores obtained from H & E slides of the colon cross-sections. **C)** Representative images of sections stained for COX-2 (magnification – 400X). **D)** Immunoreactivity score (IRS) of COX-2 from IHC staining. **E)** Clinical Disease Index (CDI) accounts for weight loss, blood in stool and stool consistency (n=8). Values represent mean ± SEM. One-way ANOVA followed by Dunnett's test was used for comparison between samples. p-value when compared to DSS group is indicated by: * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 March 2018 doi:10.20944/preprints201803.0146.v1

2.3. Panaxynol targets macrophages for DNA damage in vitro

In an effort to identify the mechanism of action of Panaxynol, we studied the structure and observed that Panaxynol is a hydrophobic compound with several sites of potential modification that could convert it to a DNA reactive alkylating agent (**Fig S3)**. The hydroxide at the 3-position (C3) can be converted to an α, ß unsaturated aldehyde, which is a Michael acceptor, while the double bond between the 9 and 10 position could potentially be converted to an epoxide. Furthermore, the hydroxide group on C3 can react with the amino group of nucleic acids and alkylate DNA. We, therefore, screened multiple cell types for Panaxynol-induced DNA damage. Strikingly, Panaxynol caused DNA damage, as identified by γ-H2AX expression. However, γ-H2AX induction only occurred in macrophages. These included mouse macrophages (ANA-1, **Fig 3A**, **B**; RAW264.7, **Fig 3C**), primary peritoneal mouse macrophages (**Fig 3D**), and human macrophages (U-937, **Fig 3E**). For all non-macrophage cells (**Fig 3F-K**), γ-H2AX induction was not seen up to 10 μM Panaxynol treatment. As well, when U-937 human monocytes were not differentiated to macrophages, γ-H2AX induction was also not seen until 10 μM Panaxynol treatment (**Fig 3K**) when compared to induction at 1 μM in U-937 cells differentiated into macrophages (**Fig 3E**). This indicated the specificity of DNA damage to mature macrophages and not monocytes. Panaxynol also induced phosphorylation of p53 at Ser15 in ANA-1 cells, further confirming DNA damage (**Fig S4**)

Macrophage cell lines



Non-macrophage cell lines

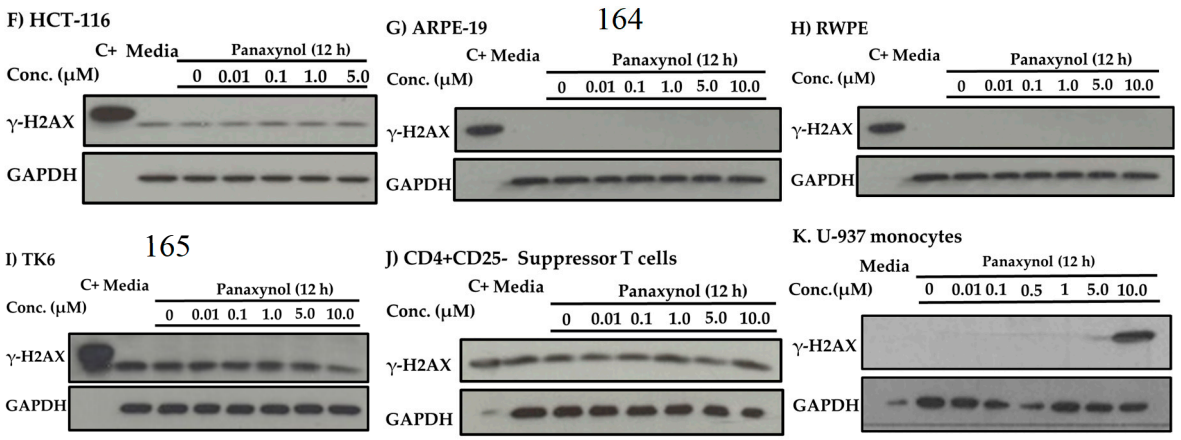


Figure 3. Panaxynol induces γ-H2AX in macrophages, but not in other cell types. All cell types were treated with Panaxynol at specified doses for 12 hours. Activated macrophages were generated by treating with IFNγ (10 ng/ml for 8 hours) prior to Panaxynol treatment. U-937 cells were treated with 10 ng/ml PMA for 24 hours for differentiation into macrophages. **A-E)** Macrophages showed increased DNA damage with doses starting from 1 μM, as shown by the increase in the expression of γ-H2AX, a sensitive marker of DNA damage. **F-J)** Non-macrophage cell lines, including other

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 March 2018 doi:10.20944/preprints201803.0146.v1

immune cells (i.e. lymphoblasts and T cells) and epithelial cell lines, did not show any change in the protein expression of γ-H2AX and **K)** U-937 cells which are monocytes were more sensitive than U-937 macrophages.

2.4. Panaxynol selectively targets macrophages for apoptosis in vitro and in vivo

Based on the understanding that DNA damage is associated with apoptosis, we hypothesized that Panaxynol can selectively cause apoptosis in macrophages. Results are consistent with this hypothesis in two macrophage cell lines (**Fig 4A – C**). Apoptosis was minimal in other non-macrophage cells, including HCT-116 cells (**Fig 4D**) and mouse embryonic fibroblasts (MEFs) (**Fig 4E**). To examine whether Panaxynol selectively causes apoptosis in macrophages in the presence of other cell types, we carried out a co-culture experiment with ANA-1 macrophages and colon cancer cells (HCT-116). Figure 4F shows that Panaxynol causes apoptosis in ANA-1 macrophages at significantly higher levels than in HCT-116 cells. This property of Panaxynol would distinguish it from broadly immunosuppressive drugs that are currently on the market for the treatment of UC.

To confirm that PA targets mΦ *in vivo*, we used colons from the DSS-induced colitis experiment to perform IHC for mΦ. We used a CD11b antibody, which is a surface marker for mΦ and we saw that PA-treated colons have lower expression of CD11b when compared to the vehicle group, indicating that PA treatment decreased the number of mΦ *in vivo* **(Fig S5)**.

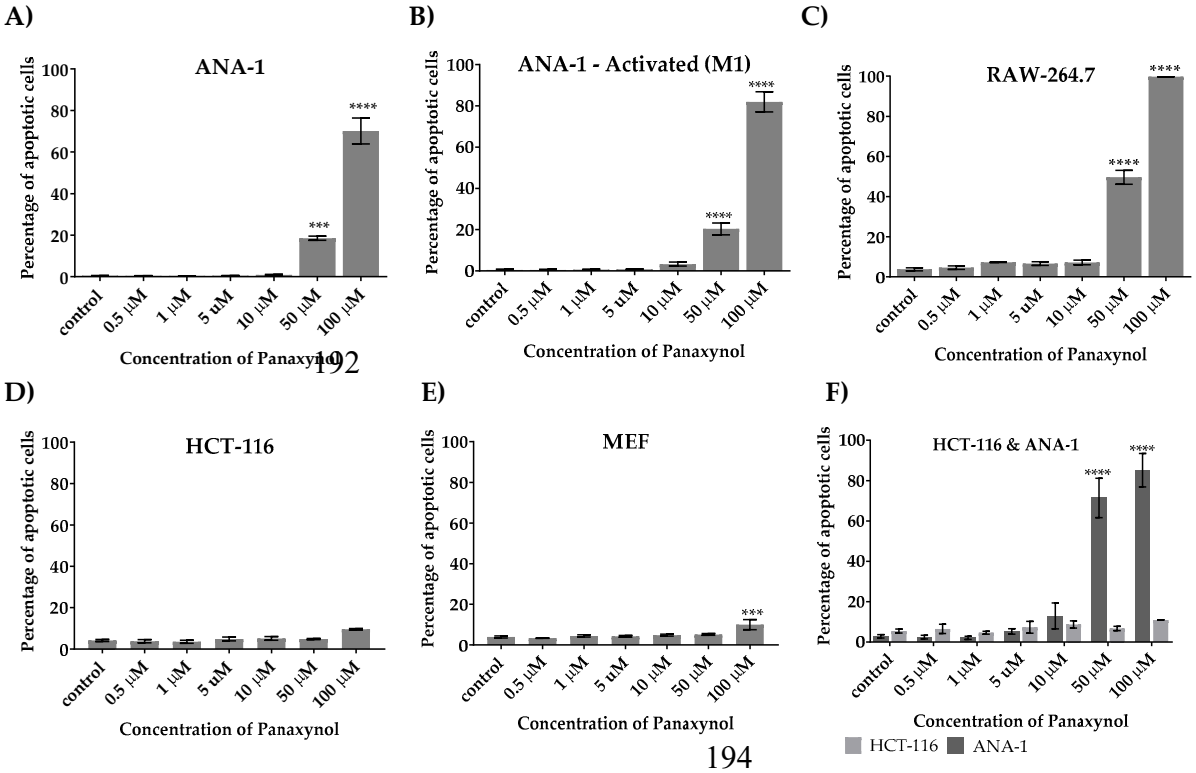


Figure 4: Panaxynol induces apoptosis in macrophages, but not in HCT-116 and MEF cells. Cells were treated with Panaxynol for 12 hours with indicated doses. Panaxynol significantly increased the percentage of apoptotic cells in **A**) unstimulated ANA-1 cells at 50 μM (18%) and 100 μM (70%), **B**) IFNγ stimulated ANA-1 cells at 10 μM (3.3%) and **C**) RAW264.7 cells at 50 μM (50%) and 100 μM (99%). **D**) Panaxynol had no significant apoptotic effect on HCT-116. **E**) Panaxynol induced apoptosis in MEFs only at a high dose of 100 μM (9.5%).**F)** In a co-culture experiment, Panaxynol caused apoptosis only in ANA-1 cells, but not HCT-116 cells. p-value indicated by; * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

 doi:10.20944/preprints201803.0146.v1

3. Discussion

Currently available treatments for UC have multiple side effects and affect major organs like kidneys, liver (hepatitis), and pancreas (pancreatitis) [24]. Furthermore, immune targeting drugs, e.g., infliximab that targets the TNF pathway, are broadly immunosuppressive thereby weakening the immune system, and making the body more susceptible to other infections like tuberculosis. We have shown that AG treats colitis in mice; however, it is composed of multiple ingredients with diverse effects, making it unfit for use as a mainstream drug. Upon examining the different extracts of AG, we identified HAG to be the most effective fraction in the treatment of colitis. Further analysis examined the various components of HAG. Panaxynol, apart from being the most abundant molecule in HAG, is also more effective than the whole HAG in suppressing iNOS expression in macrophages that are polarized to M1 (pro-inflammatory) type. Hence, testing Panaxynol for the treatment of colitis is a natural step towards the identification of the bioactive component to treat colitis and prevent colon cancer.

Consistent with our previous studies with AG and UC, we used DSS-induced mouse colitis model for studying the effect of Panaxynol on an inflammatory disease. We found that Panaxynol treats DSS-induced colitis in the mouse, as seen by decreased CDI, inflammation, COX-2 expression, and the halted weight loss in treated mice. There was no toxicity even at higher doses, as observed by the insignificant weight changes. In future experiments, we will examine the effect of Panaxynol on liver and kidneys to further rule out toxicity.

One of the mechanisms by which AG and HAG treat colitis is by targeting immune cells for apoptosis [11,13]. We also examined the structure of Panaxynol and identified it to be a hydrophobic compound, is a potential DNA-reactive alkylating agent. Panaxynol and its derivative, falcarindiol, have previously been shown to be protein-alkylating agents [25]. Furthermore, it has been shown that Panaxynol causes DNA damage in CaCo2 cells [26]. It can be reasoned that the mechanism of action of Panaxynol can be via the induction of DNA damage. Our preliminary results show that Panaxynol causes DNA damage in multiple cell lines and that macrophages are especially sensitive to DNA damage induced by Panaxynol. We predict that one possible anti-inflammatory mechanism of action of Panaxynol is targeting macrophages for DNA damage and apoptosis. This is an interesting finding with this property being unique to Panaxynol. Furthermore, we also show that macrophages are more sensitive to apoptosis by Panaxynol. This indicates activation of p53 as a result of DNA damage and the activated p53 can then induce apoptosis or cause growth arrest. In this case, p53 is possibly causing activation of apoptosis pathway. This further indicates that γ-H2AX was possibly induced because of DNA damage, and not the disintegration of DNA resulting from apoptosis.

Panaxynol, however, did not prevent colitis in mice. Furthermore, treatment with the highest dose of Panaxynol slightly increased the inflammation score when compared to the untreated mice. The resident macrophages in the lamina propria of the intestine are anti-inflammatory and important for the maintenance of homeostasis. They clear any microbes and other stimuli that cross the epithelial cell barrier, mainly by phagocytosis, but do not secrete any cytokines [27]. Since Panaxynol targeted macrophages before induction of colitis in the prevention model, the disease was more severe and Panaxynol was ineffective. This is consistent with previous studies that showed that depletion of macrophages prior to induction of colitis resulted in exacerbated DSS-induced colitis [28]. However, upon initiation of UC, there is increased accumulation of pro-inflammatory macrophages that secrete cytokines to enhance the inflammatory response. An overactive response by the macrophages to the enteric microbiota at this stage greatly contributes to the pathogenesis of colitis [29]. Treatment with Panaxynol to target macrophages at this stage was highly effective in suppressing colitis.

 doi:10.20944/preprints201803.0146.v1

The reason for the macrophages being specifically targeted by Panaxynol is not completely understood. Future directions will explore the mechanisms of DNA damage and a possible defect in DNA repair. Investigating whether Panaxynol can prevent colon cancer is the next natural step, as macrophage depletion not only decreases inflammation but also suppresses tumorigenesis in AOM-DSS-induced model of colitis induced colon cancer in mice [30]. Panaxynol is effective in the treatment of colitis and does so by targeting macrophages for DNA damage and apoptosis. We have tested a range of doses (0.01 mg/kg – 1 mg/kg), and demonstrate that Panaxynol is very effective at 0.1 mg/kg, which would translate to 6 mg for an average patient weighing 60 kg. This is an extremely low dose when compared to the immunosuppressive drugs currently available, placing Panaxynol a step above the other treatments for UC.

4. Materials and Methods

4.1. Identification and isolation of Panaxynol

Characterization of HAG and extraction of Panaxynol were carried out by our collaborator, Dr. Anthony Windust at the National Research Council (Ottawa, ON, Canada). The method for characterization and analysis of HAG has been described in detail previously [12]. Briefly, for characterization of bioactive components of HAG, this fraction was sub-fractionated through preparative, reverse-phase HPLC, where the HAG was divided into 5 sub-fractions based on elution time (4 minutes each). The fractions were collected over 6 repeat runs (6 x 50 mg injected) and evaporated to dryness. A comparative analysis by analytical scale LC-UV of both the whole and each sub-fraction was performed to confirm identities of constituents in each sub-fraction.

Panaxynol was isolated and purified from *Panax quinquefolius* grown on the Harper Ranch, Kamloops, BC, Canada. The method of extraction and purification of Panaxynol has been previously described [23]. Briefly, dried root of 4-year-old AG was dissolved in ethanol and the organic layer was concentrated using vacuum centrifuge to yield dark brown oil. This extract was further separated using flash chromatography and the fractions containing Panaxynol were dried to yield crude Panaxynol. The crude Panaxynol was then subjected to multiple passes of chromatography and the purity of the final extract was validated using liquid chromatography with UV diode array detection (LC-UV-DAD). Purified Panaxynol was dissolved in 95% ethanol for use in *in vitro* and *in vivo* experiments.

4.2. Cell lines and reagents

All cells were maintained in appropriate media recommended by ATCC supplemented with 10% New Born Calf serum (NBCS) (Biofluids, Rockville, MD), penicillin (10 U/ml) and streptomycin (10 µg/ml, Biofluids) at 37°C in a humidified chamber with 5% CO_2 atmosphere. Experiments with Panaxynol were carried out by treating the cells with indicated concentrations of Panaxynol dissolved in appropriate media with 0.1% NBCS. For polarization to M1 type macrophages, ANA-1 cells were exposed to 10 ng/ml interferon-γ (IFNγ) for 8 hours (R&D Systems, Minneapolis, MN) either before or after the treatment with Panaxynol. For differentiation of U-937 monocytes into macrophages, cells were treated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma; P1585) for 24 hours. After replacing with fresh media containing no PMA, the cells were allowed to grow for 48 hours before treatment with Panaxynol. CD4+CD25- cells were isolated from the spleens of C57BL/6 mice as previously described [13]. Briefly, the macrophages and B cells were depleted before isolation of CD4+CD25- T cells using MACS separator along with CD4 and CD25 microbeads (Miltenyi Biotec, Auburn, CA).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 March 2018 doi:10.20944/preprints201803.0146.v1

297 *4.3. Western blot analysis and antibodies*

298 Phospho-Histone H2AX (Ser139) (cat # 9718S), phosphor-p53 (Ser15) (cat # 9284S), and GAPDH
299 (cat # D16H11) (5174S) rabbit monoclonal primary antibodies (1:1000 dilution); and horseradish
300 peroxidase conjugated anti-rabbit secondary antibody (7074S) (1:2000 dilution) were purchased from
301 Cell Signaling Technology, Danvers, MA. Primary antibody incubations were carried out overnight
302 at 4^0C Secondary antibody incubations were carried out at room temperature for 1 hour. The Western
303 blot signal was detected by Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL)
304 and developed onto Hyperfilm (GE Healthcare Life Sciences, Pittsburgh, PA) or imaged using Bio-
305 Rad ChemiDoc Imager.

306 *4.4. Flow-cytometric TUNEL analysis*

307 TUNEL labeling was performed using Fluorescein in situ cell death detection (cat # 11684795910,
308 Roche Diagnostics, IN). Briefly, cells were incubated in 0.1% NBCS supplemented media containing
309 appropriate concentrations of Panaxynol or vehicle. Cells were harvested after 12 hours of treatment
310 and TUNEL assay was performed as described by the vendor. TUNEL positive cells were detected
311 and quantified using Beckman Coulter F500 Flow Cytometer and CXP software.

312 *4.5 In vivo experiments*

313 DSS (MW 36000–50000) obtained from International Laboratories USA (San Francisco, CA) was
314 used to induce colitis in mice. 8-10 week old C57BL/6 mice were obtained from Jackson Laboratories
315 (Bar Harbor, ME) and maintained in a suitable environment according to the Institutional Animal
316 Care and Use Committee (IUCUC) standards. The care and usage of the mice were monitored by
317 Animal Resource Facility (ARF) at the University of South Carolina, Columbia. This study was
318 approved by IACUC (Animal Use Protocol # 2178).

319 For the prevention model of colitis, mice were given Panaxynol, once daily, at different doses
320 (0.01 mg/kg, 0.1 mg/kg, 0.5 mg/kg and 1 mg/kg diluted in ddH_2O) by oral gavage for two weeks. The
321 lowest dose was calculated based on our previous experiments with AG and HAG. The Panaxynol
322 dose was equated to reflect the percentage composition of Panaxynol in HAG. Starting on day 7, mice
323 were given 2% DSS in drinking water to induce colitis. For the colitis treatment experiments, mice
324 were given 2% DSS in their water for 2 weeks. Starting on day 7, mice were given Panaxynol at the
325 same doses as the prevention experiments (0.01 mg/kg, 0.1 mg/kg, 0.5 mg/kg and 1mg/kg) by oral
326 gavage. Control mice were given ddH_2O by oral gavage. The weight of mice was monitored over the
327 duration of the experiment. The mice were sacrificed on day 14 and colons were harvested, length
328 was measured and processed for further analysis.

329 Blood in stool was detected using Hemoccult (Beckman Coulter) fecal immunochemical test.
330 Immediately before sacrifice, stool consistency (0-fully formed stool; 2-loose stool; 4-diarrhea) and
331 blood in the stool (0-no blood; 2-detected using Hemoccult; 4-rectal bleeding) were scored, and these
332 measurements were used along with the weight difference in mice from the beginning to the end of
333 the experiment (0=no weight loss; 1= 0-5% weight loss; 2= 6-10% weight loss; 3=11-15% weight loss;
334 4=16-20% weight loss), to calculate the CDI.

335 *4.6. Inflammation scoring*

336 Paraffin-embedded colons were serially sectioned (5 μm) and one section from each mouse was
337 stained with hematoxylin and eosin. The stained slides were blindly examined under a microscope
338 by two investigators (A. Chaparala and A. Chumanevich) for histopathological changes and scored
339 according to a system previously described and extensively used by our lab and many others
340 [12,31,32]. Briefly, the histology score for inflammation accounts for four parameters – 1)

 doi:10.20944/preprints201803.0146.v1

inflammation severity (0 (no inflammation), 1 (minimal), 2 (moderate), and 3 (severe)); 2) inflammation extent (0 (no inflammation), 1 (mucosa only), 2 (mucosa and submucosa), and 3 (transmural)); 3) crypt damage (0 (no crypt damage), 1 (one-third of crypt damaged), 2 (two-thirds damaged), 3 (crypts lost and surface epithelium intact), and 4 (crypts lost and surface epithelium lost)) and; 4) percentage area of involvement (0 (0% involvement), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%)). The scores for the first three parameters are added and the sum is multiplied by the fourth parameter, giving a range of scores between 0-40.

4.7. Immunohistochemistry

Sections of paraffin-embedded colons were incubated with cyclooxygenase-2 (COX-2) (cat # 60126; Cayman Chemical Company, Ann Arbor, MI) mouse polyclonal antibody, diluted 1:10,000 in Antibody Amplifier™ (ProHisto, LLC, Columbia, SC) overnight. The slides were then processed using EnVision+ System HRP kits (DAKO, Carpinteria, CA) according to the instructions provided by the kit, which uses the chromagen, diaminobenzidine to elicit dark brown reaction to the HRP-tagged secondary antibody provided in the kit. Methyl green was used as a secondary stain. Immunoreactivity score was obtained by multiplying scores from two criteria – 1) percentage of tissue stained (0-5: 0 (0% positive staining), 1 (< 10%), 2 (11-25%), 3 (26%-50%), 4 (51%-80%), or 5 (> 80%)), and 2) staining intensity (0-3: 0 (Negative staining), 1 (Weak), 2 (Moderate), or 3 (Strong)). The scores of two parameters are multiplied, giving a range of scores between 0-15.

4.8. Statistical Analysis

Data is expressed as mean ± standard error of the mean. Mean differences among the groups were compared by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. A P-value of ≤ 0.05 was chosen for significance.

5. Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1: Schematics of *in vivo* experimental courses; Table S1. Treatments and conditions for each group. Figure S2: Panaxynol does not prevent colitis in mice; Figure S3: Structure of Panaxynol; Figure S4: Treatment with Panaxynol increases the phosphorylation of p53 at Ser15 in ANA-1 cells.

Abbreviations:

IBD – Inflammatory Bowel Disease

UC – Ulcerative Colitis

CD – Crohn's Disease

CAM – Complementary and Alternative Medicine

AG – American Ginseng

HAG – Hexane fraction of American Ginseng

DSS – Dextran Sulfate Sodium

Acknowledgments: This work was supported by National Institutes of Health Center for Colon Cancer Research, NCCAM, NIH 2 P01 AT003961- 06A1 (PN, MN, LJH), and University of South Carolina Electronic Research Administration, USCeRA grant 11110-E193 (AC). We thank Ms. Tia Davis at the USC Animal Resource Facility for the technical assistance in blood collection and Dr. Chang-uk Lim for conducting the flow cytometry.

Author Contributions: The following statements should be used A.C., M.D.W., D.L.P., M.N., P.N., and L.J.H. conceived and designed the experiments; A.C., D.P., E.E.W., A.A.C., and H.T. performed the experiments and analyzed the data; A.W. contributed Panaxynol; A.C. compiled and wrote the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Peng Yu, A.; Cabanilla, L. A.; Qiong Wu, E.; Mulani, P. M.; Chao, J. The costs of Crohn's disease in the United States and other Western countries: a systematic review. *Curr. Med. Res. Opin.* **2008**, *24*, 319–328, doi:10.1185/030079908X260790.
2. COHEN, R. D.; YU, A. P.; WU, E. Q.; XIE, J.; MULANI, P. M.; CHAO, J. Systematic review: the costs of ulcerative colitis in Western countries. *Aliment. Pharmacol. Ther.* **2010**, *31*, 693–707, doi:10.1111/j.1365-2036.2010.04234.x.
3. Kaplan, G. G. The global burden of IBD: from 2015 to 2025. *Nat. Rev. Gastroenterol. Hepatol.* **2015**, *12*, 720–727, doi:10.1038/nrgastro.2015.150.
4. Park, K. T.; Bass, D. Inflammatory bowel disease-attributable costs and cost-effective strategies in the United States: A review. *Inflamm. Bowel Dis.* **2011**, *17*, 1603–1609, doi:10.1002/IBD.21488.
5. Molodecky, N. A.; Soon, I. S.; Rabi, D. M.; Ghali, W. A.; Ferris, M.; Chernoff, G.; Benchimol, E. I.; Panaccione, R.; Ghosh, S.; Barkema, H. W.; Kaplan, G. G. Increasing Incidence and Prevalence of the Inflammatory Bowel Diseases With Time, Based on Systematic Review. *Gastroenterology* **2012**, *142*, 46–54.e42, doi:10.1053/j.gastro.2011.10.001.
6. Roda, G.; Jharap, B.; Neeraj, N.; Colombel, J.-F. Loss of Response to Anti-TNFs: Definition, Epidemiology, and Management. *Clin. Transl. Gastroenterol.* **2016**, *7*, e135, doi:10.1038/ctg.2015.63.
7. Hofseth, L. J.; Ying, L. Identifying and defusing weapons of mass inflammation in carcinogenesis. *Biochim. Biophys. Acta - Rev. Cancer* **2006**, *1765*, 74–84, doi:10.1016/j.bbcan.2005.08.005.
8. Hofseth, L. J.; Wargovich, M. J. Inflammation, cancer, and targets of ginseng. *J. Nutr.* **2007**, *137*, 183S–185S.
9. Cui, X.; Jin, Y.; Poudyal, D.; Chumanevich, A. A.; Davis, T.; Windust, A.; Hofseth, A.; Wu, W.; Habiger, J.; Pena, E.; Wood, P.; Nagarkatti, M.; Nagarkatti, P. S.; Hofseth, L. Mechanistic insight into the ability of American ginseng to suppress colon cancer associated with colitis. *Carcinogenesis* **2010**, *31*, 1734–1741, doi:10.1093/carcin/bgq163.
10. Jin, Y.; Kotakadi, V. S.; Ying, L.; Hofseth, A. B.; Cui, X.; Wood, P. A.; Windust, A.; Matesic, L. E.; Pena, E. A.; Chiuzan, C.; Singh, N. P.; Nagarkatti, M.; Nagarkatti, P. S.; Wargovich, M. J.; Hofseth, L. J.; Matesic, E.; Pena, E. A.; Chiuzan, C.; Singh, P.; Nagarkatti, M.; Nagarkatti, P. S.; Wargovich, M. J.; Ã, L. J. H. American ginseng suppresses inflammation and DNA damage associated with mouse colitis. *Carcinogenesis* **2008**, *29*, 2351–2359, doi:10.1093/carcin/bgn211.
11. Jin, Y.; Hofseth, A. B.; Cui, X.; Windust, A. J.; Poudyal, D.; Chumanevich, A. A.; Matesic, L. E.; Singh, N. P.; Nagarkatti, M.; Nagarkatti, P. S.; Hofseth, L. J. American ginseng suppresses colitis through p53-mediated apoptosis of inflammatory cells. *Cancer Prev. Res. (Phila).* **2010**, *3*, 339–47, doi:10.1158/1940-6207.CAPR-09-0116.
12. Poudyal, D.; Le, P. M.; Davis, T.; Hofseth, A. B.; Chumanevich, A.; Chumanevich, A. A.; Wargovich, M. J.; Nagarkatti, M.; Nagarkatti, P. S.; Windust, A.; Hofseth, L. J. A hexane fraction of American ginseng suppresses mouse colitis and associated colon cancer: Anti-inflammatory and proapoptotic mechanisms. *Cancer Prev. Res.* **2012**, *5*, 685–696, doi:10.1158/1940-6207.CAPR-11-0421.
13. Poudyal, D.; Cui, X.; Mai Le, P.; Davis, T.; Hofseth, A. B.; Jin, Y.; Chumanevich, A. A.; Wargovich, M. J.; Nagarkatti, M.; Nagarkatti, P. S.; Windust, A.; Hofseth, L. J. A limited role of p53 on the ability of a Hexane fraction of American ginseng to suppress mouse colitis. *J. Biomed. Biotechnol.* **2012**, *2012*, 785739, doi:10.1155/2012/785739.
14. Poudyal, D.; Cui, X.; Le, P. M.; Hofseth, A. B.; Windust, A.; Nagarkatti, M.; Nagarkatti, P. S.; Schetter, A. J.; Harris, C. C.; Hofseth, L. J. A key role of microRNA-29b for the suppression of colon cancer cell migration

by American ginseng. *PLoS One* **2013**, *8*, e75034, doi:10.1371/journal.pone.0075034.

15. Christensen, L. P. Aliphatic C(17)-polyacetylenes of the falcarinol type as potential health promoting compounds in food plants of the Apiaceae family. *Recent Pat. Food. Nutr. Agric.* **2011**, *3*, 64–77.
16. Zidorn, C.; Jöhrer, K.; Ganzera, M.; Schubert, B.; Sigmund, E. M.; Mader, J.; Greil, R.; Ellmerer, E. P.; Stuppner, H. Polyacetylenes from the Apiaceae Vegetables Carrot, Celery, Fennel, Parsley, and Parsnip and Their Cytotoxic Activities. *J. Agric. Food Chem.* **2005**, *53*, 2518–2523, doi:10.1021/jf048041s.
17. Wang, C. N.; Shiao, Y. J.; Kuo, Y. H.; Chen, C. C.; Lin, Y. L. Inducible nitric oxide synthase inhibitors from Saposhnikovia divaricata and Panax quinquefolium. *Planta Med.* **2000**, *66*, 644–647, doi:10.1055/s-2000-8624.
18. Kobaek-Larsen, M.; El-Houri, R. B.; Christensen, L. P.; Al-Najami, I.; Frett?, X.; Baatrup, G.; Shibusawa, K.; Sakakibara, R.; Oshima, Y.; Mäenpää, H.; Koss, L.; Nordling, S.; Heinonen, O. P.; Teerenhovi, L.; Hietanen, P.; Tangrea, J. A.; Virtanen, M.; Heinonen, O. P.; Askin, F. B.; Taskinen, E.; Erozan, Y.; Greenwald, P.; Huttunen, J. K. Dietary polyacetylenes, falcarinol and falcarindiol, isolated from carrots prevents the formation of neoplastic lesions in the colon of azoxymethane-induced rats. *Food Funct.* **2017**, *8*, 964–974, doi:10.1039/C7FO00110J.
19. Purup, S.; Larsen, E.; Christensen, L. P. Differential effects of falcarinol and related aliphatic C(17)-polyacetylenes on intestinal cell proliferation. *J. Agric. Food Chem.* **2009**, *57*, 8290–6, doi:10.1021/jf901503a.
20. Yang, Z.; Sun, K.; Yan, Z.; Suo, W.; Fu, G.; Lu, Y. Panaxynol protects cortical neurons from ischemia-like injury by up-regulation of HIF-1α expression and inhibition of apoptotic cascade. *Chem. Biol. Interact.* **2010**, *183*, 165–171, doi:10.1016/j.cbi.2009.09.020.
21. Nie, B. M.; Yang, L. M.; Fu, S. L.; Jiang, X. Y.; Lu, P. H.; Lu, Y. Protective effect of panaxydol and panaxynol on sodium nitroprusside- induced apoptosis in cortical neurons. *Chem. Biol. Interact.* **2006**, *160*, 225–231, doi:10.1016/j.cbi.2006.02.001.
22. Yuan, C.-S.; Wang, X.; Wu, J. A.; Attele, A. S.; Xie, J.-T.; Gu, M. Effects of Panax quinquefolius L. on brainstem neuronal activities: Comparison between Wisconsin-cultivated and Illinois-cultivated roots. *Phytomedicine* **2001**, *8*, 178–183, doi:10.1078/0944-7113-00027.
23. Qu, C.; Li, B.; Lai, Y.; Li, H.; Windust, A.; Hofseth, L. J.; Nagarkatti, M.; Nagarkatti, P.; Wang, X. L.; Tang, D.; Janicki, J. S.; Tian, X.; Cui, T. Identifying panaxynol, a natural activator of nuclear factor erythroid-2 related factor 2 (Nrf2) from American ginseng as a suppressor of inflamed macrophage-induced cardiomyocyte hypertrophy. *J. Ethnopharmacol.* **2015**, *168*, 326–36, doi:10.1016/j.jep.2015.04.004.
24. SANDBORN, W. J.; FEAGAN, B. G.; LICHTENSTEIN, G. R. Medical management of mild to moderate Crohn?s disease: evidence-based treatment algorithms for induction and maintenance of remission. *Aliment. Pharmacol. Ther*. **2007**, *26*, 987–1003, doi:10.1111/j.1365-2036.2007.03455.x.
25. Leonti, M.; Casu, L.; Raduner, S.; Cottiglia, F.; Floris, C.; Altmann, K.-H.; Gertsch, J. Falcarinol is a covalent cannabinoid CB1 receptor antagonist and induces pro-allergic effects in skin. *Biochem. Pharmacol.* **2010**, *79*, 1815–1826, doi:10.1016/j.bcp.2010.02.015.
26. Young, J. F.; Duthie, S. J.; Milne, L.; Christensen, L. P.; Duthie, G. G.; Bestwick, C. S. Biphasic Effect of Falcarinol on CaCo-2 Cell Proliferation, DNA Damage, and Apoptosis. *J. Agric. Food Chem.* **2007**, *55*, 618–623, doi:10.1021/jf0616154.
27. Steinbach, E. C.; Plevy, S. E. The role of macrophages and dendritic cells in the initiation of inflammation in IBD., doi:10.1097/MIB.0b013e3182a69dca.
28. Qualls, J. E.; Kaplan, A. M.; van Rooijen, N.; Cohen, D. A. Suppression of experimental colitis by intestinal mononuclear phagocytes. *J. Leukoc. Biol.* **2006**, *80*, 802–15, doi:10.1189/jlb.1205734.c

29. Xavier, R. J.; Podolsky, D. K. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* **2007**, *448*, 427–434, doi:10.1038/nature06005.

30. Bader, J. E.; Velazquez, K. T.; Enos, R. T.; Cranford, T. L.; Davis, J. M.; Murphy, E. A. Macrophage depletion decreases inflammation and tumorigenesis in the AOM/DSS mouse model of colon cancer. *J. Immunol.* **2016**, *196*.

31. Morteau, O.; Morham, S. G.; Sellon, R.; Dieleman, L. A.; Langenbach, R.; Smithies, O.; Sartor, R. B. Impaired mucosal defense to acute colonic injury in mice lacking cyclooxygenase-1 or cyclooxygenase-2. *J. Clin. Invest.* **2000**, *105*, 469–78, doi:10.1172/JCI6899.

32. Dieleman; Palmen; Akol; Bloemena; PENA; Meuwissen; Van Rees Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. *Clin. Exp. Immunol.* **1998**, *114*, 385–391, doi:10.1046/j.1365-2249.1998.00728.x.