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Bioactive Constituents, Metabolites, and Functions

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Bioactive Components of Polyphenol-Rich and Non-Polyphenol Rich Cranberry Fruit Extracts and Their Chemopreventive Effects on Colitis-Associated Colon Cancer

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Abstract

Cranberries contain various constituents relevant to human health. Our previous study demonstrated the chemopreventive effects of whole cranberry against colon cancer in mice. In order to determine the role of different cranberry secondary metabolites in inhibiting colon cancer, cranberry ethyl acetate extract (EAE) and polyphenol extract (PPE) extracts were obtained. The free-radical scavenging activities and chemical composition of the cranberry extracts were determined. EAE consisted of triterpenes and sterols and trace amount of proanthocyanidins. PPE mainly contained polyphenol with a trace amount of triterpenes. The chemopreventive effects of orally administered EAE and PPE on colitis-associated colon carcinogenesis were determined in mice. Dietary EAE and PPE significantly suppressed tumor metrics without noticeable adverse effects. Gene expression levels of key proinflammatory cytokines were also attenuated by EAE and PPE in the mouse colon. In conclusion, the novel cranberry extracts may offer an efficacious and safe means to prevent colonic tumorigenesis in humans.

Keywords: colon cancer, colitis, cranberry, antioxidant activity, anti-inflammatory activity

Abbreviations: AOM, azoxymethane; DSS, dextran sulfate sodium; EAE, ethyl acetate extract; PPE, polyphenol extract; Interleukin-1, IL-1; Interleukin-6, IL-6; tumor necrosis factor; TNF-α.

Introduction

Cranberry (*Vaccinium macrocarpon*) is a native fruit of North America, and is extensively cultivated in the northeastern and north-central regions of the United States. There is a great interest in identifying the potential benefits of cranberry and cranberry extracts to human health, including colon cancer prevention. Cranberry constituents including several classes of polyphenols and triterpenoids have been reported to inhibit cancer cell proliferation by a variety of mechanisms.[1,2,3]

We recently demonstrated the chemopreventive effects of whole cranberry fruit against colitis and colon cancer in murine models.[4,5] Colon cancer is the third most common type of cancer in the United States, and it is estimated that more than 104,000 new cases will be diagnosed in 2020.[6] Various lifestyle factors have been associated with increased risk for colon cancer, including lack of physical activity, overweight and obesity, smoking, excess alcohol consumption, and diets low in plant-based foods and/or high in processed meats.[6] Also, inflammatory bowel disease (IBD) patients are at a greater risk of developing carcinoma in the colon,[7] and may develop colon cancer at a younger age compared to the age-matched non-IBD cohorts.[8] The role of inflammatory cytokines and immune cells in the pathology of colon cancer has been well-recognized, in part because they create a favorable environment for the initiation and progression of colonic tumors, as well as promote metastasis of cancer cells.[9,10]

A number of epidemiological studies have suggested that dietary patterns high in fruits, vegetables, nuts, and whole grains is associated with lower generation of inflammation and risk of colon cancer.[11,12] Consequently, fruits and fruit extracts may offer an inexpensive, safe and efficient means to prevent colonic tumorigenesis in

humans, particularly for individuals with chronic inflammation.[13,14] Our results showed that whole cranberry powder significantly diminished multiple metrics of colon cancer in azoxymethane (AOM) and dextran sulfate sodium (DSS)-treated mice by modulating signaling pathways related to cell proliferation, apoptosis, and metastasis.[4] Moreover, whole cranberry efficaciously inhibited DSS-stimulated colonic inflammation in mice, and this suppression is at least partially due to its effect in alleviating gut microbiota dysbiosis.[5] Nonetheless, many commercial cranberry products are not prepared from whole fruits and thus may be lacking in various constituents, such as triterpenoids, or they may have a lower content of polyphenols. However, the chemopreventive effects of enriched cranberry extracts containing different classes of secondary metabolites remain unknown.

The chemically diverse secondary metabolites, particularly polyphenols and triterpenes present in cranberry fruit can make independent contributions against colon carcinogenesis. A previous study showed that polyphenol cranberry fraction exerted antiproliferative activity against HCT116 colon cells.[15] Cranberry proanthocyanidins (PACs) or ursolic acid could inhibit tumor colony formation in HT-29 and HCT116 human colon tumor cell lines.[16] Ursolic acid and oleanolic acid inhibited tumor cell proliferation in human colon carcinoma cell line HCT15.[17] Triterpenoid esters (*cis*- and *trans*-3-*O*-*p*-hydroxycinnamoyl ursolic acid) isolated from cranberry fruit inhibited growth of multiple tumor cell lines including HT-29 colonic cells.[18]

Cranberry products vary substantially in content of many of these polyphenol and non-polyphenol metabolites depending on fruit sources, the type of product, and the processing methods.[19] Consumers therefore would obtain significantly different amounts of polyphenol or non-polyphenol constituents depending on the product consumed. The

content of nonpolar, non-polyphenol constituents is expected to be far lower in cranberry juice or juice-derived products than in whole cranberry.[19,20] Thus, it is of great interest to assess the relative chemopreventive effects of different cranberry extracts on colon carcinogenesis.

We hypothesized that both polyphenol and terpenoid constituents can contribute to the chemopreventive properties of whole cranberries. In this study, we prepared two extracts from the whole cranberry powder containing primarily soluble polyphenols (PPE) or lipid-soluble terpenoid constituents (EAE). Furthermore, we determined their chemopreventive efficacy on colon tumor formation using the same animal model of colitis-associated colon cancer, and investigated the underlying mode of action preliminarily.

Materials and Methods

Chemicals. Reagent grade solvents acetone, ethyl acetate, glacial acetic acid, hydrochloric acid and HPLC grade methanol were purchased from Pharmco-AAPER (Brookfield, CT). ACS reagent methanol and ethanol were obtained from Fisher Scientific (Hampton, NH). HPLC grade water was purchased from Honeywell (Morristown NJ). Diaion HP-20 was provided by Supelco (Bellefonte, PA). Standards of ursolic acid (assay ≥ 90%), oleanolic acid (assay ≥ 97%) and stigmasterol (assay 95%) were purchased from Sigma-Aldrich (St. Louis, MO) along with N,N-dimethylaminocinnamaldehyde (DMAC) powder, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), reagent-grade formic acid and NMR external standard 3,5-dinitrobenzoic acid. Standard of 6-hydroxy-2,5,7,8-

tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Aldrich Chemical Company (Milwaukee, WI). Cranberry proanthocyanidins (PAC) standard was previously prepared from an isolated cranberry PAC fraction containing A-type flavan-3-ol oligomers which was characterized by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.[21] β-sitosterol (purity 83.88%) was provided from INDOFINE Chemical Company (hillsborough, NJ). Deuterated NMR solvent dimethylsulfoxide (DMSO-d_6, 99.9%) and 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) were purchased from Cambridge Isotope Laboratories (Andover, Ma). Distilled water was produced on site in the chemistry department at University of Massachusetts Dartmouth.

Plant Material. Whole cranberry fruit of cultivar Early Black was harvested in June 2011 at UMASS Cranberry Experiment Station at State Bog in Wareham, MA. All cranberry fruit were flash frozen with liquid nitrogen and stored at -20°C until use.

Sample Extraction and Preparation. Flash frozen cranberries were thawed down to room temperature. 544.74 g of cranberry fruit were repeatedly extracted by blending with 200 mL volume of methanol/acetone/water/formic acid (40:40:19:1) mixture for 1 hour with a subsequent filtration until the residue turned pale yellow in color. Approximately 700 ml of extraction solvent mixture was consumed. The filtrate was concentrated by rotary evaporation (Büchi Rotovapor R-200) to remove organic solvent at temperature of 26 degree Celsius and the remaining water was removed by freeze-drying to obtain polar crude extract. Diaion-HP20 column chromatography was performed to remove free sugars from polar crude extract to yield 3.208 grams of de-sugared polar extract (PPE). The pale-yellow solid residue was soaked with 200 ml ethyl acetate then

left overnight in the refrigerator. A subsequent extraction with ethyl acetate was performed multiple times until all color was removed from the residue. The supernatant was rotary evaporated to dryness to achieve 1.3035 grams of nonpolar ethyl acetate extract (EAE).

Proton (^{1}H) qNMR Analysis Methods. Cranberry extracts analysis was performed using a one-dimensional proton nuclear Overhauser effect spectroscopy experiment (1D ^{1}H NOESY) on Bruker AVANCE III 400 MHz NMR. Data acquisition was carried out on IconNMR™ 5.0.3 and spectra were processed in TopSpin™ 3.5. Automated identification and quantification of selected metabolites were performed in comparison to authentic standards in an NMR special database (SBASE) using Bruker Assure-RMS software version 2.0., Bruker-BioSpin (Billerica, MA). The concentration of the target compounds can be determined by matching proton peaks and comparing peak integration with the authentic standards which were analyzed in the same experimental conditions. Ursolic acid and oleanolic acid were determined by methods described by Turbitt, et al., 2019.[19] Beta-sitosterol and stigmasterol quantification methods were set up by Assure-RMS software using 3,5-dinitrobenzoic acid with a concentration of 5 mg/mL as a calibration standard according to the PULCON approach.[22] External standards were run in the same parameters as Turbitt, et al., 2019, including a sweep width of 20.0254 ppm (8,012.820 Hz) ppm, 64K number of points, acquisition time of 4.089 seconds with an adjusted relaxation delay of 20.0 seconds instead of 10.0 seconds to obtain more accurate integration ratio.[19] Both beta-sitosterol and stigmasterol standards were analyzed at a concentration of 0.1 mg/mL. Sample extracts were prepared in 600 µL DMSO-d_6 solvent with DSS as internal standard to yield sample concentration of 25 mg/mL in polar extract and 1 mg/mL in nonpolar extract. NMR samples were vortexed,

sonicated by a Fisher Scientific FS20H Sonicator (Pittsburg, PA) until fully dissolved and then centrifuged for several minutes and transferred into NMR tubes. Samples were analyzed at 298.0 K temperature with gas flow at 400 lph, and run in triplicate.

HPLC-DAD Separation and Flavonoid Analysis. HPLC-DAD analysis was performed on a Waters Millennium binary HPLC system with two Waters 515 pumps, Millennium[32] version 32.0 software, coupled with a 996 photodiode array (PDA) detector (Milford, MA) in order to analyze anthocyanins and flavonol glycosides. Absorbance was monitored from 210 nm to 600 nm. Flavonol glycosides were determined at absorbance of 355 nm and anthocyanins at absorbance of 520 nm.[21] Separation was carried out using a 100 × 4.6 mm i.d., 2.6 µm particle size Phenomenex Kinetex C18 column. The mobile phase consisted of 4% acetic acid in water (A) and 4% acetic acid in methanol (B), with a gradient program of 1-15% B between 0 and 5 min, 15-17% B between 5 and 10 min, 17% B between 10 and 20 min, 17-40% between 20 and 30 min, and 40-100% between 30 and 40 min with a flow rate of 0.8 mL/min. Samples were analyzed at room temperature and injection volume. Samples were analyzed in triplicate.

DMAC Assay. Total proanthocyanidins content was measured using the BL-DMAC method with cranberry PAC standard as previous literature described.[19,23,24] All samples were tested in triplicate.

DPPH Free-Radical Scavenging Antioxidant Assay. The determination of free-radical scavenging antioxidant activity in cranberry polar and nonpolar extracts using DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical was followed according to Brand-Williams et al., 1995 and Kraujalytė et al., 2013 with several modifications.[25,26] PPE and EAE samples were diluted with methanol to obtain a series of different concentrations in the

range of 0.0025 to 0.000155 μM and 0.1 to 0.0053 μM. 3.5 mL of 60 μM DPPH solution was mixed with 50 μL each cranberry sample dilution then added into a 1cm quartz cuvette. The absorbance of each sample at 515 nm was measured at 0,10,20,30,40 and 50 min and was recorded using UV-visible spectrophotometer. Results were expressed as EC_{50} values: the concentration required to decrease the total absorbance of DPPH radical in solution by 50%. The EC_{50} values of the samples were compared to a standard antioxidant, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

Diets. The protocol for the animal experiment was approved by Institutional Animal Care and Use Committee of the University of Massachusetts Amherst (#2014-0079). Male CD-1 mice (5-week old) were obtained from Charles River Laboratories (Wilmington, MA). Upon arrival, the mice were kept in a temperature-controlled animal room (23°C), humidity (65–70%) and day/night cycle (12 h light, 12 h dark) with *ad libitum* access to water and AIN93G diet for 1 week for acclimation. As shown in Fig. 2, mice were then randomly divided into three groups, i.e. AOM/DSS control (n = 20); PPE group (n = 20); and EAE group (n = 20). Food jars were replenished with fresh diet every two days. Mice in positive control groups were fed with AIN93G basal diet, while mice in the treatment groups were fed AIN93G diet containing 0.1% PPE and 0.05% EAE (w/w in diet), respectively, one day after AOM injection until the end of study. The dietary dose of cranberry extracts used in this study is equivalent to approximately a daily dose of 500 mg of PPE and 250 mg of EAE, respectively, for human dietary consumption in a 60 kg adult based on equivalent surface area dosage conversion factors.[27]

Animal Study Procedure. To evaluate the anti-carcinogenic effects of cranberry extracts, an AOM/DSS-induced colitis-associated colon tumorigenesis model was used.

206 At week 2, mice were given intraperitoneal injection of AOM (12 mg·kg^{-1} body weight) in
207 saline. After 1 week, 1.5% DSS (molecular weight: 36,000–50,000, International Lab,
208 Chicago, IL) was administered in the drinking water of control and treatment groups for 4
209 days followed by 1 week of tap water for recovery, and this cycle was repeated four times.
210 All mice were sacrificed by CO_2 asphyxiation at 22nd week for evaluation of tumors in the
211 colon. The liver and spleen were removed and weighed. After measurement of length and
212 weight, the entire colons were cut longitudinally, rinsed with phosphate-buffered saline
213 (pH 7.4) and then macroscopically inspected. Number and size of visible tumors in the
214 colons were documented using an ocular micrometer. The size of tumors was determined
215 by the following formula: tumor volume (mm3) = $L \times W^2 / 2$, where L is the length and W
216 is the width of the tumor. The opened colon, spread out on a glass plate sitting atop
217 crushed ice, was then gently scraped using glass microscope slides and the mucosa thus
218 obtained was stored at -80°C for qRT-PCR analysis.

219 **qRT-PCR Analyses of Pro-inflammatory Cytokines.** Real-time qRT-PCR
220 analysis was conducted as previously described.[28] Briefly, total RNA of colonic mucosa
221 was isolated by RNeasy Plus Mini Kit according to the manufacturer's instructions
222 (Qiagen, Valencia, CA). From each sample, 0.16 mg of total RNA was converted to single-
223 stranded cDNA, which was then amplified by Brilliant II SYBR Green QRT-PCR Master
224 Mix Kit, 1-Step (Agilent Technologies, Santa Clara, CA) to detect quantitatively the gene
225 expression of IL-1β, IL-6, TNF-α and β-actin (as an internal standard) using Mx3000P
226 QPCR System (Stratagene, La Jolla, CA). The primer pairs were from Integrated DNA
227 Technologies, Inc. (Coralville, IA, oligonucleotide primers used for qRT-PCR:
228 Supplementary Table 1s). A minimum of three independent experiments was carried out,

and each experiment had triplicate samples for each treatment. The copy number of each transcript was calculated with respect to the GADPH copy number, using the $2^{-\Delta\Delta Ct}$ method.[29]

Statistical Analysis. All data were presented as the mean ± standard errors (SE). Student's *t*-test was used to test the mean difference between the treatment group and control group. The tumor incidence was statistically calculated using the chi-square test. A p value < 0.05 was considered to be statistically significant.

Results and Discussion

^{1}H NMR Identification and Quantification of Triterpenoids. Ursolic acid and its isomer, oleanolic acid, are two major triterpenoids present in the outer layer of the cranberry fruit that are primarily responsible for anti-inflammatory, anti-tumor, and anti-cancer activities.[30,31] Ursolic acid, oleanolic acid, β-sitosterol and stigmasterol in the cranberry extracts were identified and quantified using Assure-RMS which incorporated specific signals of the external standard and each SBASE standard for peak fitting. Peak information for metabolites quantification was listed in Table 1.[19] Previous study by Croreau & Fagerson., 1969 and Croreau & Fagerson., 1971 confirmed that β-sitosterol as the major sterol and stigmasterol were present in the cuticular wax of cranberry and lipids from cranberry seeds by gas-liquid chromatography.[32,33] Due to the similarities of β-sitosterol and stigmasterol structures, overlapping signals were found in both NMR spectra including their quantification peaks 0.965 ppm and 0.967 ppm respectively. According to Chaturvedula et al., 2012, the signals at 0.965 ppm (s, 3H) from β-sitosterol

and 0.967 ppm (s, 3H) from stigmasterol both correspond to C29 position. Since these two sterols cannot be distinguished, quantification of the peak 0.96 ppm gives the total content of both sterols.[34] β-sitosterol and stigmasterol were detected in EAE at a concentration of 107.83 mg/g extract, along with 372.97 mg/g ursolic acid and 79.16 mg/g oleanolic acid. Ursolic acid was determined in PPE at a content of 10 mg/g extract. Oleanolic acid and both sterols were undetectable in PPE. Unpublished data shows that qNMR is unlikely to detect analytes present at concentrations below 0.03 mM. Full ^{1}H NMR spectrum of EAE at region between 0 to 10 ppm was shown in Fig. 1s (Supplementary Information). The Fig. 1a shows the expanded region between 0 to 3 ppm in the ^{1}H NMR spectrum of EAE sample used for quantification of triterpenoids and sterols. Some of the ursolic acid in the EAE may be present in the form of hydroxycinnamoyl esters.

Previous studies by Kondo and Neto et al., 2011 and Murphy et al, 2003 identified two phenolic hydroxycinnamate esters of ursolic acid in cranberries: *cis*- and *trans*-3-*O*-*p*-hydroxycinnamoyl ursolic acid which could inhibit tumor cell proliferation.[18,20] Also, according to a 2009 study by Huang and coworkers, *cis/trans*-hydroxycinnamoyl ursolic acid can be identified in the cranberry methanol extract by ^{1}H NMR analysis.[35] The chemical shifts of *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid from previous reported data by Murphy et al., 2003 and Huang et al., 2009 matched signals occurring between 5.61- 7.66 ppm including signals at 5.81 ppm (d, J = 12.8 Hz), 6.82 ppm (d, J = 8.5 Hz), 6.85 ppm (d, J = 12.8 Hz) and 7.66 ppm (d, J = 8.5 Hz) in the ^{1}H NMR spectrum of EAE sample which are consistent with the presence of *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid in EAE sample (Fig.1b).[18,35] A signal at 7.58 ppm (d, J =

8.05 Hz) is consistent with reported data on 1,4-disubstituted benzene fragment of *trans*-3-*O*-*p*-hydroxycinnamoyl ursolic acid.[35] Based on Murphy et al., 2003 and Huang et al., 2009, coupling constants were the key to distinguishing olefinic protons of the *cis* and *trans* isomers: J value of 12.8 Hz corresponded to *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid.[18,35] Additional signals with J value of 15.8 Hz corresponding to the *trans* isomer appear to be obscured by overlapping signals in these regions. However, further evidence of the *cis*- and *trans*-3-*O*-*p*-hydroxycinnamoyl ursolic acid was observed in EAE in preliminary LC-MS data (Fig. 2S, Supplementary Information). In Fig. 2s, two major peaks eluting several minutes apart are observed with $[M-H]^-$ m/z 601.4, eluting at 10.66 and 13.1 minutes, which is consistent with previously reported LC-MS data on *cis*- and *trans*-3-*O*-*p*-hydroxycinnamoyl ursolic acid.[20,35] These hydroxycinnamoyl esters were not quantified in the present study; however our previous study found that the esters comprise approximately 19% of the total ursolic acid in Early Black cultivar cranberry fruit.[20]

Based on NMR analysis data, approximately 56% of EAE is made up of these major triterpenes and sterols including 37.3% ursolic acid, 7.9 % oleanolic acid and 10.9% sterols, which have reported anti-cancer, anti-tumor and anti-inflammatory activities (Table 2).[30,36,37] A small percentage of the ursolic acid appears to be present in the form of hydroxycinnamoyl esters. Since over half the EAE is made up of triterpenoids and sterols, these compounds may contribute to the observed anti-inflammatory and anti-tumor activities. In PPE however, only trace amounts of triterpenes (1% ursolic acid) can be measured via NMR; sterols were not detected (Table 2). Signals in the 4 – 9 ppm regions of the PPE spectrum associated with cranberry polyphenols were too poorly

297 resolved to provide reliable quantitative data; thus polyphenols were determined using
298 other methods.[19]

299 **Determination of Flavonoids by HPLC-DAD Method.** Four major anthocyanins
300 and seven flavonol glycosides were identified and quantified by HPLC-DAD method.
301 Anthocyanin peaks eluted at retention times between 11.821 and 23.835 mins with elution
302 order as follow: cyanidin-3-*O*-galactoside, cyanindin-3-*O*-arabinoside, peonidin-3-*O*-
303 galactoside and peonidin-3-*O*-arabinoside at 520 nm absorbance wavelength. Flavonol
304 glycosides were determined at 355 nm absorbance with retention time between 21.074
305 and 33.277 mins. Flavonol glycosides eluted in the order: myricetin-3-galactoside,
306 myricetin-3-arabinoside, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-
307 xyloside, quercetin-3-arabinopyranoside, quercetin-3-arabinofuranoside, quercetin-3-
308 rhamnopyranoside, and finally unknown flavonol glycosides. Only PPE was found to have
309 a significant content of anthocyanins and flavonols with a concentration of 9.97% and
310 4.14% respectively, totaling approximately 14% flavonoids. No significant content of
311 anthocyanins or flavonols is found in EAE sample (Table 2).

312 **Determination of Total Proanthocyanidin Content by DMAC Assay.** The total
313 content of PACs in the PPE and EAE were 596 ± 39 mg/g extract and 2.20 ± 0.05 mg/g
314 extract respectively by DMAC assay. Both DMAC and HPLC results demonstrate the lack
315 of any significant quantity of polyphenols in the nonpolar extract EAE. From the DMAC
316 assay result, almost 60% of the PPE sample is made of PACs (Table 2). The polar extract
317 PPE has a considerable amount of phenolics containing a variety of flavonoids: flavonols
318 (quercetin glycosides and myricetin), anthocyanins and proanthocyanidins. Combined
319 with HPLC results, the estimated total polyphenol content of the extract is over 70% by

weight. The high phenolic content in PPE suggests that it will have higher antioxidant activity than EAE, due to the antioxidant activities of flavonoids.

Cranberry Extracts Free-Radical Scavenging Antioxidant Activity. The free-radical scavenging activities of PPE and EAE were determined by DPPH assay. PPE had the highest free radical quenching ability with an EC_{50} value of 3.71×10^{-4} μg/mL, compared to EAE and the standard Trolox. EAE had an EC_{50} value of 2.52×10^{-2} μg/mL and Trolox had a value of 8.76×10^{-4} μg/mL. DPPH assay results confirm that PPE is rich in antioxidants, consistent with its higher content of flavonoids and proanthocyanidins, which are well known radical scavengers that can prevent oxidative processes. Due to high polyphenol antioxidant content of PPE, these compounds may contribute to its observed anti-inflammatory and anti-cancer activities.

PPE and EAE Suppressed Colitis-Associated Colon Tumorigenesis in AOM/DSS-Treated Mice. AOM/DSS-induced colitis-associated colon cancer mouse model has been widely used to assess the chemopreventive effects of dietary components. In this model, a single injection of a colon-specific carcinogen AOM in combination with 2-4 cycles of DSS (a pro-inflammatory agent) in drinking water induce the development of colitis, colorectal dysplasia, and cancer. The clinical and histopathologic features of AOM/DSS model of inflammation-associated colon cancer resemble the early histopathologic changes that lead to colon cancer in humans, by which many cancer chemopreventive agents have been identified.[38] We have previously demonstrated the inhibitory effects of whole cranberry in AOM/DSS-treated mice.[4]

Herein, we employed the same animal model to assess the chemopreventive effects of PPE and EAE against colitis-associated colon tumorigenesis. The dietary doses

of PPE and EAE used in this study represent their portion in the powdered whole cranberry by weight. These doses of PPE and EAE were equivalent to approximately a daily dose of 500 and 250 mg of PPE and EAE, respectively, for human dietary consumption in a 60 kg adult based on equivalent surface area dosage conversion factors, which are reasonably achievable in humans.[27] Throughout the entire experimental period, body weight was monitored twice a week (Fig. 3), and no difference was found between PPE- or EAE-fed mice and mice in the control groups (final body weights were shown in the Table 3). There was no difference in the weight of liver and spleen among all three groups (Table 3), and no apparent behavioral or appearance difference was observed either, suggesting no noticeable adverse effects were associated with dietary feeding of the cranberry extracts to the mice.

Shortening of the length of the colon is one of most common symptoms associated with DSS-induced colitis, and is correlated with the severity of the disease.[39] The result showed that EAE reversed the shortening of colon significantly compared to the control group (97.0 mm vs. 88.9 mm, $p < 0.02$), whereas PPE treatment showed no effect. AOM injection in conjunction with cyclic administration of DSS led to the development of neoplastic tumors in the colon, as evidenced by 80% tumor incidence, tumor multiplicity of 7.20 ± 1.24 colonic tumors, and tumor burden of 17.7 ± 2.48 mm^3 per mouse in the control group (Table 3). Dietary administration of 0.1% PPE significantly decreased tumor incidence to 50%, tumor multiplicity to 4.95 ± 0.84 tumors per mouse, and tumor burden of 8.20 ± 1.71 mm^3 per mouse; and 0.05% EAE decreased tumor incidence to 75%, tumor multiplicity to 3.40 ± 0.73, and tumor burden to 9.42 ± 2.73. These suppression on tumor metrics were statistically significant ($p < 0.05$), except that EAE treatment did not

significantly reduce the tumor incidence compared to the control. These findings indicate that these two cranberry extracts substantially attenuated the development of colitis-associated colon cancer, while minimum side-effect was observed, providing a basis for further mechanistic investigations.

Previously, we found that whole cranberry powder resulted in a 38, 53 and 74% reduction in tumor incidence, multiplicity and burden, respectively.[4] Compared to whole fruit, PPE exerted same degree of suppression on tumor incidence, EAE showed comparable level of suppression on tumor multiplicity, while whole cranberry powder had the strongest effects on other tumor metrics. Our finding demonstrated that polyphenol and terpenoid constituent of cranberry each contribute to the anti-tumor effects of whole cranberry we observed previously, and different constituents may work synergistically to achieve the best chemopreventive efficacy. Overall, consuming whole fruit might be the best approach, however, many commercial cranberry products are not prepared from whole fruits and thus may be lacking in various constituents. Thus, this study suggests that consumers who choose to take these cranberry products may receive comparable health benefits with respect to colon cancer prevention if either polyphenols or terpenoids are present in quantities similar to whole fruit. Our previous study however suggests that many commercial products such as supplements are lacking in one or both of these important classes of constituents.[19]

PPE and EAE Attenuated AOM/DSS-Induced Gene Expression of Pro-Inflammatory Cytokines. A large number of studies have demonstrated that colonic inflammation, including obesity-induced low-grade inflammation and that occurred in IBD could greatly elevate the risk for colon cancer in various experimental settings.[7,40,41]

Inflammatory cytokines and chemokines promote tumor growth, hinder the differentiation of tumorous cells, and support their survival.[42] Therefore, we next evaluated the anti-inflammatory effects of the cranberry extracts in the colon of AOM/DSS-treated mice by real-time qRT-PCR analysis. As shown in Fig. 4, PPE and EAE intervention significantly diminished the levels of pro-inflammatory cytokines in the colon, in comparison to the mice on standard diet. Specifically, PPE and EAE led to significant decreases in the mRNA levels of IL-1β by 26 and 28% compared to the control group, respectively. For IL-6, PPE and EAE reduced its mRNA level by 59 and 39%, respectively; for TNF-α, PPE and EAE reduced those by 90 and 58%, respectively. Comparing PPE and EAE cranberry extracts, PPE had slightly stronger inhibitory effects on these pro-inflammatory cytokines (especially TNF-α) than EAE.

Both animal and human studies have shown that pro-inflammatory cytokines, such as IL-6 and TNF-α play a critical role in the initiation and progression of CRC and other malignancies by inducing pro-carcinogenic signal pathways, including signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB). These pro-inflammatory cytokines facilitate tumorigenesis by enhancing cellular proliferation, tumor invasiveness and resistance to apoptosis.[43] In addition to inflammation-associated cancer, it is estimated that 3 of 5 people die of chronic inflammatory diseases such as stroke, chronic respiratory diseases, heart disorders, obesity, and diabetes.[44] Interventions to reduce or reverse inflammation can be expensive and focus on prescription medicines, such as statins and NSAIDs, which while effective may have their own risks when used long-term (e.g. gastric bleeding, kidney and stomach problems).[45] Thus, creating dietary interventions that are effective, inexpensive, and safe (with little or no side effects) holds

great potential for inflammation prevention. In the present study, we revealed that despite the different compounds present in each extract, both extracts exerted a potent anti-inflammatory efficacy on biochemical inflammation in the colon in AOM/DSS-treated mice. This finding also suggests these cranberry extracts may play a role in the prevention of other chronic inflammation-associated diseases, which warrants further investigation.

To sum up, this study demonstrated that multiple secondary metabolites in cranberry fruit contributed to the chemopreventive effects of whole cranberry against colon tumorigenesis through attenuating colonic inflammation. The novel cranberry extracts may offer an efficacious, safe and inexpensive means to prevent colonic tumorigenesis in humans, particularly for individuals with chronic inflammation.

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561 **Table 1** ^{1}H NMR Signals Used for Identification and Quantification of Terpenes and
562 Sterols

Compound	**δ (ppm) Value(s)**	**Splitting**	**Coupling Constant(s) (J, Hz)**
Ursolic acid	0.775	Singlet	-
	0.820-0.860	Doublet	6.63
Oleanolic acid	0.745	Singlet	-
Beta-sitosterol	0.965	Singlet	-
Stigmasterol	0.967	Singlet	-

563

564 **Table 2.** Chemical Composition of PPE and EAE [a]

Group	PPE (% w/w)	EAE (% w/w)
Ursolic acid	1%	37.3%
Oleanolic acid	ND	7.9%
Sterols	ND	10.9%
Flanonoids	14%	Non detectable
PACs	59.6%	0.2%

565

566 [a] The results are expressed as percent weight by weight.

567 **Table 3** Final Physiologic and Colon Assessment [a]

Group	Control	PPE	EAE
Treatment	AOM/DSS	AOM/DSS/0.1% PPE	AOM/DSS/0.05% EAE
Body weight (g)	47.99 ± 1.71	48.10 ± 1.10	50.60 ± 1.53
Colon length (mm)	88.90 ± 3.46	91.62 ± 2.48	97.00 ± 3.16 *
Tumor incidence	80%	50% *	75%
Tumor multiplicity	7.20 ± 1.24	4.95 ± 0.84 *	3.40 ± 0.73 *
Tumor burden	15.83 ± 3.29	8.20 ± 1.71 *	9.42 ± 2.73 *
Liver weight/body weight	0.044 ± 0.002	0.046 ± 0.002	0.051 ± 0.001
Spleen weight/body weight	0.059 ± 0.002	0.0069 ± 0.002	0.0056 ± 0.001

568

569 [a] Data are shown as the mean ± SE. Student's *t*-test was used to test the mean
570 difference between the treatment group and control group. * Indicates statistically
571 significant differences from the control group ($p < 0.05$).

572

573

574 **FIGURE CAPTIONS**

575 **Fig. 1a.** Expanded region (1-3 ppm) of the ^{1}H NMR spectrum of EAE (1mg/mL) with
576 selected regions of interest labeled. **1b.** Expanded region (5-8 ppm) of the ^{1}H NMR
577 spectrum of EAE (10mg/mL) with selected regions of interest labeled.

578 **Fig. 2.** Animal study design.

579 **Fig. 3.** Weekly body weight of AOM/DSS-treated mice.

580 **Fig. 4.** Effects of EAE and PPE on the mRNA levels of IL-1β, IL-6, and TNF-α in the
581 colonic mucosa of AOM/DSS-treated mice. Data are shown as the mean ± SE. The
582 amount of IL-1β, IL-6, and TNF-α mRNA expression was normalized to that of β-actin.
583 Student's *t*-test was used to test the mean difference between the treatment group and
584 control group. * Indicates statistically significant differences from the positive control
585 group ($p < 0.05$, n = 3).

586 **Fig. 1a**

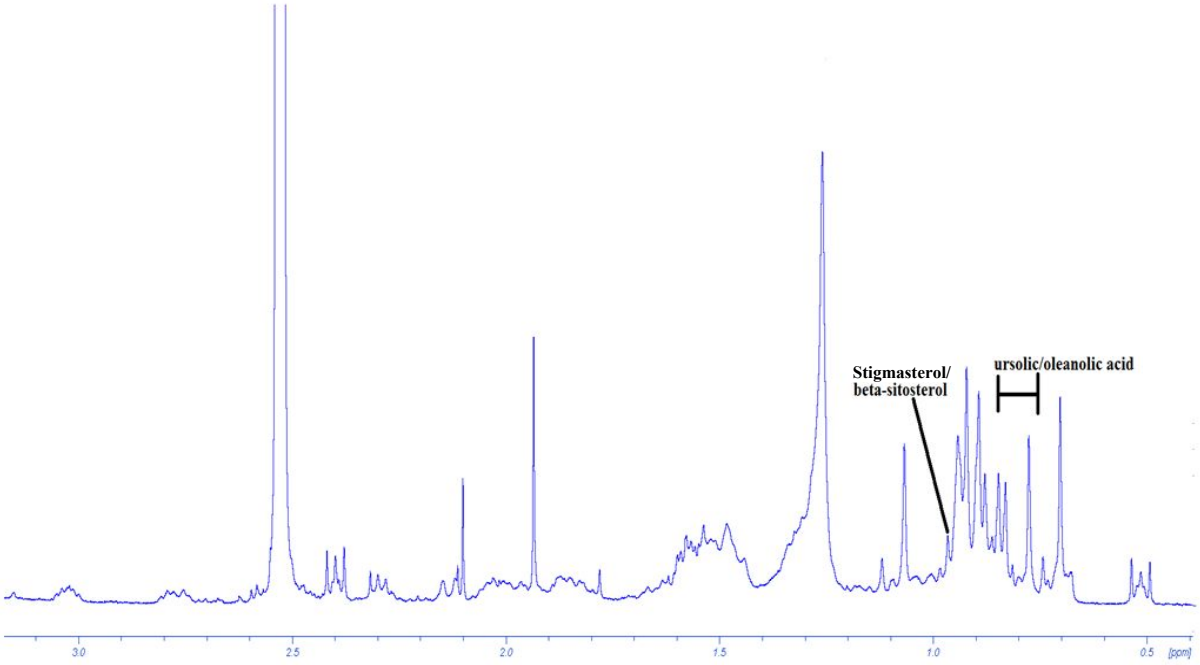
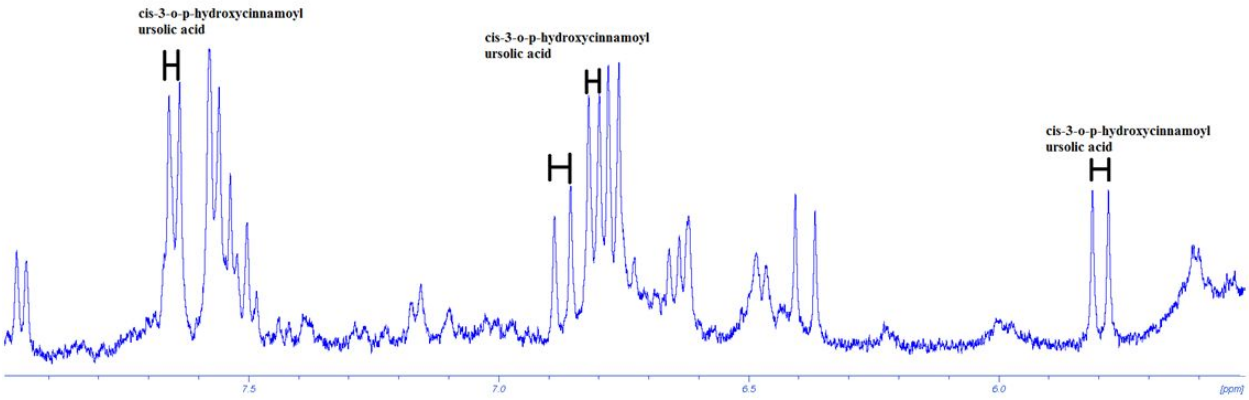
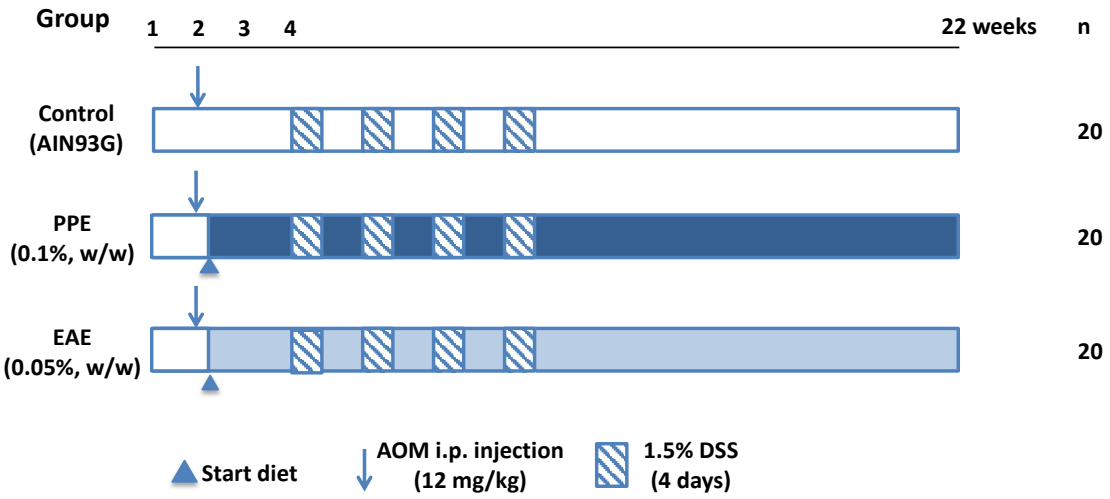


Fig. 1b



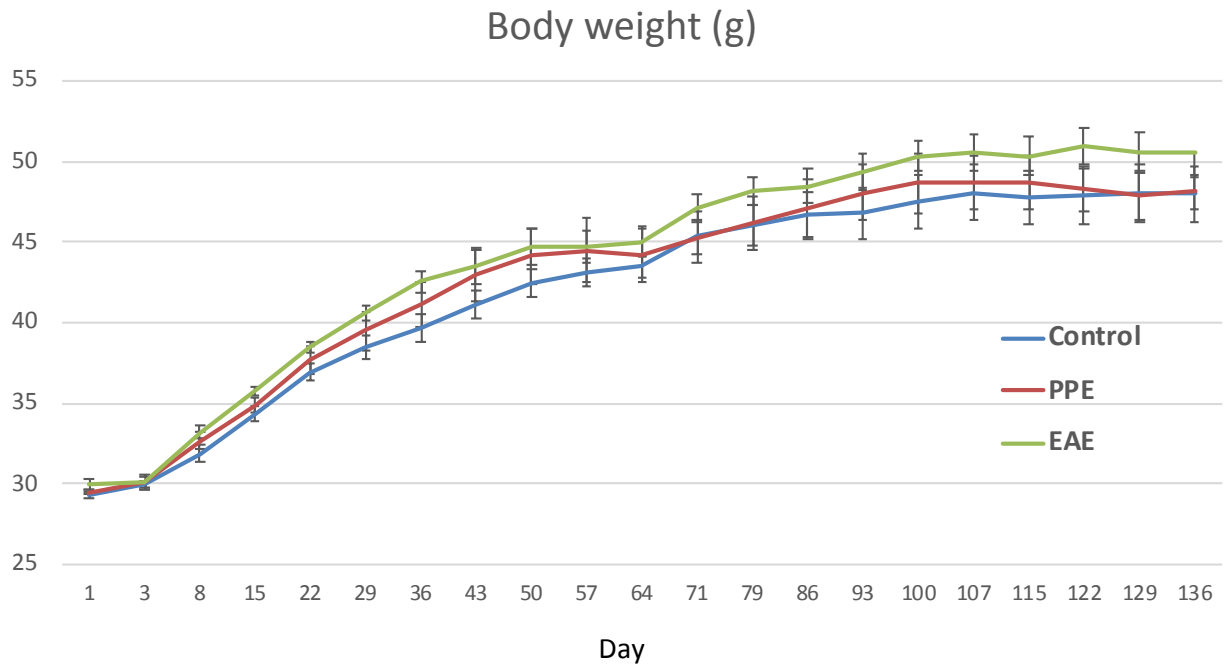
587

588 **Fig. 2**


Group
1
2
3
4
22 weeks
n
Control
(AIN93G)
20
PPE
(0.1%, w/w)
20
EAE
(0.05%, w/w)
20
Start diet
AOM i.p. injection
(12 mg/kg)
1.5% DSS
(4 days)

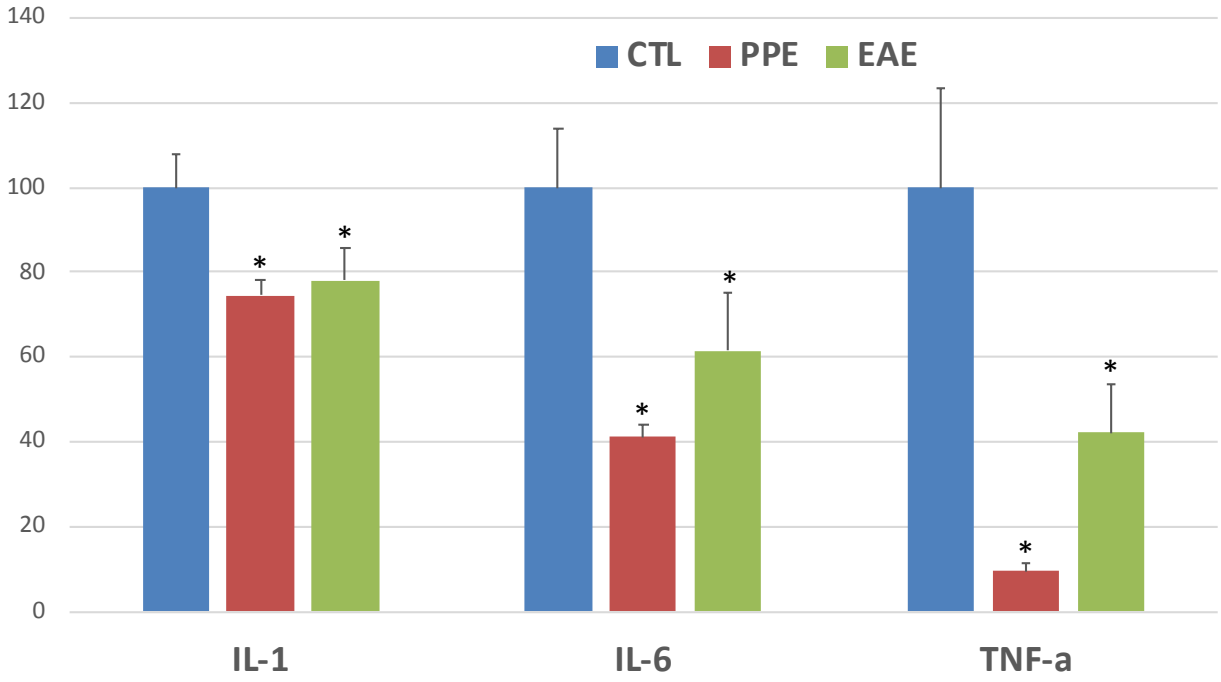
589

590 **Fig. 3**


Body weight (g)
55
50
45
40
35
30
25
1
3
8
15
22
29
36
43
50
57
64
71
79
86
93
100
107
115
122
129
136
Day
Control
PPE
EAE

591

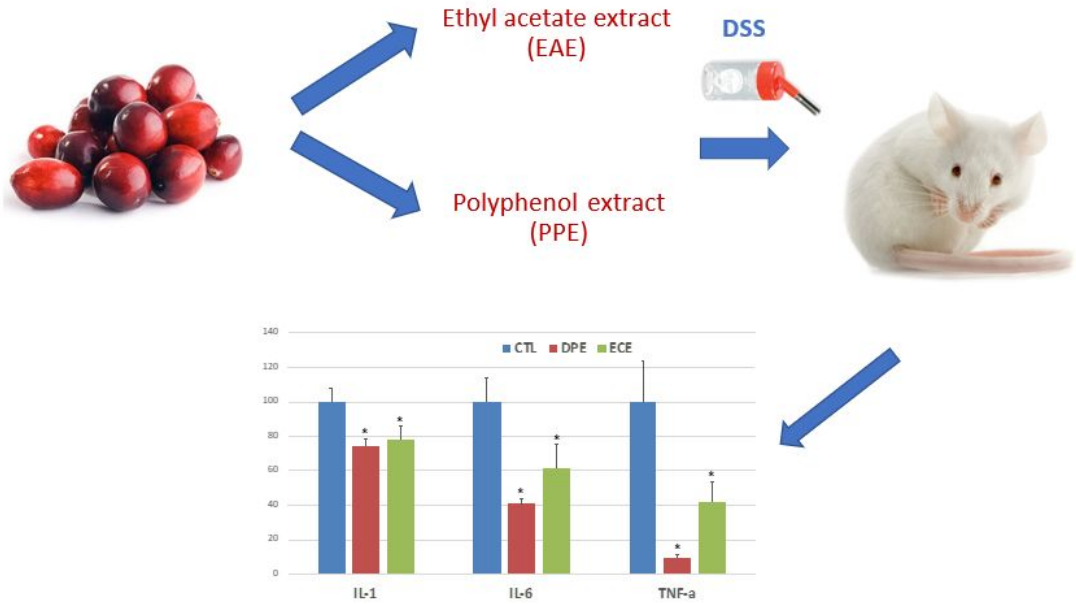
592 **Fig. 4**



593

594

595 **TOC Graphic**



596

597 In this study, we characterized two novel cranberry extracts and determined their

598 chemopreventive effects on colon tumorigenesis in mice.