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Pomegranate prevents binge alcohol-induced gut leakiness and hepatic inflammation by suppressing oxidative and nitrative stress

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ABSTRACT

Alcoholic liver disease (ALD) is a major chronic liver disease worldwide and can range from simple steatosis, inflammation to fibrosis/cirrhosis possibly through leaky gut and systemic endotoxemia. We investigated whether pomegranate (POM) protects against binge alcohol-induced gut leakiness, endotoxemia, and inflammatory liver damage. After POM pretreatment for 10 days, rats were exposed to 3 oral doses of binge alcohol (5 g/kg/dose) or dextrose (as control) at 12-h intervals. Binge alcohol exposure induced leaky gut with significantly elevated plasma endotoxin and inflammatory fatty liver by increasing the levels of oxidative and nitrative stress marker proteins such as ethanol-inducible CYP2E1, inducible nitric oxide synthase, and nitrated proteins in the small intestine and liver. POM pretreatment significantly reduced the alcohol-induced gut barrier dysfunction, plasma endotoxin and inflammatory liver disease by inhibiting the elevated oxidative and nitrative stress marker proteins. POM pretreatment significantly restored the levels of intestinal tight junction (TJ) proteins such as ZO-1, occludin, claudin-1, and claundin-3 markedly diminished after alcohol-exposure. In addition, the levels of gut adherent junction (AJ) proteins (e.g., β-catenin and E-cadherin) and desmosome plakoglobin along with associated protein α-tubulin were clearly decreased in binge alcohol-exposed rats but restored to basal levels in POM-pretreated rats. Immunoprecipitation followed by immunoblot analyses revealed that intestinal claudin-1 protein was nitrated and ubiquitinated in alcohol-exposed rats, whereas these modifications were significantly blocked by POM pretreatment. These results showed for the first time that POM can prevent alcohol-induced gut leakiness and inflammatory liver injury by suppressing oxidative and nitrative stress.

1. Introduction

Chronic and/or binge alcohol (ethanol) consumption is known to cause alcoholic liver disease (ALD), which is one of the leading causes of hepatic diseases and liver-related death worldwide [1–3]. The clinical spectrum of ALD includes alcoholic fatty liver (steatosis), steatohepatitis (inflammation), fibrosis/cirrhosis, and increased risk of hepatocellular carcinoma [1,3]. Alcohol can directly affect the physiological functions of different liver cells including hepatocytes, Kupffer cells and stellate cells. In addition, alcohol can indirectly stimulate the liver injury by changing gut microbiome accompanied with the impaired gut barrier function, leading to increased leaky gut and translocation of intestinal bacteria with elevated plasma levels of bacterial endotoxin (e.g., lipopolysaccharide, LPS) [4–6]. Many recent reports showed that circulating LPS can reach the liver via portal vein and interact with hepatic toll-like receptor-4 (TLR4) to initiate the inflammatory cascades for the accelerated development of ALD [4–6]. Alcohol-induced gut leakiness is critically important in the progression to more severe ALD such as fibrosis/cirrhosis since the elevated levels of endotoxin are correlated with the development of liver cirrhosis [7].

Under the influence of ethanol, the expression and the levels of many genes and proteins are altered through transcriptional and post-transcriptional mechanisms, including epigenetics [8]. The ethanol-inducible cytochrome P450-2E1 (CYP2E1) is one of the well-characterized proteins that are induced and/or activated by chronic and/or binge alcohol intake [9]. Under relative low amounts of alcohol, hepatic and

Abbreviations: POM, pomegranate; ALD, alcoholic liver disease; TJ, tight junction; AJ, adherent junction; CYP2E1, ethanol-inducible cytochrome P450-2E1; iNOS, inducible nitric oxide synthase; nitroxidative stress, oxidative and nitrative stress; KI mice, knock-in mice; KO mice, knock-out mice; UA, urolithin A; EA, Ellagic acid; CMZ, chlormethiazole; BAC, blood alcohol concentration; ER, endoplasmic reticulum; TLR4, toll-like receptor-4; PERK, protein kinase-like endoplasmic reticulum kinase; eIF2α, eukaryotic translation initiation factor-2α; PDI, protein disulfide isomerase; PTM, post-translational modification; ROS, reactive oxygen species; TEER, trans-epithelial electrical resistance; FITC-D4, FITC-labeled 4-kDa dextran; IP, immunoprecipitation; JNK, c-Jun *N*-terminal protein kinase; LPS, lipopolysaccharide

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gastric alcohol dehydrogenases usually metabolize alcohol. However, after long-term alcohol drinking or under high blood alcohol concentration (BAC), CYP2E1 becomes involved in the ethanol metabolism (K_m about ~ 10 mM ethanol), resulting in the production of acetaldehyde and reactive oxygen species (ROS), which can negatively affect many cellular components and can cause cell death [10]. Death of parenchymal cells can activate liver-resident inflammatory cells such as Kupffer cells or infiltrated neutrophils that can significantly promote inflammatory ALD and severe disease [2,9,11]. Indeed, the specific involvement of CYP2E1 in ALD has been demonstrated by utilizing CYP2E1 overexpressed transgenic (Tg), knock-out (KO), and knock-in (KI) mice [2,12,13]. Recently, our group reported that CYP2E1 is also involved in n-6 fatty acid containing western-style high-fat diet induced non-alcoholic steatohepatitis [14] and aging-related liver and kidney damage [15,16] through increasing the oxidative and nitrative (nitroxidative) stress. Furthermore, both intestinal and hepatic CYP2E1 induced (i.e., via protein stabilization) by chronic alcohol drinking [17] or binge alcohol exposure seems critically important in promoting alcohol-mediated increased nitroxidative stress, gut leakage, and endotoxemia, contributing to apoptosis of hepatocytes and steatohepatitis [18,19].

Pomegranate (POM, *Punica granatum*) is one of the edible fruits and mainly grown in middle east, India, southern Asia and Mediterranean Europe, as well as in warm climate areas of the world including Americas [20]. It is a rich source of polyphenolic compounds like anthocyanidins (delphinidin, cyanidin and pelargonidin) and hydrolysable tannins (such as punicalagin, pedunculagin, punicalin, gallagic, ellagic acid esters of glucose) [21] against a variety of diseases including cancer [22], cardiovascular disorders [23], diabetes [24], Alzheimer's disease [25], aging [26], and AIDS [27]. Recently, it was shown to prevent development of non-alcoholic fatty liver disease in rats [28] and ethanol-induced toxicity in HepG2 human hepatoma cells [29]. Acute and sub-chronic toxicity studies with rodents revealed that oral administration of its extracts for up to ~ 4330 mg/kg/day is considered safe with no observable adverse effects [30–32].

Based on our recent results on alcohol-induced gut leakiness [18,19], we hypothesized that POM, with various antioxidants and anti-inflammatory ingredients, can also prevent alcohol-mediated oxidative stress critically important in promoting gut leakiness and inflammatory liver injury. In this present study, we have evaluated the beneficial effects of POM on alcohol-induced gut leakiness and inflammatory liver injury in rats and investigated the protective mechanisms in rats as well as cultured hepatocytes and T84 colonic cells.

2. Material and methods

2.1. Materials

All chemicals, urolithin A (UA) and ellagic acid (EA) used in this study were from Sigma Chemical (St. Louis, MO, USA). POM extracts (Bulk Supplements; 40% EA) were obtained from Amazon. T84 human colonic cell line (CCL-248) and AML12 normal mouse liver cells (CRL-2254) were purchased from the ATCC (American Type Culture Cells, Manassas, VA). Other materials not described here were the highest grades available and/or the same, as recently described [19,33,34].

2.2. Animal treatments

All animal experimental procedures were carried out by following the National Institutes of Health (NIH) guidelines for small animal experiments and approved by the NIAAA Institutional Animal Care and Use Committee. All rats were maintained under controlled lighting (12-h light/dark cycle) with food and water provided ad libitum. Age-matched 7-weeks old female Fischer 344 wild-type (WT) rats were orally administered a daily dose of 600 mg POM/kg, based on the safety and effective dosages of POM extracts [30–32,35,36]. Control rats were orally administrated a vehicle (water). After POM pretreatment for 10 days, some rats (n ≥ 4/group) were exposed to 3 oral doses of binge alcohol (5 g/kg/dose) or dextrose (as control) at 12-h intervals and euthanized 1-h after the last ethanol dose.

2.3. Culture of liver cells and colon cells

The AML12 liver cells were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic solution, 0.1 μM dexamethasone, and insulin-transferrin-selenium (Invitrogen). Cells were grown at 37 °C under 5% CO_2. AML12 cells were treated with 100 mM ethanol in the absence or presence of the selective inhibitor of UA (15 μM), EA (100 μM), or chlormethiazole (CMZ, 15 μM) for 24 h. T84 colon cells were grown in a humidified incubator under 95% air and 5% CO_2 at 37 °C in Ham's F-12 medium, supplemented with 10% FBS, 1% antibiotic-antimycotic solution. Confluent monolayers appeared 6–14 day after plating. T84 monolayer cells were exposed to 40 mM ethanol in the absence or presence of the selective inhibitor of UA (15 μM), EA (100 μM), or CMZ (15 μM) for 8 h prior to harvest for subsequent analyses.

2.4. Histological analysis and serum ALT measurement

Rats were briefly sedated to carbon dioxide gas followed by decapitation and immediate collection of trunk blood, small intestine, and liver from each rat. In this study, part of the largest liver lobe or small intestine from each rat exposed to POM pretreatment or control with or without binge ethanol exposure was fixed in neutral formalin. Paraffin-embedded blocks of formalin-fixed individual liver or small intestine sections were cut at 4 μm, stained with hematoxylin/eosin (H/E) by American Histolabs, Inc. (Gaithersburg, MD). To further support fat accumulation, frozen liver samples embedded in optimal cutting temperature compound were cut (10 μm) and stained with Oil Red O by American HistoLabs, Inc. The plasma ALT level in each rat was determined by using the standard end-point colorimetric assay kit (TECO Diagnostics, Anaheim, CA), as described [34].

2.5. Endotoxin assay

Plasma endotoxin levels were determined using the commercially available endpoint LAL Chromogenic Endotoxin Quantitation Kit with a concentration range of 0.015–1.2 EU/mL (Thermo Fisher Scientific, Waltham, MA), as described [19].

2.6. Triglyceride determination in liver

The amounts of hepatic triglyceride (TG) were assessed by using a commercially available kit (Thermo Fisher Scientific) [19,34].

2.7. Determination of the levels of hydrogen peroxide, nitrate/nitrite, lipid peroxidation and superoxide dismutase (SOD) activity in liver extracts

The levels of hepatic hydrogen peroxide, nitrate/nitrite, lipid peroxidation and superoxide dismutase (SOD) activity were assessed by using commercially available kits, respectively (Cayman; Abcam; Oxford Biomedical Research; Cayman).

2.8. Determination of plasma ROS levels

The amounts of plasma ROS were determined by visualization with 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Thermo Fisher Scientific). Following incubation with DCFH-DA at 37 °C for 20 min, the DCFH-DA fluorescence was then determined by the method, as recently described [33].

2.9. CYP2E1 activity assay

CYP2E1 activities in the liver homogenates were measured, as described previously [34].

2.10. Enzyme-linked immunosorbent assay (ELISA)

The liver lysates prepared from individual POM-pretreated or control rats in the absence or presence of binge ethanol exposure were analyzed by using the respective ELISA kit for TNF-α or MCP-1 (Abcam, Cambridge, United Kingdom) by following the manufacturers' protocols. The protein concentration was measured with the BCA reagent (BioRad, Hercules, CA) to use the equal amounts of protein for the ELISA. Duplicate samples from each lysate (n = 4/group) were used for ELISA, which was repeated twice.

2.11. Immunoblot analysis and immunoprecipitation

Parts of liver tissue and small intestine from each rat were homogenized with RIPA buffer. Same amounts (50 μg protein equally pooled from different rat samples within the same group) were separated by SDS/PAGE and transferred to nitrocellulose membranes. These membranes were incubated with the respective rabbit polyclonal antibody against CYP2E1 (1:5000 dilution; Abcam), p-JNK (1:1000 dilution; Cell Signaling), 3-NT (1:5000 dilution; Abcam), iNOS (1:5000 dilution; Abcam), P-PERK (1:1000 dilution; Santa Cruz Biotechnology), p-eIF2α (1:1000 dilution; Santa Cruz Biotechnology), PDI (1:1000 dilution; Santa Cruz Biotechnology), cleaved caspase-3 (1:1000 dilution; Cell Signaling), ZO-1 (1:5000 dilution; Abcam), claudin-1 (1:1000 dilution; Thermo Fisher), cladin-4 (1:1000 dilution; Thermo Fisher), or occludin (1:5000 dilution; Abcam). Respective mouse monoclonal antibody against JNK (1:1000 dilution; Cell Signaling), Bax (1:1000 dilution; Santa Cruz Biotechnology), β-catenin (1:1000 dilution; Santa Cruz Biotechnology), E-cadherin (1:1000 dilution; Santa Cruz Biotechnology), Plakoglobin (1:1000 dilution; Santa Cruz Biotechnology), α-tubulin (1:1000 dilution; Santa Cruz Biotechnology), ubiquitin (1:1000 dilution; Cell Signaling), or β-actin (1:10,000 dilution; Santa Cruz Biotechnology) was also used to detect the specific antigen target, as indicated. After washing the nitrocellulose membranes with PBS three times at 10 min intervals, horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology) was used as the secondary antibody at 1:5000 dilution. Relative protein images were determined by using HRP-conjugated secondary antibodies and ECL substrates (Thermo Fishers). The intensities of the immuno-reactive bands were quantified by densitometry using ImageJ software (National Institutes of Health).

Immunoprecipitation of small intestinal proteins was carried out, as described previously [19,34]. Briefly, 0.5 mg proteins of small intestinal proteins equally pooled from individual control rats or POM-pretreated groups with or without ethanol exposure were incubated with the specific antibody to claudin-1 protein followed by addition of protein A/G-Sepharose for 4 h at 4 °C with constant head-to-tail rotations. Sepharose beads were pre-blocked with 2% BSA in PBS before being used for immunoprecipitation. After incubation, proteins bound to Sepharose beads were washed twice with the lysis buffer, four times with ice-cold PBS and mixed with 2x SDS-PAGE gel loading buffer. The immunoprecipitated proteins were resolved by SDS-PAGE for subsequent immunoblot analyses using the antibody to each target protein, as indicated in the actual figure.

2.12. Immunofluorescence and confocal microscopy

For detecting CYP2E1 and ZO-1 by immunofluorescence, AML12 liver and T84 colonic cells were initially plated onto chamber slides, as described previously [19]. Briefly, the cells were fixed for 30 min at room temperature with 4% paraformaldehyde. After removal of the blocking solution, the cells were incubated with the specific antibody, as indicated, at 4 °C overnight. Anti-CYP2E1 or Anti-ZO-1 antibody was used at 1:300 dilution. For immunofluorescence detection, the cells were incubated with Alexa Fluor 488-labeled anti-rabbit secondary antibody (Thermo Fisher Scientific). For nuclear staining, the cells were counter-stained with 1 mg/mL 4′,6′-diamino-2-phenylindole (DAPI) for 5 min. The cells were washed and fixed with VECTASHIELD mounting solution (Vector Laboratories, Burlingame, CA). Fluorescence images were collected by using a confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.13. TUNEL assay

Small intestine and liver specimens were fixed overnight in 10% buffered formalin and embedded in paraffin. The ApopTag peroxidase in situ apoptosis detection kit (Millipore, Billerica, MA) was used to identify apoptotic enterocytes or hepatocytes by the TUNEL analysis, as recently described [19].

2.14. RNA extraction and real-time analysis

Total RNA was extracted from the individual rat livers using Trizol (Thermo Fisher Scientific), with overnight precipitation at 20 °C to increase the yield of RNA. For real-time analysis, cDNA was transcribed from a total of 600 ng of DNase I–treated RNA using the cDNA reverse-transcription kit and random primers (Thermo). Real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed using a Mx3000p System. To determine relative mRNA expression, housekeeping gene (β-actin) and apoptosis marker gene with SYBR green I (SYBR Advantage qPCR Premix) were used.

2.15. Trans-epithelial electrical resistance and FITC-D4 permeability analysis

Colonic T84 cells were grown in transwell inserts with a surface area of 0.33 cm^2 and 0.4 μm pore size (Thermo Fisher Scientific), as recently described [19]. The rates of trans-epithelial electrical resistance (TEER) of monolayer cells were measured with an epithelial volt-ohm-meter in each insert and multiplied by the membrane surface area (0.33 cm^2), corrected by subtracting background resistance of the blank membrane (i.e., no cells). Data were collected from triplicates inserts per treatment in two independent experiments and expressed as percentage of basal TEER (i.e., 600 Ω cm^2) obtained before treatment. At the end of TEER measurements, FITC-labeled 4-kDa dextran (1 mg/mL FITC-D4; Sigma) was added to the apical side of cells. Monolayer permeability was assessed by measuring the fluorescence of FITC-D4 in the basal medium compartment spectrophotometrically using a microplate reader at excitation and emission spectra of 485 and 540 nm, respectively. Data were reported as relative fluorescent units (% baseline).

2.16. Statistical analysis and other methods

Statistical significance was determined using one-way ANOVA and Dunnet's multiple comparison post-tests were used to compare the means of different groups. All the experiments were repeated at least twice unless otherwise stated. Data were presented as mean ± SD. Different letters in actual figures stand for significant difference between various treatments at $p < 0.05$ by one-way ANOVA. GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA) was used for analysis. Other methods not described in this study were the same as recently reported [19,33,34].

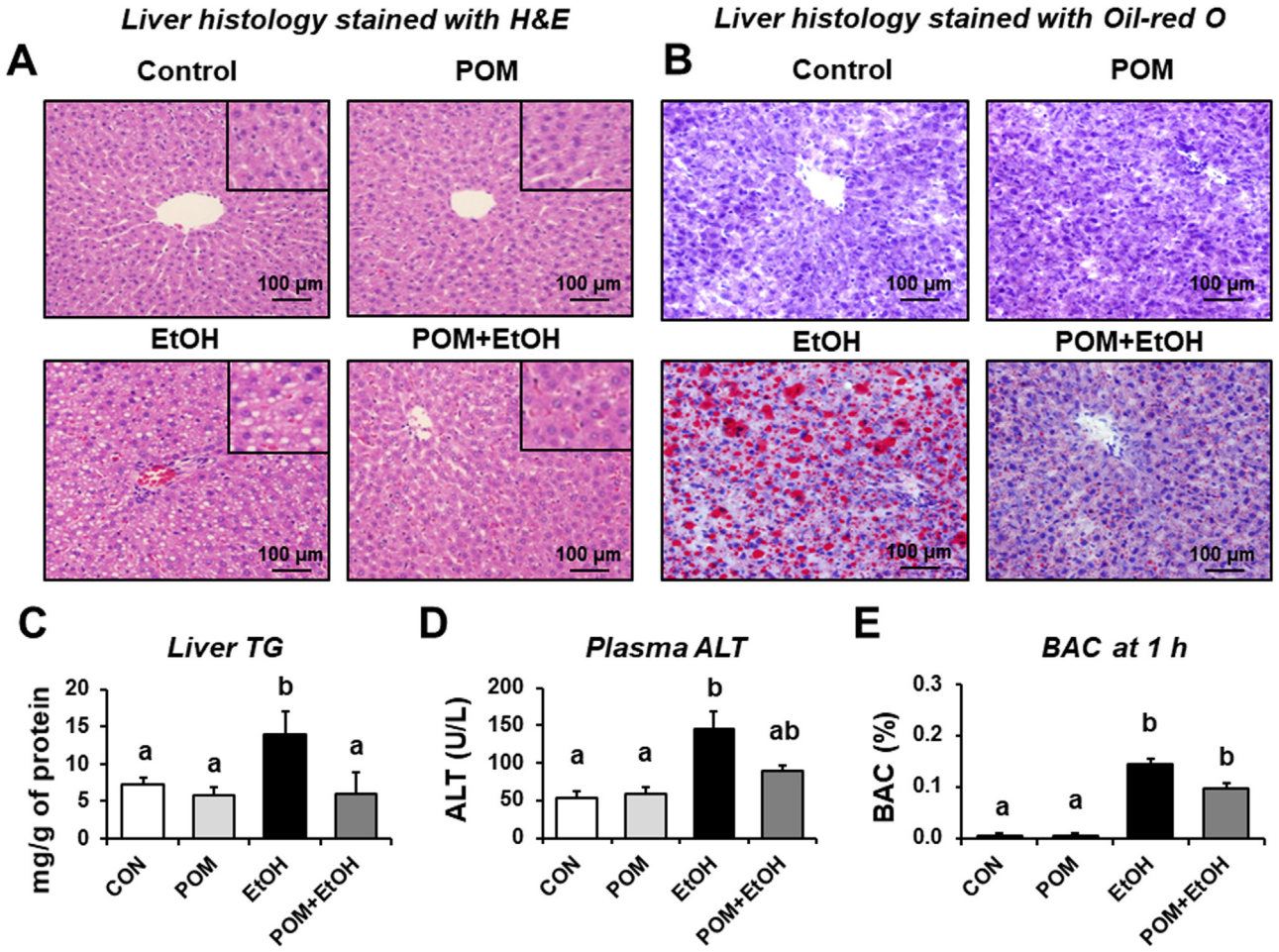

Fig. 1. POM prevented binge alcohol-induced fatty liver and injury in rats. (A and B) Representative H/E or Oil Red O staining of formalin-fixed or frozen liver sections for control (CON), pomegranate (POM), ethanol (EtOH), or POM + EtOH-exposed rats. Inset within each figure represents a higher magnification image. The levels of (C) hepatic triglyceride (TG), (D) plasma ALT, and (E) BAC. Data represent means ± SD. Different letters stand for significant difference between various treatments at $p < 0.05$ by one-way ANOVA. Labeled characters without a common letter represent significant differences from the other group(s).

3. Results

3.1. Pomegranate decreased the alcohol-mediated hepatic fat accumulation, plasma ALT, and hepatic triglyceride levels

Based on the safety data [30–32] and previous reports by other scientists [35,36], rats were treated with oral gavages of POM (600 mg/kg/day) to determine its protective effects against alcoholic fatty liver. Our results showed that there was no significant difference in the initial mean body weights of rats among the different groups (i.e., no change in body weight gains among different groups, data not shown). Histological analyses with H/E and oil red O staining showed slightly but significantly elevated fat accumulation in the liver after binge alcohol-exposure compared to that of dextrose-control (Fig. 1A and B, respectively). In contrast, POM pretreatment reduced the hepatic lipid droplets elevated after binge alcohol-exposure (Fig. 1A and B). The liver H/E-stained histology data are consistent with the significant changes in the levels of hepatic triglyceride (TG) (Fig. 1C) and plasma ALT (Fig. 1D), where whereas POM pretreatment decreased these increments. However, POM pretreatment did not significantly reduce the elevated BAC at 1 h after the last oral ethanol administration (Fig. 1E). These results suggest that the effects of POM are likely resulted from modulating other factors but not by affecting the BAC.

3.2. Pomegranate decreased the hepatic oxidative stress, ER stress, and apoptosis marker proteins in binge alcohol-exposed rats

The CYP2E1 is one of the major contributors in alcohol-induced oxidant stress, contributing to alcohol-induced liver injury [9,12,13,18]. Our data showed that the levels of hepatic CYP2E1 protein expression and activity were significantly increased in alcohol-exposed rats (Fig. 2A and Supplemental Fig. 1). In contrast, POM reduced the alcohol-mediated increase in the levels of CYP2E1 protein expression and activity. In addition, the elevated amounts of inducible nitric oxide synthase (iNOS) protein following alcohol exposure were significantly decreased in POM-pretreated rats (Fig. 2A). The elevated amounts of nitrated proteins reflected by 3-NT immunoreactivity were significantly decreased in POM-pretreated rats compared to the corresponding alcohol-exposed rats (Fig. 2A). The increased amounts of hepatic hydrogen peroxide and nitrate/nitrite were significantly decreased in POM-pretreated rats compared to the corresponding alcohol-exposed rats (Supplemental Fig. 2A and B). Additionally, the decreased hepatic superoxide dismutase (SOD) activities in alcohol-exposed rats were restored in POM-pretreated rats (Supplemental Fig. 2C).

CYP2E1 induction or activation is usually associated with increased production of reactive oxygen species (ROS). Elevated ROS production is likely to increase lipid peroxidation products. Our results showed that the elevated levels of plasma ROS and lipid peroxidation (MDA + HNE) in alcohol-exposed rats were significantly decreased in POM-pretreated rats (Supplemental Fig. 3).

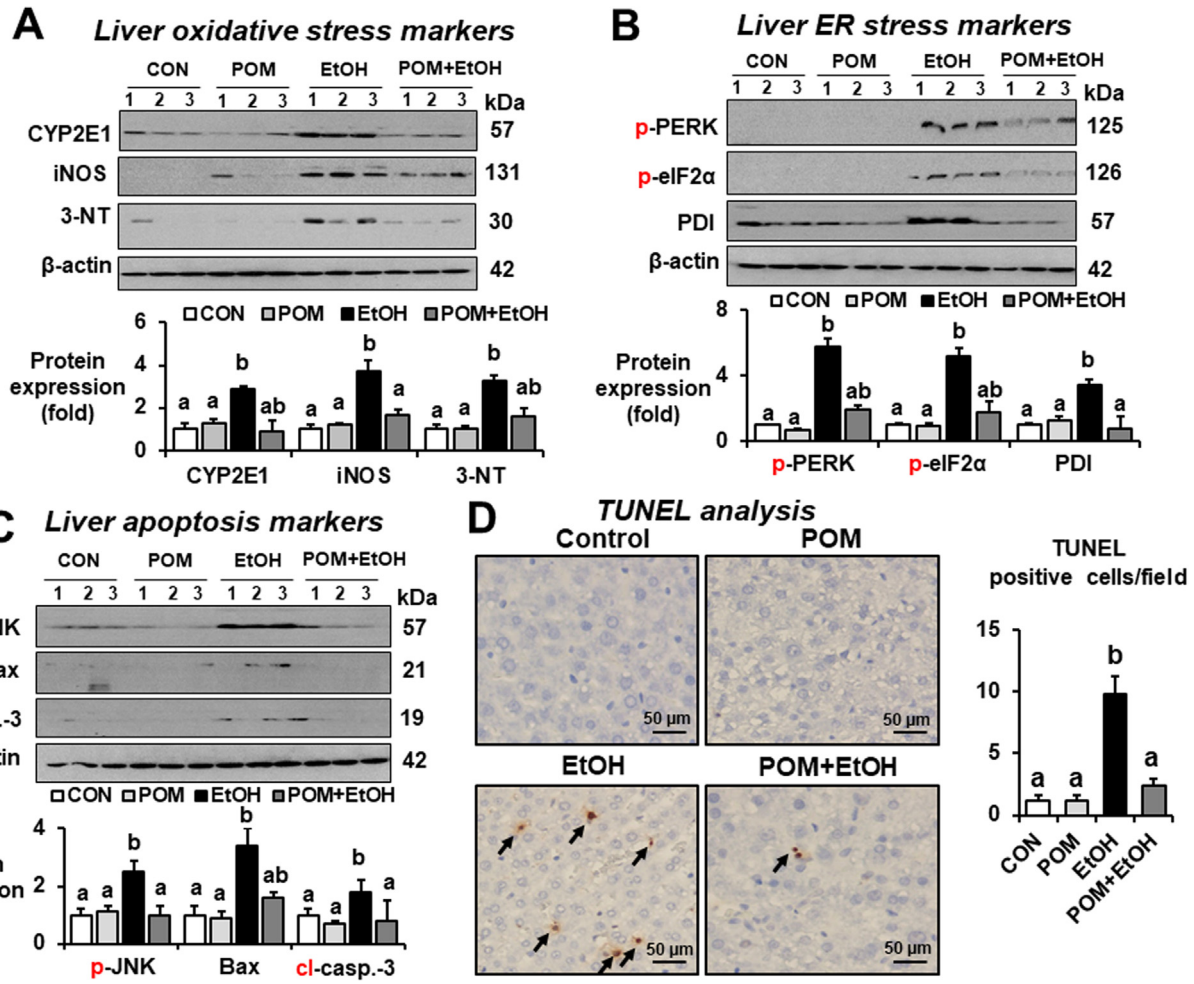

Fig. 2. POM prevented hepatic oxidative stress, ER stress, and apoptosis marker proteins in binge alcohol-exposed rats. (A) The levels of hepatic CYP2E1, iNOS, and nitrated proteins detected by anti-3-NT antibody, (B) liver ER stress marker proteins p-PERK, p-eIF2α, and PDI, (C) liver apoptosis marker proteins p-JNK, Bax, and cleaved-caspase-3 and (D) TUNEL analyses in the indicated groups are presented. The arrows represent TUNEL positive cells. Densitometric quantitation of each immunoreactive protein relative to β-actin, used as a loading control, is shown. Data represent means ± SD. Significant difference between various treatments was determined by one-way ANOVA. Labeled characters without a common letter represent significant differences from the other group(s).

To determine whether POM-mediated prevention of alcohol-induced hepatic injury is promoted through decreased ER stress, we evaluated the hepatic levels of ER stress markers such as phospho-protein kinase-like endoplasmic reticulum kinase (PERK), phospho-eukaryotic translation initiation factor-2α (eIF2α), and protein disulfide isomerase (PDI). Alcohol-exposure increased the levels of hepatic p-PERK, p-eIF2α, and PDI. POM pre-treatment significantly attenuated ER stress as indicated by the significant reduction of the increased amounts of p-PERK, p-eIF2α, and PDI in alcohol-exposed rats (Fig. 2B).

To further test whether POM-mediated prevention of alcohol-induced hepatic injury is promoted through decreased levels of hepatic apoptosis, we determined the expression of apoptosis-related protein markers. We observed that binge alcohol increased the amounts of hepatic p-JNK, Bax, and cleaved caspase-3 proteins and that POM pretreatment significantly decreased the alcohol-mediated increments of these proteins (Fig. 2C). Furthermore, TUNEL analyses followed by quantitation revealed that ethanol-exposure significantly increased apoptosis of hepatocytes while this elevation was markedly reduced by POM pretreatment (Fig. 2D). Taken together, these results revealed that POM significantly attenuated oxidative/nitrative stress, ER stress, and hepatic apoptosis in alcohol-exposed rats.

3.3. Pomegranate decreased the hepatic inflammation response in binge alcohol-exposed rats

Binge alcohol consumption can stimulate gut leakiness, contributing to increased hepatic steatosis and inflammation [18,19]. Therefore, we evaluated the levels of inflammation marker proteins. ELISA analysis showed that alcohol exposure elevated the hepatic contents of TNF-α and MCP-1, both of which were significantly decreased by POM-pretreatment (Fig. 3A and B). In addition, immunoblot analysis showed that hepatic levels of inflammation-associated proteins TLR-4, TNF-α, IL-1β and MCP-1 were significantly elevated in alcohol-induced liver injury but decreased in POM-pretreated rats (Fig. 3C). These results indicated that POM significantly decreased the expression of hepatic inflammation-associated cytokine/chemokine in alcohol-exposed rats.

3.4. Urolithin A and ellagic acid decreased the oxidative stress and inflammation related genes in AML12 liver cells

EA and UA are natural polyphenols contained in POM with anti-proliferative and antioxidant properties [20–29]. Based on the prevention of alcohol-induced liver injury by POM (Figs. 1–3), we further studied the mechanisms of the beneficial effects by its individual components UA and EA on oxidative stress and inflammation-related mRNAs upregulated after binge alcohol exposure. Based on the

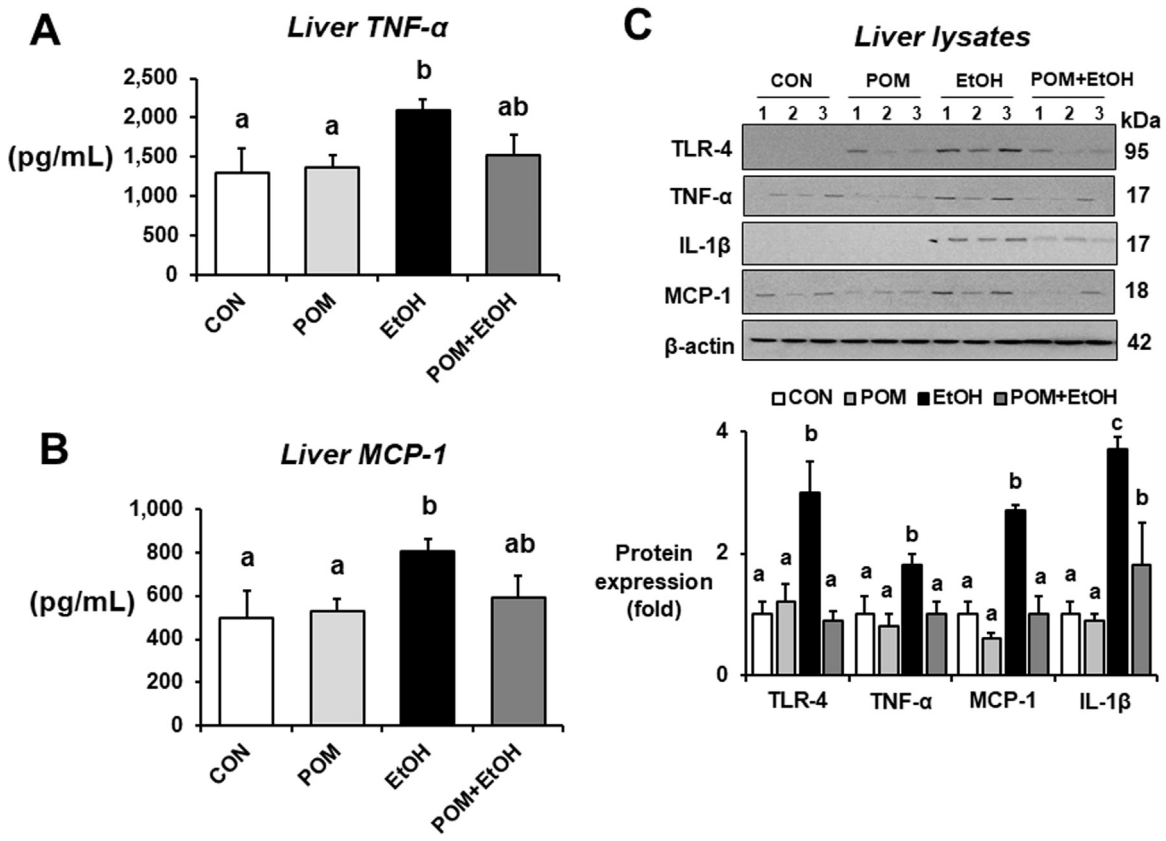

Fig. 3. POM prevented increased hepatic inflammation marker proteins in binge alcohol exposed rats. (A and B) ELISA results for TNF-α and MCP-1 in the liver lysates from control (CON), pomegranate (POM), ethanol (EtOH), or POM + EtOH-exposed rats are presented. (C) The levels of hepatic TLR4 and inflammation-associated proteins (TNF-α, IL-1β, and MCP-1) in the indicated groups are presented. Significant difference between various treatments was determined by one-way ANOVA. Labeled characters without a common letter represent significant differences from the other group(s).

previous studies with UA [37] and EA [38], AML12 normal liver cells were treated with 100 mM ethanol in the absence or presence of UA, EA, or CMZ for 24 h. Confocal microscopy image showed that binge alcohol significantly increased the CYP2E1 expression. However, pretreatment with UA or EA at the indicated concentration decreased the CYP2E1 expression in ethanol-exposed AML12 cells, as comparable to that treated with CMZ, a specific inhibitor of CYP2E1 transcription (Fig. 4A). Immunoblot analysis demonstrated that pretreatment with UA or EA significantly decreased the amounts of CYP2E1, iNOS, and nitrated proteins in alcohol-exposed AML12 liver cells (Fig. 4B). Furthermore, real-time RT-PCR analysis revealed that UA or EA pretreatment significantly suppressed the elevated mRNA levels of inflammation markers NLRP3, IL-1β, and TNF-α in alcohol-exposed AML12 cells (Fig. 4C).

3.5. Pomegranate decreased the plasma endotoxin and intestinal TNF-α protein in binge alcohol-exposed rats

Since hepatic inflammation could be indirectly stimulated by increased leaky gut and endotoxemia [4–7], the levels of plasma endotoxin and intestinal TNF-α were measured to determine whether the POM-mediated prevention of alcoholic liver injury could result from the inhibition of endotoxemia. H/E stained histology slides revealed disorganization and detachment of many intestinal epithelial cells with abnormal shapes in alcohol-exposed rats compared to those of dextrose-controls (Fig. 5A). POM pretreatment prevented these abnormal shapes and restored their contours. Morphometric analysis of H/E stained sections showed that alcohol-exposed rats had significantly shorter villus height compared to control rats (Fig. 5B). Consistently, binge alcohol exposure markedly elevated the plasma endotoxin concentration compared to the control rats, whereas POM pretreatment significantly prevented this increase (Fig. 5C). Additionally, elevated levels of intestinal TNF-α and MCP-1 in alcohol-exposed rats were significantly decreased by POM pretreatment (Fig. 5D and E).

3.6. Pomegranate reduced the intestinal oxidative stress, nitration, acetylation, and ubiquitination in binge alcohol-exposed rats

Increased nitroxidative stress with elevated levels of CYP2E1, iNOS and nitrated proteins are the major factors in alcohol-mediated intestinal barrier dysfunction [18,19]. Binge alcohol markedly elevated the amounts of intestinal CYP2E1 and iNOS whereas POM pretreatment significantly prevented the increments by binge alcohol (Fig. 6A).

Alcohol exposure was recently shown to promote post-translational protein modifications (PTMs) including methylation, nitration, phosphorylation, and acetylation [8,10]. Immunoblot results showed that binge alcohol markedly elevated the intestinal protein nitration, lysine acetylation and ubiquitin-conjugated proteins. In contrast, POM pretreatment significantly inhibited the alcohol-mediated increments of these modified proteins (Fig. 6B–D). Therefore, POM pretreatment prevented alcohol-mediated gut leakiness by at least partially suppressing the CYP2E1, iNOS, elevated nitration, acetylation, and ubiquitination-conjugation of many intestinal proteins in alcohol-exposed rats.

3.7. Pomegranate restored the altered levels of intestinal tight junctions, adherent junctions, and apoptosis marker proteins in binge alcohol-exposed rats

Gut leakiness and endotoxemia can be resulted from decreased

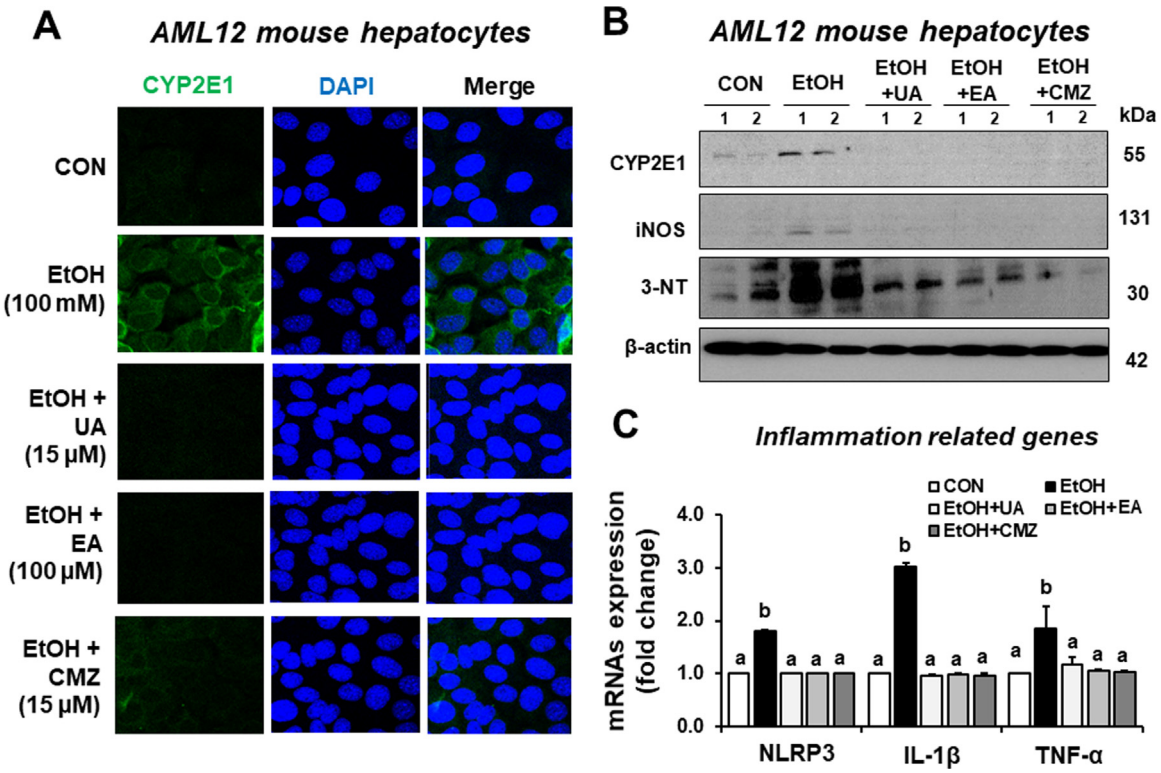

Fig. 4. Urolithin A and Ellagic acid suppressed the CYP2E1 amount, oxidative stress and inflammation maker proteins in alcohol-exposed AML12 liver cells. AML12 liver cells were treated with culture media (CON) or 100 mM ethanol (EtOH) in the absence or presence of 15 μM Urolithin A (UA), 100 μM Ellagic acid (EA), or 15 μM chlormethiazole (CMZ) for 24 h, as indicated. (A) Confocal image showing the reduction of CYP2E1 in ethanol-exposed AML12 cells with or without UA, EA, or the CYP2E1 inhibitor CMZ. Cell nuclei were counter-stained with DAPI. (B) Immunoblot analyses for CYP2E1, iNOS, and nitrated proteins detected by anti-3-NT antibody in AML12 cell lysates for the indicated groups. Densitometric quantitation of the immunoblots for each protein, relative to the loading control β-actin, is shown where each lane represents the protein samples equally combined from 2 specimens within the same groups (n = 4/group). (C) The levels of mRNA transcripts for NLRP3, IL-1β, and TNF-α in the indicated groups are presented (n = 4/group). Significant difference between various treatments was determined by one-way ANOVA. Labeled characters without a common letter represent significant differences from the other group(s).

intestinal tight junction (TJ) and adherent junction (AJ) proteins and elevated apoptosis of intestinal enterocytes [19]. Therefore, the levels of intestinal epithelial TJ and AJ were examined to study the underlying mechanisms by which POM prevents the gut barrier dysfunction. Binge alcohol exposure significantly decreased the levels of a few typical TJ proteins ZO-1, occludin, claudin-1, and claudin-3 (Fig. 7A). However, POM significantly restored the basal levels of these TJ proteins markedly suppressed in alcohol-exposed rats (Fig. 7A). Similarly, binge alcohol exposure significantly decreased the levels of AJ proteins β-catenin, E-cadherin, plakoglobin, and α-tubulin whereas POM pretreatment prevented these decrements and restored their normal levels comparable to those of dextrose-controls (Fig. 7B).

To further investigate the mechanisms for the decreased TJ and/or AJ proteins in alcohol-exposed rats, we performed immunoprecipitation (IP) followed by immunoblot (IB) analysis. Our IP and IB analysis showed that nitration of claudin-1, used as an example of TJ protein, was significantly elevated in binge alcohol-exposed rats (Fig. 7C). In addition, the amounts of ubiquitin-conjugated protein were increased in immunoprecipitated claudin-1, suggesting its decreased levels through proteolytic degradation in binge alcohol-exposed rats. However, POM pretreatment reduced the amounts of nitrated and ubiquitin-conjugated claudin-1, resulting in the restoration of its level and prevention of the leaky gut in alcohol-exposed rats.

To further study the preventive mechanism of POM against alcohol-mediated gut leakiness, we also determined the levels of apoptosis-associated proteins such as p-JNK, Bax and cleaved (activated) caspase-3 in different rat intestines as indicated. Immunoblot results showed binge alcohol exposure significantly increased these apoptosis marker proteins, whereas POM pretreatment prevented their increments in alcohol-exposed rat intestines (Fig. 7D). In addition, TUNEL analyses followed by quantitation (not shown) showed significantly elevated apoptosis of intestinal enterocytes in alcohol-exposed rats compared to that of dextrose-control, whereas this elevation was markedly reduced by POM pretreatment (Fig. 7E). These results demonstrate that POM restored intestinal TJ and AJ proteins while decreasing apoptosis of enterocytes, contributing to prevention of alcohol-induced gut leakiness.

To support our in vivo animal data, epithelial cell permeability of T84 colonic cells and the underlying mechanisms were evaluated in the absence or presence of 40 mM ethanol exposure with or without UA, EA, or CMZ for 8 h. Confocal image analysis showed that alcohol exposure disrupted the normal distribution of ZO-1, used as an example of TJ protein (Fig. 8A) while it decreased viability of T84 colon cells (Fig. 8B). However, the alcohol-mediated changes in confocal image and cell viability were significantly prevented by treatment with UA, EA, or CMZ (Fig. 8B). Furthermore, binge alcohol exposure significantly decreased the rates of TEER (Fig. 8C) with elevated permeation of FITC-D4 (Fig. 8D). However, co-treatment with UA, EA, or CMZ significantly blocked abnormal changes of ZO-1 TJ protein, viability and epithelial cell permeability in alcohol-exposed T84 colon cells (Fig. 8). Confocal image analyses of immunoreactive CYP2E1 revealed that UA, EA, or CMZ treatment reduced the elevated levels of CYP2E1 following ethanol exposure (Supplemental Fig. 4). These in vitro results are consistent with the in vivo rat results and further support the important roles of CYP2E1 and oxidative stress in promoting epithelial barrier dysfunction following alcohol-exposure.

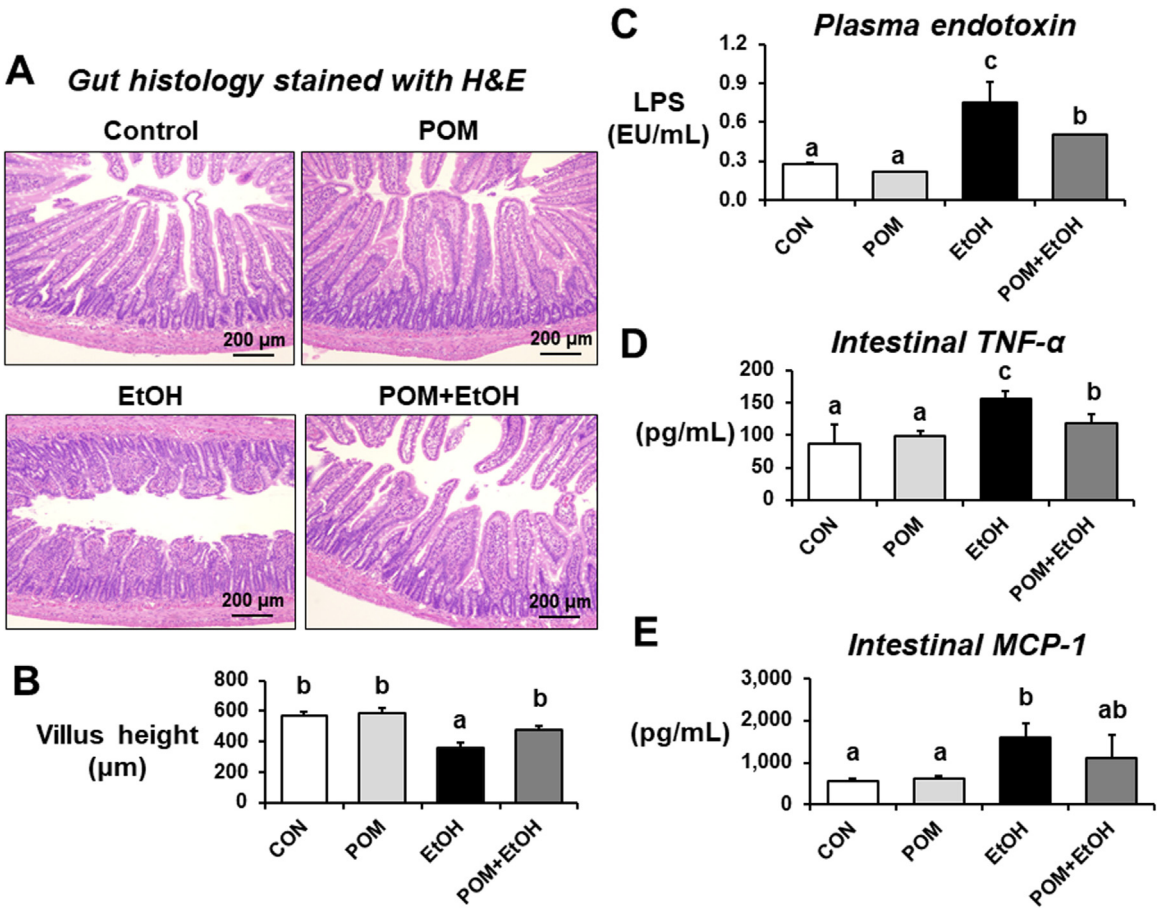

Fig. 5. POM prevented binge alcohol-mediated gut damage, endotoxemia, and intestinal TNF-α and MCP-1 production in rats. (A) Representative histology slides of rat small intestines stained with H/E, (B) villus height (μm), (C) the levels of plasma endotoxin, (D) TNF-α and (E) MCP-1 in the intestinal lysates from the indicated groups are presented. Significant difference between various treatments was determined by one-way ANOVA. Labeled characters without a common letter represent significant differences from the other group(s).

4. Discussion

It is well-established that chronic and/or excessive amounts of binge alcohol intake can damage many cells or organs. One of the main causes of alcohol-induced organ damage is the elevated oxidative and nitrative (nitroxidative) stress through activating pro-oxidant enzymes/genes while suppressing the antioxidant levels including glutathione [39,40]. Some of the pro-oxidant enzymes induced or activated after alcohol exposure are CYP2E1 and iNOS, which are responsible for production of reactive oxygen and nitrogen species, respectively, contributing to elevated nitroxidative stress [9–19]. In fact, alcohol-induced CYP2E1 protein expression has been directly linked to the development of both steatosis and inflammation [2,9,15]. For instance, *Cyp2e1*-KO or *iNOS*-KO mice were resistant to alcohol-induced liver injury [39,41] and non-alcoholic substance such as n-6 fatty acid containing western-style high fat diets [14]. In contrast, transgenic mice with over-expressed CYP2E1 [42], or *Cyp2e1*-KI mice [43] were sensitive to tissue damage to alcohol [12] or non-alcoholic substances [44,45]. CYP2E1 and iNOS are also expressed in the intestine and upregulated by chronic [17] and binge alcohol exposure [18,19,46]. Subsequently, increased nitroxidative stress, produced from elevated CYP2E1 and iNOS, can increase PTMs such as oxidation, nitration, phosphorylation, acetylation, adduct formation, etc. of hepatic and intestinal proteins, as reported [40,47–51], contributing to mitochondrial dysfunction, ER stress, and cell/tissue injury.

It is well-established that increased ER stress is an important causal factor in alcoholic (ALD) and non-alcoholic fatty liver disease (NAFLD) [52–55]. Further, ER stress is closed associated with activation of the apoptotic pathways after failure to adapt to ER stress [56]. Consistent with these reports, our current results showed that binge alcohol significantly increased the hepatic levels of ER stress marker proteins such as p-PERK, p-eIF2α, and PDI, with increased levels of apoptosis marker proteins including p-JNK, Bax, and cleaved caspase-3. In addition, the hepatic levels of inflammatory cytokines TNF-α and IL-1β as well as chemokine MCP-1 were significantly elevated in alcohol-exposed rats compared to those of dextrose-control. These results demonstrate that significantly increased hepatic ER stress, inflammatory cytokines/chemokines and apoptosis of hepatocytes played important roles in promoting inflammatory hepatic injury in alcohol-exposed rats.

In addition to direct insults to the liver, excessive amounts of alcohol can indirectly stimulate hepatic damage by activating the leaky gut and endotoxemia via the gut-liver axis [4–7]. Epithelial TJ and AJ proteins confer the epithelial mucosal barrier function in the intestine and other tissues. For instance, the junctional complex proteins represent multi-protein complexes consisting of transmembrane proteins such as junctional adhesion molecules and adapter proteins which interact with many other proteins including actin-binding proteins [57]. Recent reports showed the contributing roles of increased gut leakiness and endotoxemia in promoting inflammatory injury in many tissues or disease states including alcoholic and non-alcoholic fatty liver disease with inflammation [4–7,58,59]. In addition, elevated gut leakiness and endotoxemia are implicated in many other diseases or potentially toxic conditions, such as burn injury [60], radiation [61], autism [62], and Gulf War illness [63], although the underlying mechanisms of each disease state are not fully understood. Many different mechanisms of alcohol-induced gut leakiness and inflammatory liver injury have been

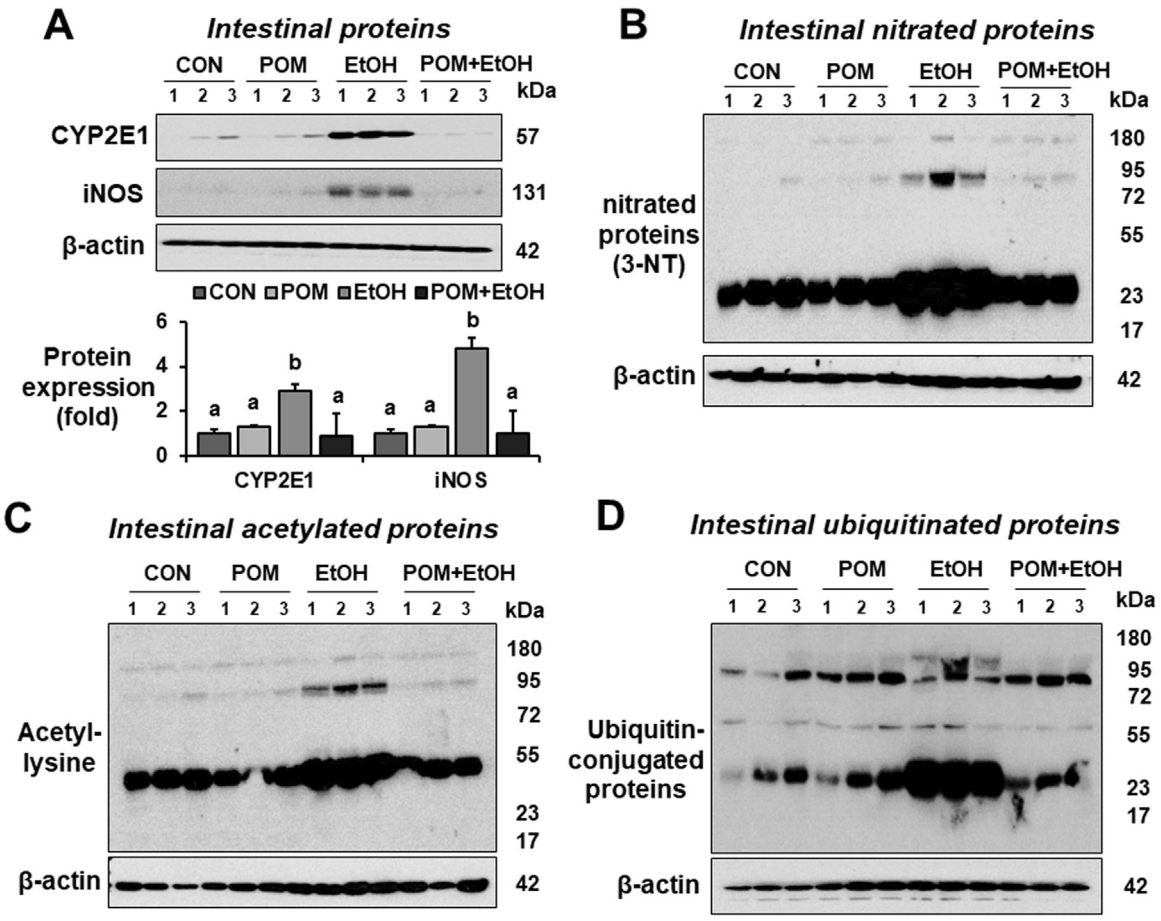

Fig. 6. POM prevented elevated oxidative stress and protein nitration, acetylation, and ubiquitination of intestinal proteins in binge alcohol-exposed rats. (A) Intestinal CYP2E1 and iNOS in the indicated groups are presented. Densitometric quantitation of each immunoreactive protein, relative to β-actin, is shown. (B–D) Intestinal nitrated proteins detected by anti-3-NT antibody, acetylated protein, and ubiquitin-conjugated proteins in the indicated groups are presented. Significant difference between various treatments was determined by one-way ANOVA. Labeled characters without a common letter represent significant differences from the other group(s).

proposed. One of the leading mechanisms of alcohol-induced intestinal barrier dysfunction is the altered composition of gut microbiome (enteric dysbiosis) with decreased abundance of *Bacteroidetes* and *Firmicutes* phyla with proportionally elevated levels of the gram negative *Proteobacteria* and gram positive *Actinobacteria* phyla [64–67] while pomegranate intake can alter gut microbiota with elevated growth of probiotic bacteria and increased production of short chain fatty acids, including propionate and butyrate [68,69]. However, it is still poorly understood how different gut bacterial composition or dysbiosis stimulates leaky gut on molecular levels. Our recent data showed that elevated apoptosis of enterocytes and nitration of the junctional complex proteins contributed to alcohol-induced gut leakiness in rats, mice and humans [19]. In this case, the levels of CYP2E1, iNOS and nitrated proteins were elevated and contributed to increased gut barrier dysfunction in alcohol-exposed rodents whereas pretreatment with a general anti-oxidant or the specific inhibitor of CYP2E1 or iNOS significantly prevented alcohol-induced gut leakiness. Furthermore, *Cyp2e1*-null mice were quite resistant to alcohol-induced gut leakiness and inflammatory liver injury despite the exposure to extremely high dosages of alcohol (3 consecutive doses of 6 g/kg/dose at 12-h intervals), demonstrating the critical roles of CYP2E1 and oxidative stress in alcohol-mediated gut leakiness. These results are consistent with the many direct and indirect roles of CYP2E1, which was shown to be involved in oxidative stress and various PTMs including protein nitration [40,47,70,71].

POM contains many polyphenols and anti-oxidants, including EA and UA. Therefore, POM extracts and/or juice have been widely used in alleviating or preventing various conditions of many disease models as a safe dietary supplement [72–74]. However, its preventive effects on alcohol-induced gut leakiness and inflammatory liver injury are relatively unknown, although POM juice also exhibited protective effects against hepatic oxidative stress in mice [75] possibly through suppression of CYP2E1 by EA [35]. Several reports also showed that POM extract or juice attenuated inflammatory conditions through inhibition of proinflammatory cytokines and chemokines [28,76–79]. Based on our recent results about the causal roles of CYP2E1 and nitroxidative stress in alcohol-induced gut leakiness [18,19] and the anti-oxidant properties of POM, we studied the protective effects of POM against alcohol-induced gut leakiness and liver inflammation. We also elucidated the underlying mechanisms of POM-mediated protection against alcohol-induced gut and liver injury. In the current study, we provided direct *in vivo* and *in vitro* evidence that POM protected the alcohol-induced gut leakiness and inflammatory liver injury through suppressing the nitroxidative stress with elevated levels of CYP2E1, iNOS and nitrated proteins in the intestines and livers of the alcohol-exposed rats. Our data revealed that binge alcohol increased hepatic injury, fat accumulation, nitroxidative stress, nitrated proteins, ER stress and apoptosis marker proteins as well as inflammation response in the liver. Under these conditions, POM pretreatment significantly prevented changes in all these parameters with efficient blockade of gut leakiness and hepatic injury in alcohol-exposed rats (Fig. 9). In addition, pretreatment with an individual compound of POM EA or UA significantly

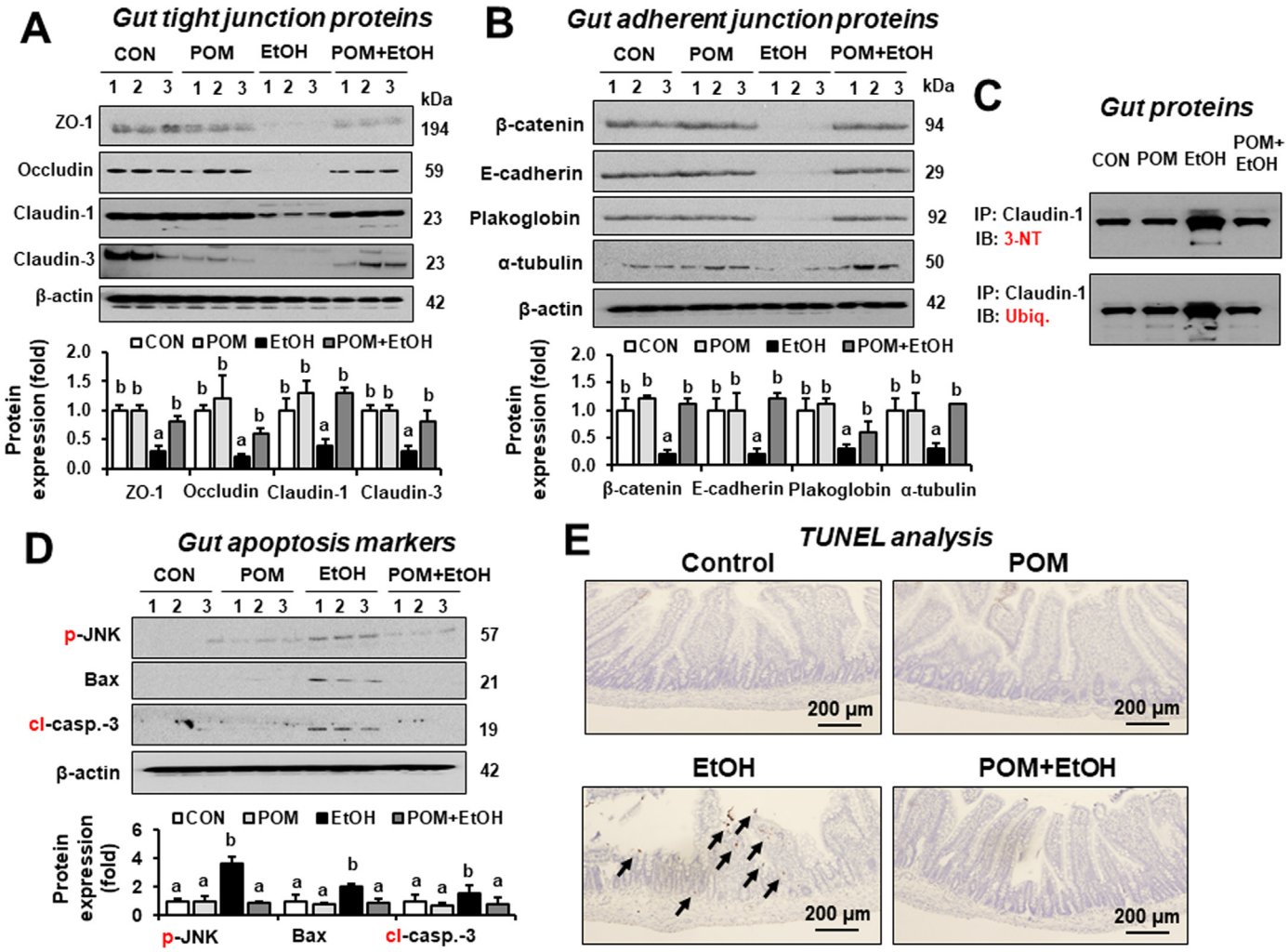

Fig. 7. POM prevented the changes in gut TJ/AJ proteins and apoptosis marker proteins in binge alcohol-exposed rats. Representative levels of (A) gut TJ proteins ZO-1, claudin-1, claudin-3, and occludin, (B) AJ proteins (β-catenin and E-cadherin), plakoglobin and α-tubulin, (C) nitrated or ubiquitin-conjugated proteins, (D) apoptosis marker proteins p-JNK, Bax, and cleaved caspase-3, and (E) TUNEL analyses in the indicated groups are shown. The arrows indicate TUNEL positive cells. Densitometric quantitation of the immunoblots for each protein relative to β-actin is shown. (C) The same amounts of intestinal proteins equally combined from 4 rats/group were immunoprecipitated with the specific antibody to claudin-1 and then subjected to immunoblot analysis with the specific antibody to 3-NT (top panel) and ubiquitin (bottom). Significant difference between various treatments was determined by one-way ANOVA. Labeled characters without a common letter represent significant differences from the other group(s).

reduced the elevated levels of CYP2E1, iNOS and nitrated proteins as well as the mRNA transcripts of NLRP3, IL-1β, and TNF-α in alcohol-exposed AML12 liver cells. Therefore, these studies indicate that the beneficial effects of POM could result from suppressed inflammation in alcohol-exposed hepatocytes and rats. Consistently, POM pretreatment suppressed the plasma endotoxin and restored the decreased intestinal TJ and AJ proteins, leading to prevention of alcohol-induced gut leakiness (Fig. 9). Furthermore, we showed the preventive effects of EA and UA against disruption or decreased TJ protein claudin-1 or ZO-1 with altered TEER and FITC-D4 transport rates in alcohol-exposed T84 colon cells, although the beneficial effects of the individual polyphenols of POM need to be studied in *in vivo* models. Based on the previous results [18,19,46], we believe that decreased CYP2E1 in POM-pretreated rats could at least partly be an underlying mechanism by which POM prevented gut leakiness, endotoxemia and inflammatory liver injury. Alternatively, POM pretreatment may also improve the dysbiosis by decreasing the endotoxin producing bacteria, although this needs further characterization.

Our detailed mechanistic studies also demonstrated that the protection of the intestinal barrier dysfunction by POM pretreatment was achieved in part through suppression of elevated nitroxidative stress and subsequent PTMs with the restoration of the basal levels of intestinal TJ and AJ proteins that were markedly decreased after alcohol-exposure. First, POM pretreatment prevented the reduced amounts of gut TJ proteins such as ZO-1, occludin, claudin-1 and claudin-3 in alcohol-exposed rats. Second, POM pretreatment also prevented the decreased levels of AJ proteins like β-catenin, E-cadherin, plakoglobin, and α-tubulin. Third, POM pretreatment prevented apoptosis marker proteins such as p-JNK, Bax, and cleaved caspase-3 in intestinal enterocytes in alcohol-exposed rats. Confocal image analyses also demonstrated that POM component EA or UA decreased the levels of CYP2E1 and prevented disorganization of a ZO-1 TJ protein in T84 colon cells. Therefore, POM pretreatment may prevent the abnormal distribution of TJ and AJ proteins as well as the activation of apoptosis of enterocytes. Finally, IP followed by immunoblot analyses confirmed increased nitration and ubiquitin conjugation of claudin-1, indicating ubiquitin-dependent proteolytic degradation of this TJ protein in alcohol-exposed rat intestines, as we recently reported [18,19,46]. Although we did not further study the levels of nitration and ubiquitin-conjugation of many other TJ or AJ proteins [57], it is highly likely that other junctional complex proteins might undergo similar modifications, based on the decreased amounts of intestinal TJ or AJ proteins with

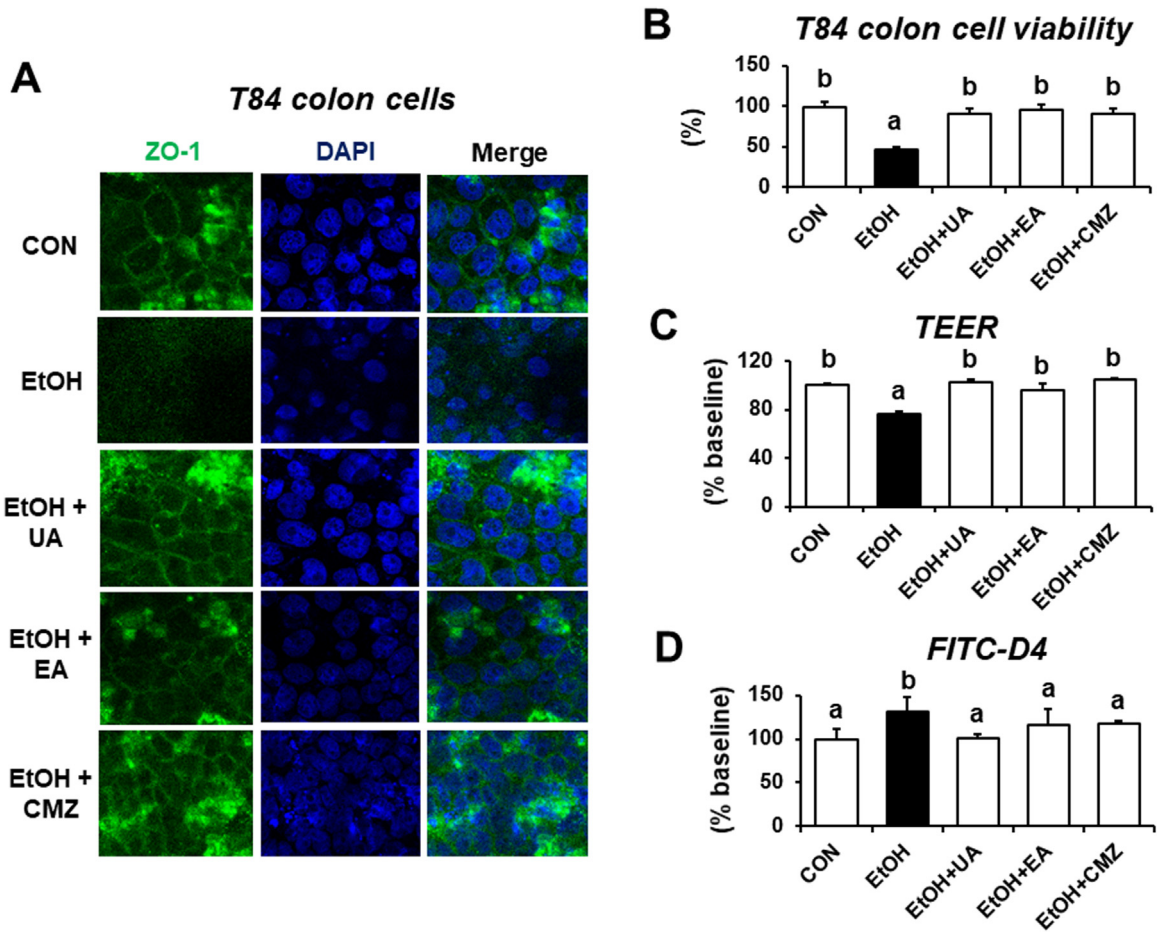

Fig. 8. Urolithin A and Ellagic acid prevented epithelial cell barrier dysfunction in alcohol-exposed T84 colon cells. T84 cells were treated with culture media (CON) or 40 mM ethanol (EtOH) in the absence or presence of 15 μM UA, 100 μM EA, or 15 μM CMZ for 8 h, as indicated. (A) Confocal image showing the disorganized ZO-1 in ethanol-exposed T84 cells but restored ZO-1 after treatment with UA, EA, or the CYP2E1 inhibitor CMZ. Cell nuclei were counter-stained with DAPI. (B) Cell viability of the indicated groups is also presented. (C–D) Representative levels of TEER (C) and permeability to FITC-D4 after 3 h pretreatment without or with the specific agent, UA, EA, or CMZ. Data indicate means ± SD of triplicate wells from two separate experiments. Significant difference between various treatments was determined by one-way ANOVA. Labeled characters without a common letter represent significant differences from the other group(s).

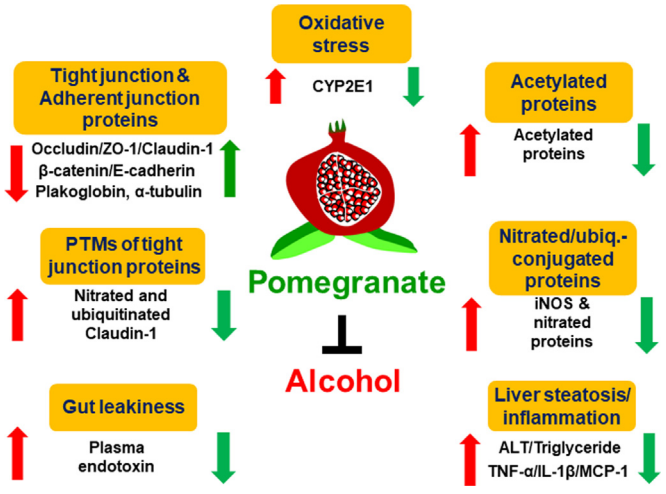

Fig. 9. Summary of the preventive effects of pomegranate against alcohol-Induced oxidative stress, gut leakiness, endotoxemia and inflammatory fatty liver. The effects of alcohol (ethanol) are shown in red while the benefits of pomegranate are marked in green, as indicated. The up and down arrows indicate an increment and decrease of each indicated parameter, respectively (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

elevated nitrated proteins in alcohol-exposed rats compared to those in dextrose controls, as similar to the recent report [57]. Therefore, POM pretreatment restored the basal levels of TJ/AJ proteins and prevented elevated apoptosis of enterocytes, contributing to protection against alcohol-induced gut leakiness, endotoxemia and inflammatory liver injury. In conclusion, our current results demonstrated for the first time, to our knowledge, that POM can reduce plasma endotoxin and protect against alcohol-induced gut leakiness and inflammatory liver injury. Our mechanistic studies further revealed that the elevated levels of CYP2E1, iNOS and nitrated proteins and ER stress along with hepatic apoptosis and inflammatory markers in alcohol-exposed rats were significantly decreased by POM pretreatment. In addition, POM pretreatment restored the basal levels of the intestinal junctional complex proteins and prevented apoptosis of enterocytes, leading to prevention of alcohol-mediated leaky gut and endotoxemia (Fig. 9). Histological and confocal imaging analyses showed that POM could restore the gut shapes by preventing the loss or disruption of various intestinal TJ/AJ proteins with normalization of colon epithelial cell barrier function and suppression of apoptosis of enterocytes in alcohol-exposed rats and T84 colon cells. These findings provide a potentially novel prevention or treatment of alcohol-induced gut leakiness and inflammatory liver injury by POM and/or its components.

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Competing interests

The authors declare no competing financial interests.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2018.07.012.

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