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Polysaccharides from Shiitake Culinary-Medicinal Mushroom *Lentinus edodes* (Agaricomycetes) Suppressed pMLKL-mediated Necroptotic Cell Death and Colitis in Mice

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SHORT TITLE: *Lentinus edodes* Polysaccharides Suppressed Necroptotic Cell Death

ABSTRACT: The incidence of inflammatory bowel disease (IBD) has continued to increase worldwide, and a caspase-independent, pro-inflammatory form of programmed cell death termed necroptosis has been observed to actively play an important role in its pathogenesis. Recent studies have indicated that polysaccharides from edible mushrooms suppressed colitis. However, there is a lack of information on the effect of mushrooms polysaccharides on colitis-associated necroptosis. In this study, the anti-inflammatory activity of polysaccharides from *Lentinus edodes* (*L. edodes*) and its impact on colitis-associated necroptosis was investigated using both *in vivo* and *in vitro* models. Polysaccharides extracted from *L. edodes* was administered to DSS-induced colitis mice prior to and during colitis induction. Caco-2 cells model of necroptosis was used to investigate the anti-necroptosis activity of the polysaccharides sample in *in vitro* system. We found that polysaccharides from *L. edodes* suppressed colitis in mice in a dose-dependent manner, and inhibited necroptotic cell death in Caco-2 cells. Interestingly, the polysaccharides extract exerted a remarkable inhibitory effect on the RIPK1-RIPK3-MLKL necroptosis signaling cascade which resulted in a decreased level of phosphorylated MLKL (pMLKL) in the colon of colitis mice. Notably, the anti-inflammatory and anti-necroptosis activities of the polysaccharides sample were found to be dependent on the carbohydrate-rich fraction of the polysaccharides. These results suggested that the inhibitory effect of the polysaccharides from *L. edodes* on necroptotic cell death in the colon may partly be responsible for its anti-inflammatory activity against ulcerative colitis. Therefore, this study has provided evidence for the anti-necroptosis and anti-inflammatory activity of *L. edodes* polysaccharides extract to support its use as an alternative source of therapeutic agent against ulcerative colitis.

KEY WORDS: *Lentinus edodes,* IBD, necroptosis, polysaccharides, cell death, medicinal mushrooms.

ABBREVIATIONS: **DAMPs**, damage-associated molecular patterns; **DMSO**, Dimethyl Sulfoxide; **DSS**, dextran sodium sulfate; **ECL**, enhanced chemiluminescence; **FBS,** fetal bovine serum; **IBD**, **i**nflammatory bowel disease; **IECs,** intestinal epithelial cells; **MLKL**, mixed lineage kinase domain-like pseudokinase; **MTT**, (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide; **NEAA**, non-essential amino acids; **PBS**, phosphate-buffered saline; **PVDF**, polyvinylidene fluoride; **rhTNF-α**, recombinant human tumor necrosis factor-α; **RIPA**, radioimmunoprecipitation; **RIPK1**, receptor interacting protein kinase 1; **RIPK3**, receptor interacting protein kinase 3; **TBST**, Tris-buffered saline containing 0.1% Tween 20; **TCA**; trichloroacetic acid; **TLR3** and **TLR4**, toll-like receptor 3 and 4; **TNF-α**, tumor necrosis factor-α; **TNFR1**, tumor necrosis factor receptor 1; **TRAIL**, tumor necrosis factor-related apoptosis-inducing ligand; **zVAD**, a pan-caspase inhibitor.

I. INTRODUCTION

The intestine is a specialized organ in the human body, playing an initial and crucial role in nutrient absorption and energy generation for the normal functioning of the body. Being an organ responsible for food digestion and absorption, it is constantly in contact with harmful environmental chemicals and various pathogenic and non-pathogenic microorganisms, such as fungi, bacteria, and viruses, accompanying food intake.[1,2] Ingested toxic environmental chemicals do not only affect the activities and composition of microbiota in the lumen, but also cause serious damage to intestinal epithelial cells.[3-5] The intestinal epithelial cells (IECs) comprise three major cell types, namely the absorptive enterocytes, goblet cells, and the Paneth cells, which form both physical and chemical barrier between the immune cells in the lamina propria and the microbiota and food substance in the gut lumen. These cells are responsible for food absorption, secretion of mucin glycoproteins and mucus lining, and antimicrobial peptides, which play important functions in maintaining the intestinal epithelial homeostasis.[6] Alterations in the composition and activities of microbiota, and damage to IECs resulting from toxic environmental chemicals and food-borne microorganisms have been reported as a major cause of chronic intestinal inflammation. Damaged IECs undergo cell death and result in disruption of the mucosal barrier leading to an increased permeability to gut microbiota, and ultimately homeostatic imbalance and systemic spread of microorganisms (sepsis).[7-10] Furthermore, a compromised mucosal barrier consequently results in aberrant response of innate immune cells in the lamina propria to microbiota and food substances leading to increased secretion of proinflammatory cytokines and chronic intestinal inflammation.[7,11-13]

Inflammatory bowel disease (IBD) is a generalized term used to describe two kinds of disorders that is characterized by the chronic inflammation of the gastrointestinal tract, with an

77 increasing global incidence and prevalence.[14,15] It affects people of all age groups, and negatively
78 impacts on every aspect of lives of the victims.[16,17] It is subdivided into ulcerative colitis and
79 Crohn's disease, characterized with severe abdominal pain, diarrhea, weight loss, rectal bleeding,
80 narrowed gut lumen, and shortened colon length.[18,19] There is currently no cure for the disease,
81 and the pathogenesis is not completely understood.[19] However, researchers have attributed a
82 number of mixed interacting factors ranging from, genetic predisposition, aberrant reactions of
83 immune cells in the lamina propria to microbiota, luminal antigens and food factors, and
84 environmental factors. The interactions of these factors result in cell death, disruption of intestinal
85 epithelial mucosa barrier, and ultimately chronic inflammation of the gut.[20]

86 Recent reports have implicated necroptosis in the pathogenesis of IBD, and it is actively
87 involved in colitis and other inflammatory conditions.[21-23] It is a caspase-independent
88 immunogenic form of programmed cell death.[24] It is an alternative path of cell death when
89 apoptosis is inhibited in cells, and it is usually triggered in cells by the stimulation of cell surface
90 receptors such as TNFR1, TLR3, TLR4, Fas, and TRAIL by their respective exogenous ligands in
91 the absence or pharmacologic inhibition of caspase-8, or by recognition of viral or endogenous
92 RNA by Z-DNA/RNA binding protein 1 (ZBP1).[25-28] Being caspase-independent, it is mediated
93 by the activities of receptor interacting protein kinases known as RIPK1, RIPK3, and MLKL
94 (mixed lineage kinase domain-like pseudokinase). Stimulation of cell surface death receptors
95 results in the phosphorylation and activation of RIPK1, RIPK3, and MLKL.[27,29] Phosphorylated
96 MLKL (pMLKL), known as the necroptosis executor, undergoes oligomerization and translocate
97 to the plasma membrane where it binds to phosphatidylinositol phosphates to induce
98 necroptosis.[30,31]

Edible mushrooms have recently been reported to possess therapeutic properties, ranging from anti-inflammatory, anti-cancer, hepatoprotective, cholesterol-lowering, anti-oxidant, and immunomodulatory properties.[19,32-35] One of the prominent components of edible mushrooms which has attracted significant attention is a group of bioactive polysaccharides known as β-glucans. Many studies have reported that β-glucans, such as lentinan (a β-1,3;1,6-glucan, derived from *Lentinus edodes* (*L. edodes*), is able to suppress intestinal inflammation by modulating the activities of microbiota and reducing the expression of pro-inflammatory cytokines, such as TNF-α, IL-8, IL-1β, IFN-γ, and IL-6 by binding to Dectin-1, a C-type lectin-like receptor, expressed on the cell surface of IECs and innate immune cell, such as macrophages, dendritic cells, and neutrophils.[36-40] However, no study has yet investigated the effect of polysaccharides derived from *L. edodes* on necroptosis during colitis. Therefore, in this study, the anti-inflammatory effect of polysaccharides from *L. edodes* and its effect on necroptosis were investigated using *in vivo* and *in vitro* models.

II. MATERIAL AND METHODS

A. Reagents

DEAE-sepharose CL-6B was purchased from GE Healthcare Bio-sciences AB (Sweden). Dextran sodium sulphate (DSS, 36-50 kDa) was purchased from MP Biomedicals (Canada). Dulbecco's Modified Eagle Medium (DMEM) containing glutamine and glucose (4.5 g/L) was purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). RPMI 1640 medium and MEM non-essential amino acids (NEAA) were purchased from Nissui Pharmaceutical (Tokyo, Japan) and Gibco (Grand Island, NY, USA) respestively. FBS (fetal bovine serum) was purchased from

Biological Industries (Beit, Israel). Recombinant human tumor necrosis factor-α (rhTNF-α) was purchased from PeproTech (Rocky Hill, NJ, USA). Necrostatin-1 (Nec-1) and zVAD-fmk (pan-caspase inhibitor) were purchased from Abcam (ab141053) and Selleck (Tokyo, Japan) respectively. MTT was purchased from Nacalai Tesque (Kyoto, Japan), mouse anti-β-actin antibody was from Santa Cruz Biotechnology (Delaware Avenue, CA), rabbit monoclonal antibody against human pMLKL (ab187091) and mouse pMLKL (ab196436) were purchased from Abcam. Mouse HRP-conjugated anti-IgG and rabbit HRP-conjugated anti-IgG were purchased from R&D Systems (Minneapolis, USA) and Cell Signaling Technology (Danvers, MA, USA) respectively. Every other chemicals and reagents were standard guaranteed commercial products.

B. Used Mushroom and Extraction of Polysaccharides

L. edodes strain MH009105, was gifted by a mushrooms' cultivation company in Japan (Hokuto Corporation: Hokuto's Kinoko). The polysaccharides extraction was carried out according to the method of Mizuno et al.[41] with little modifications. The fresh mushroom samples (fruit body) were pulverized in liquid nitrogen using an Ace homogenizer (AM_7, Nihonseiki Kaisha Ltd), and lyophilized to obtain the dried powdery sample. Twenty grams (20 g) of the dried powdery sample was extracted in 600 mL of distilled water at 100 °C for 6 h. The extracts were obtained by centrifugation at 3500 rpm, 25 ºC for 20 min, and the pellet was discarded. Ethanol (100%) in the ration of 3:1 was added to the supernatant and allowed to stand overnight at 4 °C to precipitate the polysaccharides. The supernatant and precipitate were separated by centrifugation at 3500 rpm, 4 °C for 20 min, and the pellets were washed twice with distilled water and centrifuged again at 3500 rpm, 4 °C for 20 min. Pellets obtained were lyophilized to obtain the dried crude polysaccharides used for this study.

C. Chromatographic Separation of Polysaccharides Samples

Anion exchange column chromatographic technique was used to separate the polysaccharides obtained from *L. edodes*. A slurry of DEAE-sepharose CL-6B in distilled water was packed in a column (2.4 cm × 18 cm) at a flow rate of 1 mL/min, and washed four times with distilled water to the ethanol used for preservation. The column was equilibrated with distilled water and loaded with 20 mL of 5 mg/mL of polysaccharides sample. The sample was eluted in a step wise manner with 200 mL of NaCl solution of increasing ionic strength (0.0, 0.5, and 1.0 M) at a flow rate of 1 mL/min, and eluate collected at 10 mL/tube. Total carbohydrate content of the eluate in each tube was monitored at 490 nm using the phenol-sulfuric acid method.[42] The same peak fractions were pooled and dialyzed for 24 h in distilled water and lyophilized, and the two different fractions, fraction 1 and 2 were name LeP1 and LeP2. The dried polysaccharides were stored at 4 ºC for subsequent use.

D. Total Carbohydrate Content Determination

The carbohydrate content of the samples was determined by the phenol-sulfuric acid method.[42] Briefly, 0.4 mL of sample was mixed with 0.5 mL of 5% phenol and vortexed, followed by addition of 1 mL of concentrated sulfuric acid (H_2SO_4), vortexed, and allowed to stand for 20 min at room temperature (25 ºC). Absorbance was measured using a microplate reader (SH-9000, Corona electric, Japan) at 490 nm. D-glucose (0-100 μg/mL) was used as standard to calculate the total carbohydrate content.

E. Total Protein Content Determination

Total protein content of the samples was determined by Lowry's method[43] with slight modification. Briefly, 0.2 mL of sample was mixed with 1 mL of Lowry's solution, vortexed, and incubated at 25-30 ºC for 15 min, followed by addition of 100 µL of 1 N Folin's phenol reagent (prepared fresh), vortexed, and incubated at 25-30 ºC for 30 min. Absorbance was measured at 750 nm with a microplate reader (SH-9000, Corona Electric, Japan). Bovine serum albumin (BSA) was used as standard for calculating total protein content. Lowry's solution was prepared by mixing solution A (4 mg/mL NaOH and 20 mg/mL Na_2CO_3 in water) and solution B (10 mg/mL of potassium sodium tartrate and 5 mg/mL $CuSO_4$ in water) in 50:1 ratio.

F. Deproteination of Polysaccharides Sample

Polysaccharides sample was deproteinated with trichloroacetic acid (TCA) according to the method of Trakoolpolpruek et al.[44] Briefly, 2 mg/mL of polysaccharides was mixed with 20% TCA and stored at 4 ºC overnight. Precipitated protein was removed by centrifugation at 13,000 g for 20 min. TCA was removed from supernatant by dialysis in distilled water for 24 h with fresh water change every 2 h. Polysaccharides were precipitated by adding three-fold of 100% ethanol overnight at 4 ºC. Precipitate was recovered by centrifugation at 3500 rpm for 20 min, washed with 100% ethanol, and lyophilized.

G. Cell Death Assay

1. Cell culture

Caco-2 cells (intestinal epithelial cell line) were cultured in a 75 cm^3 plastic flask containing 10 mL of DMEM (4.5 g/L glucose) supplemented with 10% FBS, 1% MEM-NEAA, 100 µg/mL streptomycin, and 100 U/mL penicillin. The cells were incubated at 37 ºC in a 5% CO_2 incubator. New culture medium was changed every other two days. Cells were passaged at 80% confluence. Trypsin was used to release cells from the bottom of the flask after washing with phosphate-buffered saline (PBS), and re-plated in a new flask.

2. Cell death induction

Caco-2 cells were cultured in DMEM (4.5 g/L glucose) with fresh medium change every two days until the cells reached 80% confluence. The methods of Dong et al.[45] and Lou et al.[46] were adopted with modifications. Briefly, the cells were harvested with trypsin and 100 µL of 2 $\times 10^5$ cells/mL were seeded onto 96-well plates and incubated overnight at 37 ºC and 5% CO_2. Cell death was induced with increasing concentration of TNF-α (20, 50, and 100 ng/mL). For necroptotic cell death induction, Caco-2 cells were incubated with 50 µM zVAD, 50 µM zVAD plus 20 µM necrostatin-1 (Nec-1), 50 µM zVAD plus 200 µg/mL or 500 µg/mL LeP1 2 h prior to TNF-α stimulation for an additional 24 h. Cell death was determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay.

3. Measurement of cell death

Cell death analysis was performed by MTT assay. Briefly, 100 µL of Caco-2 cells (2 × 10^5 cells/mL) in RPMI 1640 (supplemented with 10% FBS, 100 µg/mL streptomycin, and 100 U/mL penicillin) were seeded in 96-well plate and incubated overnight at 37 ºC and 5% CO_2. Media was

replaced with 100 µL of fresh media containing the different treatments, followed by additional incubation for 24 h at 37 ºC and 5% CO_2. Fresh media containing MTT at a final concentration of 0.25 mg/mL was added, after removal of the old media, and incubated for 4 h at 37 ºC and 5% CO_2. The MTT-containing media was then replaced with Dimethyl Sulfoxide (DMSO, 100 µL/well) and incubated again at 37 ºC and 5% CO_2 for 20 min. Absorbance was measured at 570 nm with a microplate reader (SH-9000, Corona electric, Japan). Percentage cell survival was calculated with the formula: Cell survival (%) = $(A_a - A_o)/ (A_b - A_o) \times 100$, where A_a is absorbance of the test well, A_b is absorbance of the control well, and A_o is the absorbance of blank well.

H. Animal Study

1. Mice

Eight weeks old female C57BL/6 mice, purchased from Japan SLC (Shizuoka, Japan) were used for this study. Mice were acclimatized for 7 days with free access to water and food (*ad libitum*), housed in a room with controlled temperature at 23 ± 2 ºC, humidity of 50 ± 10%, and 12 h light/ dark cycle at the Life Science Laboratory, Kobe University. This study was duly approved by Kobe University Animal Care and Use Committee (approval number: 28-10-04-R1), and was carried out according to the Institution's Animal Experimentation Regulations.

2. Colitis/ *In vivo* necroptosis induction

Eight weeks old female C57BL/6 mice were used for this experiment. 2.5% (w/v) DSS (dextran sodium sulfate: 36-50 kDa), in drinking water (*ad libitum*), was used to induce colitis in mice for

7 days. Mice were treated with polysaccharide samples for 7 days prior to treatment with 2.5% DSS and continued during DSS treatment for additional 10 days. Body weight of mice were recorded daily and mice were sacrificed at the end of treatment/administration. Colon tissues were excised, washed in ice-cold PBS and used for Western blot analysis after measuring their length.

3. Western blot analysis

Colon tissue samples (4-5 mg) were homogenized in ice-cold RIPA (radioimmunoprecipitation) buffer containing PMSF and a cocktail of protease and phosphatase inhibitors (aprotinin, leupeptin, Na Fluoride, and DTT). Protein quantification was carried out by Lowry's method, and equal amounts of proteins were separated with SDS/PAGE (10% gels) under reducing conditions. Proteins were transferred onto PVDF (polyvinylidene fluoride) membranes after electrophoresis in running buffer with 10% methanol. The membrane was washed four times in TBST (Tris-buffered saline containing 0.1% Tween 20) for 5 min each. Non-specific sites were blocked with 4% (w/v) BSA (bovine serum albumin) in TBST for 1 h 30 min at room temperature (25 °C). The membranes were then incubated overnight at 4 °C with anti-pMLKL (dilution of 1:1000) after washing four times with TBST for 5 min each, and anti-β-actin (dilution of 1:5000) was used as loading control. After four washes with TBST for 5 min each, the membranes were incubated with horseradish-peroxidase conjugated secondary antibodies for 2 h in TBST (dilution of 1:5000). Protein bands were visualized using enhanced chemiluminescence (ECL) Plus Western blot detection kit. ImageJ software was used to determine the relative density of the bands.

I. **Statistical Analysis**

All data are presented as mean ± SD. Statistical analysis was performed by Tukey-Kramer and two-tailed standard *t* tests. Statistical significance was defined as *p < 0.05, **p < 0.01, and ***P < 0.001.

III. RESULTS

A. Polysaccharides from *L. edodes* Suppressed Colitis in Mice

To investigate the effect of polysaccharides extracted from *L. edodes* on colitis, a DSS-induced colitis mice model was used. DSS is a relatively water soluble, negatively charged sulphated polysaccharide which is usually used as a colitogenic agent to induce acute colitis in mice. The DSS-induced colitis model is considered to be a simple model and have many similarities with human ulcerative colitis, making it a useful model for studying chronic intestinal inflammatory conditions.[47] In this study, mice treated with DSS developed colitis with loss in body weight, bloody diarrhea, and shortened colon length. However, in contrast to DSS-treated mice, oral administration of 500 μg/mouse polysaccharides extracts from *L. edodes* (which showed no sign of toxicity to mice, Fig. 1A) significantly prevented colon length shortening (Fig. 1D and E) and suppressed the severity of the disease in mice (Fig. 1C). Disease Activity Index was determined according to the method of Jeengar et al.[48] There was no significant difference in body weight of DSS-treated mice and mice orally treated with polysaccharides extract probably due to insufficient dose of the active component of the polysaccharides extract (Fig. 1B). These results suggest that polysaccharide from *L. edodes* possessed inhibitory activity against colitis in mice.

B. Anti-inflammatory Activity of *L. edodes* Polysaccharides is Dependent on the Carbohydrate-rich Fraction

The results above on polysaccharides from *L. edodes* indicated that it possessed anti-inflammatory properties against colitis in mice (Fig. 1D and E), and chromatographic separation of the polysaccharides using DEAE-sepharose CL-6B suggested that it contained two different polysaccharides, LeP1 and LeP2 (Fig. 1F). Carbohydrate and protein content of LeP1 and LeP2 were carried out by phenol-sulfuric acid method and Lowry's method respectively.[42,43] The results in Fig. 2A and B, indicate that LeP1 is a carbohydrate rich component while LeP2 is a protein rich component. To determine the component which exerted the anti-inflammatory activity above, colitis was again induced in C57BL/6 mice treated with LeP1 or LeP2 using 2.5% DSS for seven (7) days. Although both LeP1and LeP2 were unable to significantly prevent body weight loss in mice (Fig. 2C), probably due to insufficient dose, their oral administration to mice resulted in a significant suppression of colon length shortening, a marker for colitis, with LeP1 showing a much stronger ability to prevent colitis in mice (Fig. 2D and E). Furthermore, deproteination of polysaccharides from *L. edodes* did not abrogate its anti-inflammatory activity (Fig. 2F-I) against colitis in mice, indicating that the anti-inflammatory activity did not depend on the protein component. Taken together, these data demonstrate that the anti-inflammatory activity of *L. edodes* polysaccharides is dependent on the carbohydrate-rich component. However, other non-carbohydrate and non-protein compounds which may be present in the carbohydrate-rich fraction may have also contributed to the anti-inflammatory activity observed.

C. Anti-inflammatory Activity of LeP1 from *L. edodes* is Dose-dependent

The results in Fig. 2 above, showed that LeP1 strongly suppressed colitis, but was unable to significantly prevent loss of body weight of colitis mice. To determine whether the anti-inflammatory activity of LeP1 was dependent on the dose. We treated colitis mice with different doses (110, 200, and 300 μg/mouse) of LeP1. In this study, we observed that the ability of LeP1 to suppress loss in body weight of colitis mice was dose-dependent (Fig. 3A). Furthermore, shortened colon length in colitis mice was significantly prevented, in a dose-dependent manner, with the treatment of LeP1 (Fig. 3B and C). These data suggest that the anti-inflammatory activity of LeP1 against intestinal inflammation is dose-dependent.

D. LeP1 from *L. edodes* Suppressed Necroptosis in Mice

Recent studies on the pathophysiology of intestinal inflammation and other inflammatory disease conditions have shown that a type of cell death which is caspase-independent and morphologically different from apoptosis, known as necroptosis, is actively involved in the pathogenesis of inflammatory diseases. Necroptosis leads to the disruption of intestinal mucosal barrier which results from the death of IECs.[22] IECs death results in an increased leakage of the barrier to luminal antigens and microbiota into the lamina propria. Interactions of immune cells in the lamina propria with luminal antigens and microbiota results in the release of pro-inflammatory cytokines and consequently exacerbates intestinal inflammation.[49,50] To determine whether necroptosis was involved in DSS-induced colitis, we analysed the level of the necroptosis executor, pMLKL (phosphorylated MLKL), which is widely used as a marker for necroptosis. Consistent with recent reports that necroptosis is actively involved in inflammatory bowel disease conditions, we found that DSS treatment to mice resulted in an increased level of pMLKL, indicating that the necroptosis pathway was activated during colitis (Fig. 4A-D). However, LeP1 significantly prevented

necroptosis by suppressing the phosphorylation of MLKL in a dose-dependent manner (Fig. 4A-D). These data indicate that LeP1 prevented necroptosis in mice by suppressing the level of pMLKL, and this may partly account for its anti-inflammatory activity against colitis in mice.

E. LeP1 from *L. edodes* Prevented Necroptotic Cell Death *In Vitro*

To further study the anti-necroptosis effect of polysaccharides from *L. edodes*, an *in vitro* necroptosis model comprising Caco-2 cells was used. Apoptotic cell death was induced in Caco-2 cells with increasing concentration of TNF-α, and the result obtained indicate that apoptotic cell death by TNF-α is dose-dependent (Fig. 5A). The reports of Nishitani et al.[19] and Zhang et al.[51] indicated that TNF-α was highly expressed during colitis suggesting that it played a significant role in the associated necroptotic cell death observed in our study. Therefore, in this study, TNF-α and zVAD (A pan-caspase inhibitor) was used to induce necroptotic cell death in Caco-2 cells. We observed that treatment with zVAD-fmk, an apoptosis inhibitor, did not prevent TNF-α-induced cell death in Caco-2 cells, indicating that cell death was caspase-independent (Fig. 5B). However, both 200 μg/mL and 500 μg/mL of LeP1 significantly inhibited TNF-induced necroptotic cell death in Caco-2 cells (Fig. 5C). Furthermore, Necrostatin-1 (Nec-1), an inhibitor of necroptosis, also significantly prevented TNF-induced cell death in Caco-2 cells. Nec-1 is an allosteric inhibitor of the kinase activity of RIPK1, an important player in the RIPK1-RIPK3-MLKL necroptosis pathway (Fig. 5C). These data demonstrate that polysaccharide from *L. edodes* prevented necroptotic cell death in an intestinal epithelial cell line, Caco-2 cells, *in vitro*.

F. LeP1 from *L. edodes* May Be Acting on RIPK1 to Exert Its Anti-necroptosis Effect

RIPK1 is capable of inducing both apoptotic and necroptotic cell death through its kinase activity, and its inhibition results in blockage of its two downstream effects, apoptosis and necroptosis.[52-55] Having demonstrated that our polysaccharides sample prevented necroptotic cell death in Caco-2 cells and colon tissues of colitis mice (Fig. 4A-D; Fig. 5C), we next investigated whether the polysaccharides sample (LeP1) could prevent apoptotic cell death in the same manner. Surprisingly, we observed that the polysaccharides sample significantly blocked TNF-α-induced cell death in Caco-2 cells (Fig. 5D). These data suggest that the polysaccharides sample may be exerting its effect on RIPK1 to suppress both necroptosis and apoptosis.

IV. DISCUSSION AND CONCLUSIONS

Necroptosis is a caspase-independent, pro-inflammatory form of programmed necrotic cell death. Unlike apoptosis, it is characterized by cell swelling, lysis, and release of intracellular contents into surrounding tissues. It is known as immunogenic because of the release of pro-inflammatory cell contents such as interleukins and damage-associated molecular patterns (DAMPs) into extracellular medium. Recent reports indicated that it is actively involved and associated with colitis and other inflammatory disease conditions. Studies have suggested that lentinan, a β-1,3-1,6-glucan, suppressed intestinal inflammation by suppressing gene expression of pro-inflammatory cytokines through binding to dectin-1 on the surface of IECs.[19] However, necroptotic cell death plays an important role in the pathogenesis and progression of intestinal inflammation, and inhibitors of necroptosis have shown promising clinical results in preventing intestinal inflammation.[51] No study has yet reported any effect of polysaccharides from edible mushrooms against necroptosis. In this study, consistent with reports of other researchers, we found that necroptosis was associated with DSS-induced colitis.[51] However, we demonstrated that

363 polysaccharides extract from *L. edodes* significantly suppressed colitis and necroptosis in a dose-
364 dependent manner.

365 An *in vitro* necroptosis model consisting of Caco-2 cells, previously described by Dong et
366 al.[45] and Lou et al.[46] was utilized in this study. First, we performed *in vitro* TNF-α-induced cell
367 death studies using Caco-2 cells, and we found that Caco-2 cells died by necroptotic cell death in
368 the presence of caspase inhibitor (zVAD-fmk) when stimulated with TNF-α, and also responded
369 to TNF-α-induced cell death in a dose dependent manner. Interestingly, we also observed that
370 LeP1 from *L. edodes* significantly protected Caco-2 cells from TNF-α-induced necroptotic cell
371 death *in vitro*.

372 RIPK1 is capable of inducing both apoptosis and necroptosis (when caspase-8 is
373 inactivated) in cells through its kinase activity, indicating that inhibition of the kinase activity
374 could effectively block both necroptosis and apoptosis.[52-55] Notably, we found that the
375 polysaccharide sample was able to prevent both TNF-α-induced necroptosis and TNF-α-induced
376 extrinsic apoptosis in Caco-2 cells, suggesting that it may be exerting its suppressive effect on
377 RIPK1, which in turn resulted in the reduced level of its downstream effector, pMLKL
378 (necroptosis executor), as observed in this study. Furthermore, we showed that the anti-
379 inflammatory and anti-necroptosis activity of polysaccharides from *L. edodes* was majorly
380 dependent on the carbohydrate-rich fraction (LeP1), and not on the protein-rich fraction.

381 Necroptosis leads to disruption of intestinal mucosal barrier as a direct consequence of
382 IECs death.[22] IECs death results in an increased leakage of the barrier to luminal antigens and
383 microbiota into the lamina propria. Interactions of immune cells in the lamina propria with luminal
384 antigens and microbiota results in the release of pro-inflammatory cytokines and consequently
385 exacerbates intestinal inflammation.[49,50] Therefore, these findings strongly suggest that the

inhibition of necroptosis by polysaccharides derived from *L. edodes*, as demonstrated in this study, may partly be responsible for its activity against intestinal inflammation. This study is the first to report that polysaccharides from *L. edodes* prevented necroptosis in both *in vivo* and *in vitro* studies, and also suppressed DSS-induced colitis in mice. Moreover, this study has provided a useful and novel insight on the therapeutic mechanism of *L. edodes* polysaccharides against gastrointestinal inflammation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Fig. 1 Polysaccharides from *L. edodes* prevented colitis in mice**.** DSS (2.5%, w/v) was administered to crude polysaccharides-treated mice for 7 days. (A) Body weight of mice administered with crude polysaccharides from *L. edodes*. (B) Body weight of colitis mice treated with crude polysaccharides from *L. edodes*. (C) Disease Activity index (DAI) of colitis mice treated with polysaccharides from shiitake. (D, E) Colon length of colitis mice treated with *L. edodes* polysaccharides. (F) DEAE-sepharose CL-6B column chromatographic separation of *L. edodes* polysaccharides. Values are presented as Mean ± SD (n = 4-5). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Fig. 2 Anti-inflammatory activity of *L. edodes* polysaccharides is dependent on the carbohydrate-rich fraction (LeP1). DSS (2.5%, w/v) was administered to polysaccharides (110 μg/mouse LeP1 or 365 μg/mouse LeP2)-treated mice for 7 days in drinking water. Mice were pre-treated with LeP1 or LeP2 for 7 days before colitis induction and continued for 7 days during DSS administration and an additional 3 days after DSS treatment. Also, DSS (2.5%, w/v) was administered to deproteinated-polysaccharides pre-treated mice for 7 days before colitis induction

and continued for 7 days during DSS administration and an additional 3 days after DSS treatment. (A and B) Total carbohydrate and protein analysis of LeP1 and LeP2. (C) Body weight change of colitis mice treated with LeP1 or LeP2. (D and E) Colon length of colitis mice treated with LeP1 or LeP2. The different doses administered were determined from their percentage yield after chromatographic separation and lyophilization (LeP1:22% of 500 μg = 110 μg, LeP2: 73% of 500 μg = 365 μg) to represent the equivalence of 500 μg/mouse crude polysaccharides previously administered. (F) Carbohydrate and protein content of deproteinated polysaccharides from *L. edodes*. (G) Body weight of colitis mice treated with deproteinated polysaccharides from *L. edodes*. (H and I) Colon length of colitis mice treated with deproteinated polysaccharides from *L. edodes*. Values are presented as Mean ± SD (n = 3-4). *p < 0.05 and **p < 0.01.

Fig. 3 Anti-inflammatory activity of LeP1 from *L. edodes* against colitis is dose-dependent. Mice were pre-treated with LeP1 (110, 200, or 300 μg/mL) for 7 days before colitis induction and continued for 7 days during DSS administration and an additional 3 days after DSS treatment. (**A**) Body weight change of colitis mice treated with different doses of LeP1. (B and C) Colon length of colitis mice treated with different doses of LeP1. Values are presented as Mean ± SD (n = 5). *$p < 0.05$ and **$p < 0.01$.

Fig. 4 LeP1 from *L. edodes* suppressed necroptosis in mice in a dose-dependent manner. Mice were administration with different doses of polysaccharides for 7 days prior to 2.5% DSS treatment for 7 days with a co-administration of different doses of polysaccharides for 10 days. (A-B) Western blot analysis of necroptosis marker, pMLKL, in the colon of mice treated with DSS +

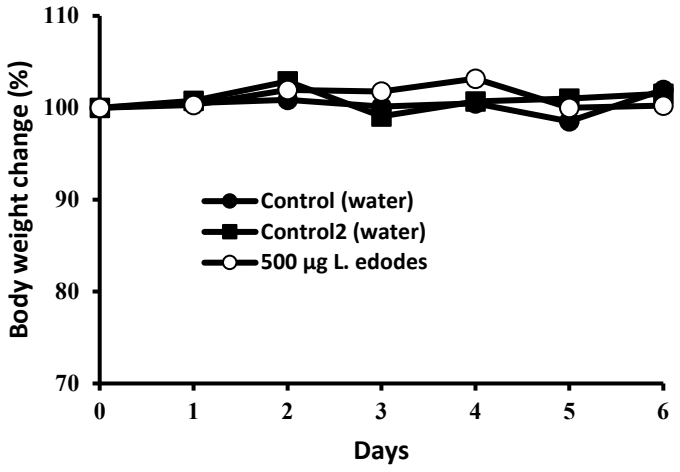
607 crude polysaccharides (500 μg/mouse), LeP1 (110 μg/mouse), and LeP2 (365 μg/mouse). (C-D)
608 Representative Western blot of the necroptosis marker, pMLKL, with anti-pMLKL antibody in
609 colon of mice treated with DSS + increasing doses of LeP1 (110, 200, or 300 μg/mouse). Values
610 are presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01.

611

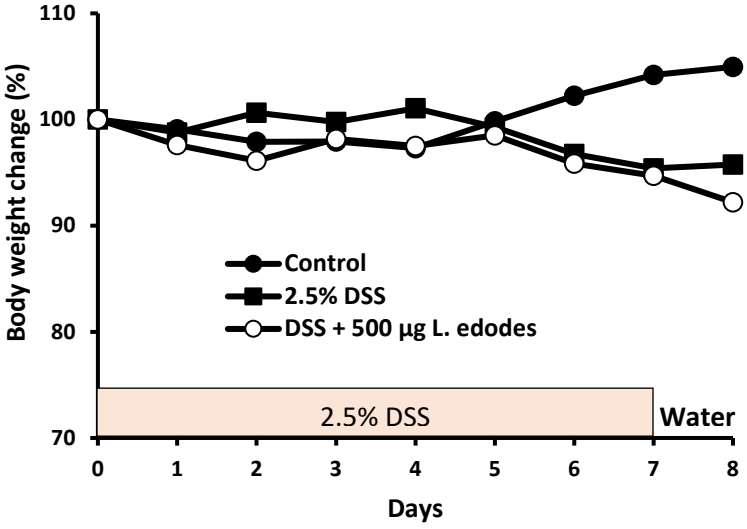
612 **Fig. 5** LeP1 from *L. edodes* inhibited necroptotic cell death *in vitro*. Caco-2 cells were treated with
613 50 μM zVAD (caspase inhibitor), 50 μM z VAD+20 μM Nec-1 (necroptosis inhibitor), or 50 μM
614 zVAD+200 μg/mL or 500 μg/mL of polysaccharides for 2 h prior to stimulation with 20 ng/mL
615 rhTNF-α or not for another 24 h. (A) Induction of cell death with increasing concentration of TNF-
616 α (20, 50, or 100 ng/mL) for 24 h. (B) Induction of necroptotic cell death with 20 ng/mL TNF-α +
617 50 μM zVAD. (C) Inhibition of necroptotic cell death (induced with 20 ng/mL TNF-α+50 μM
618 zVAD) with 20 μM Nec-1 or different doses of LeP1 (200 μg/mL or 500 μg/mL). (D) Inhibition
619 of TNF-induced cell death by LeP1 (Caco-2 cells were treated with 200 μg/mL or 500 μg/mL
620 polysaccharides for 2 h prior to stimulation with 100 ng/mL rhTNF-α or not for another 24 h). Cell
621 viability was determined by MTT assay. Values are presented as mean ± SD (n = 3-4). *$p < 0.05$,
622 **$p < 0.01$.

FIGURE 1

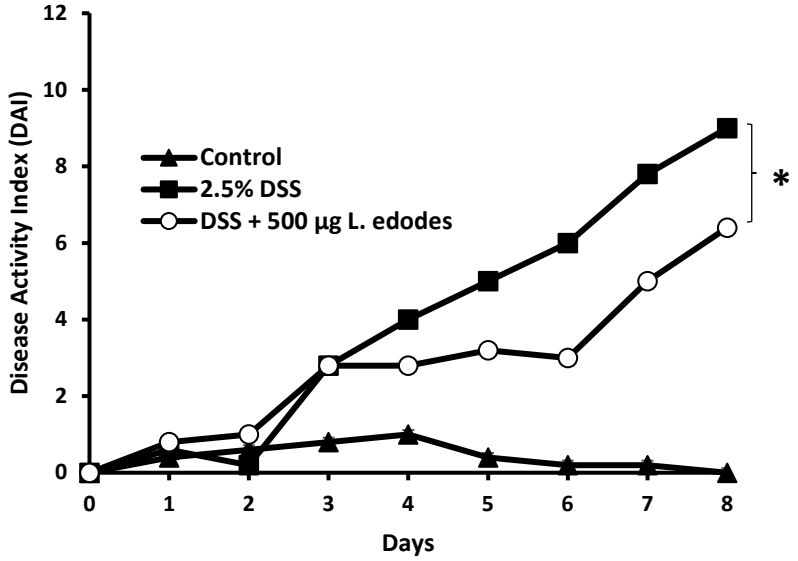
A


110
100
90
80
70
Body weight change (%)
Control (water)
Control2 (water)
500 µg L. edodes
0
1
2
3
4
5
6
Days

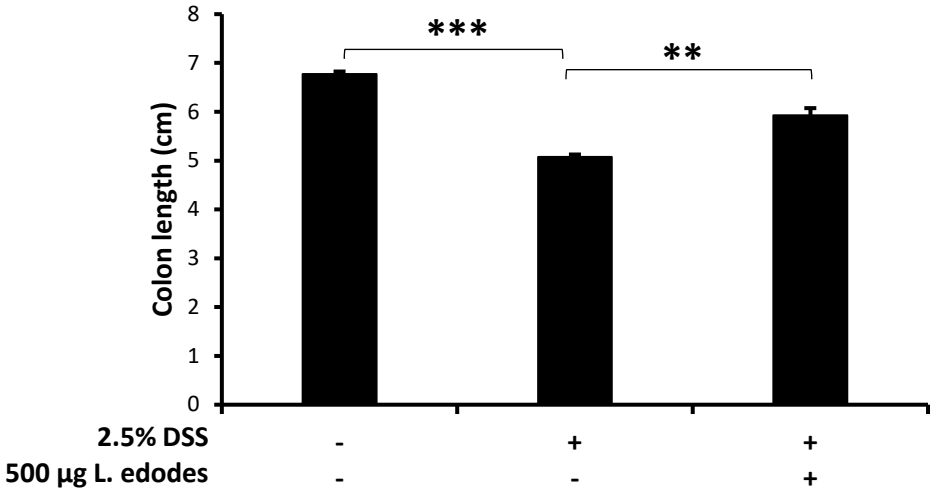
B


110
100
90
80
70
Body weight change (%)
Control
2.5% DSS
DSS + 500 µg L. edodes
2.5% DSS
Water
0
1
2
3
4
5
6
7
8
Days

C

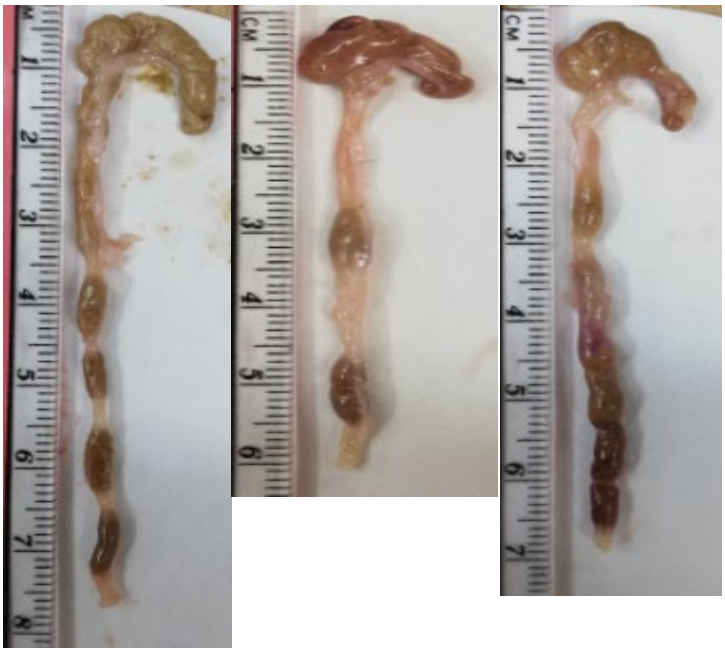

Disease Activity Index (DAI)
12
10
8
6
4
2
0
Control
2.5% DSS
DSS + 500 µg L. edodes
*
0
1
2
3
4
5
6
7
8
Days

D


Colon length (cm)
8
7
6
5
4
3
2
1
0
***
**
2.5% DSS
500 µg L. edodes
-
-
+
-
+
+

E

2.5% DSS	**-**	**+**	**+**
500 µg L. edodes	**-**	**-**	**+**



F

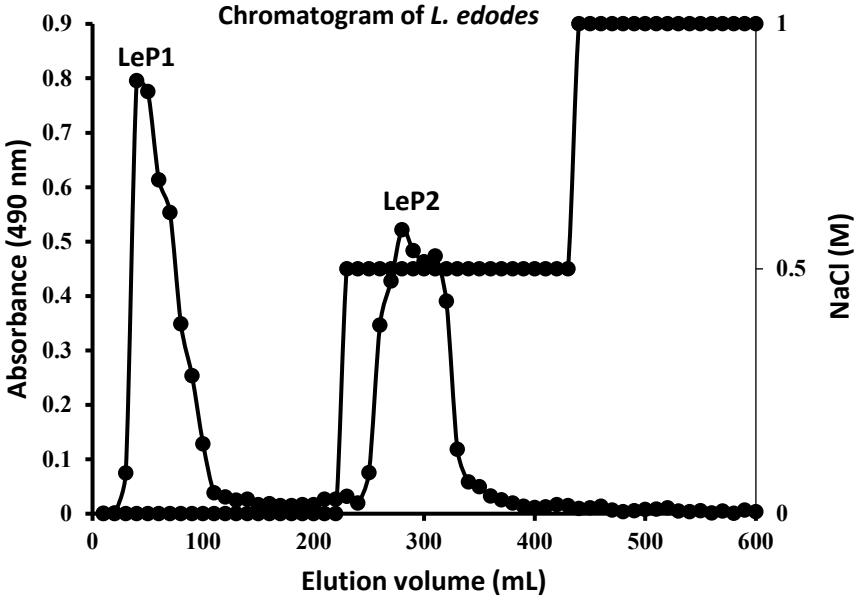
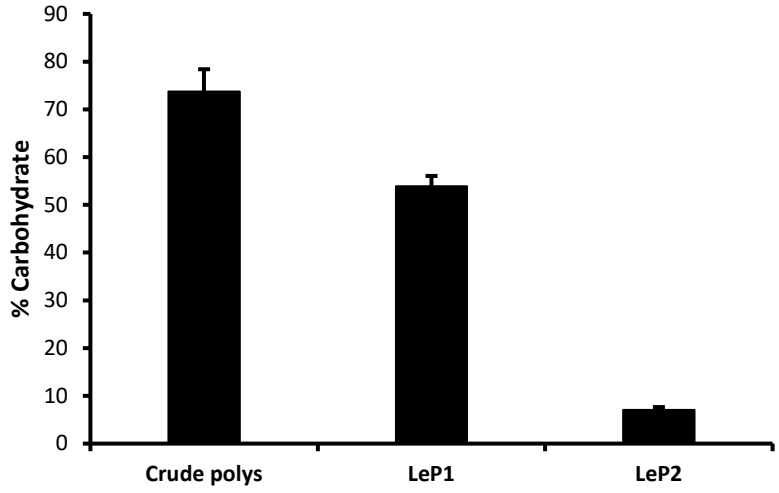
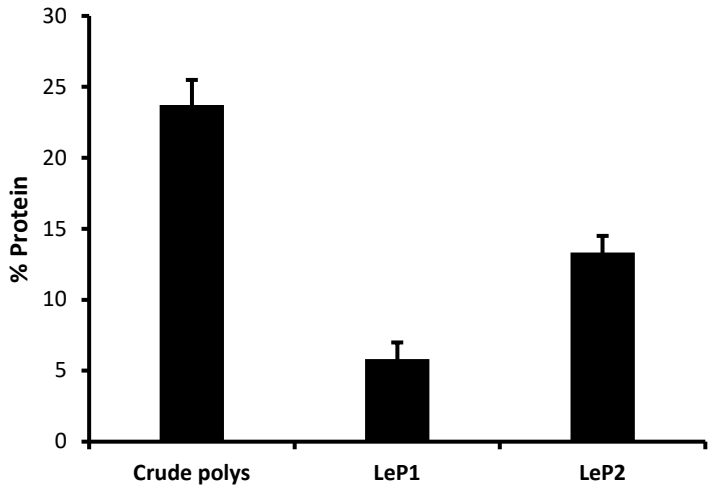


FIGURE 2

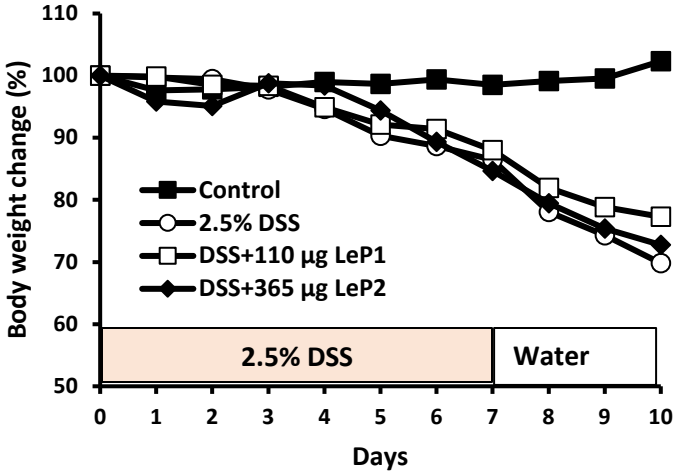
A


90
80
70
60
50
40
30
20
10
0
% Carbohydrate
Crude polys
LeP1
LeP2

B

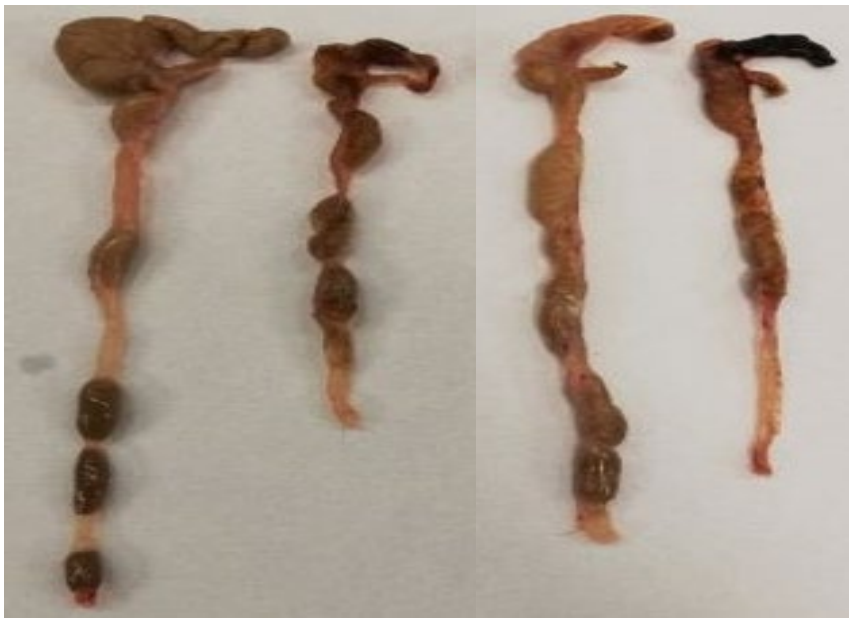

30
25
20
15
10
5
0
% Protein
Crude polys
LeP1
LeP2

C

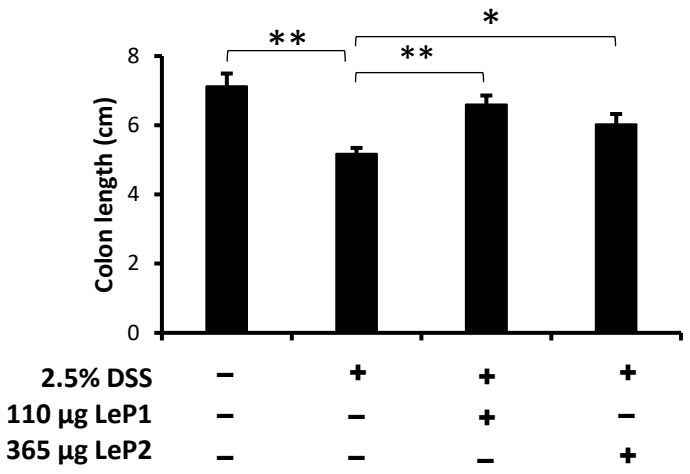


D

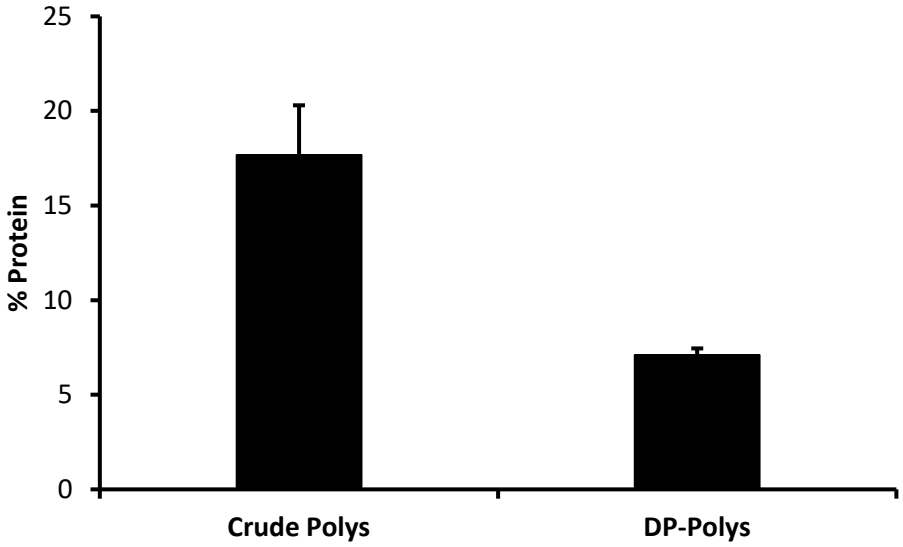
2.5% DSS	-	+	+	+
110 µg LeP1	-	-	+	-
365 µg LeP2	-	-	-	+



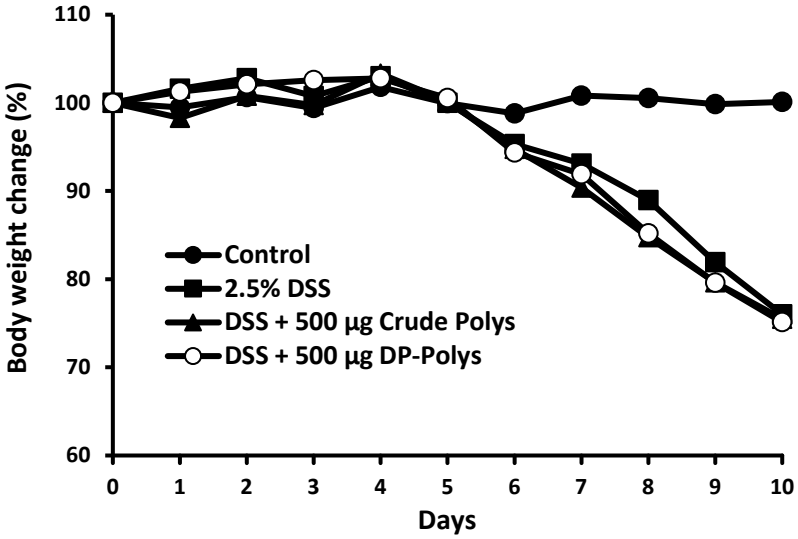
E


**
**
*
Colon length (cm)
8
6
4
2
0
2.5% DSS – + + +
110 µg LeP1 – – + –
365 µg LeP2 – – – +

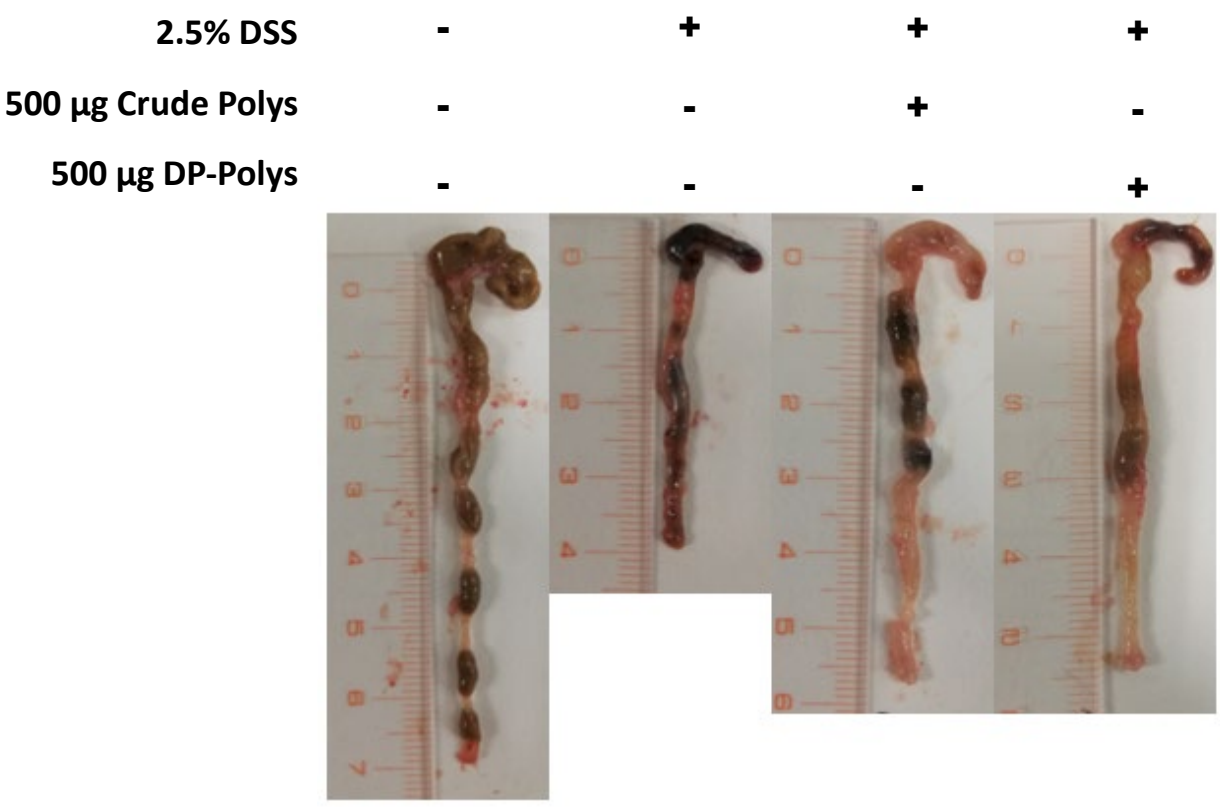
F


% Protein
25
20
15
10
5
0
Crude Polys
DP-Polys

G


110
100
90
80
70
60
Body weight change (%)
0
1
2
3
4
5
6
7
8
9
10
Days
Control
2.5% DSS
DSS + 500 µg Crude Polys
DSS + 500 µg DP-Polys

H


2.5% DSS - + + +
500 µg Crude Polys - - + -
500 µg DP-Polys - - - +

I

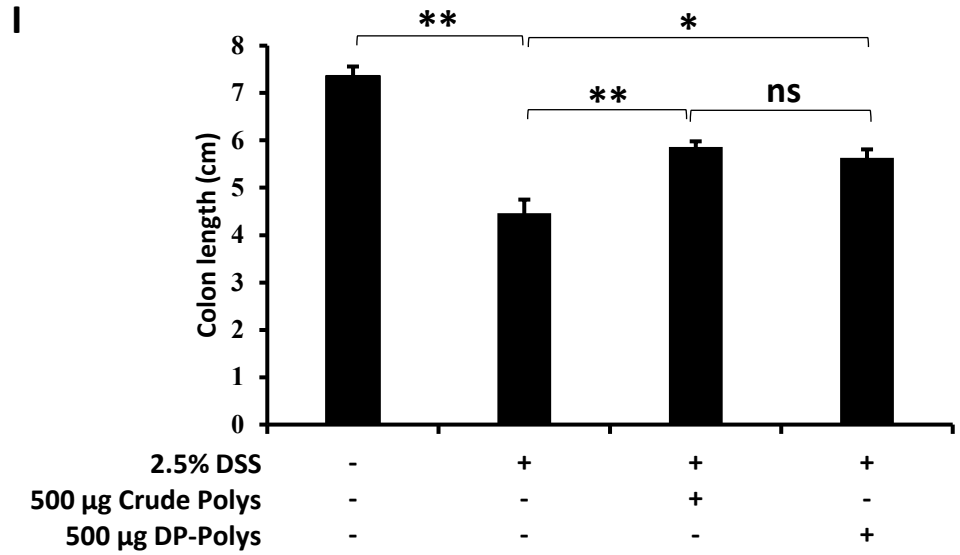
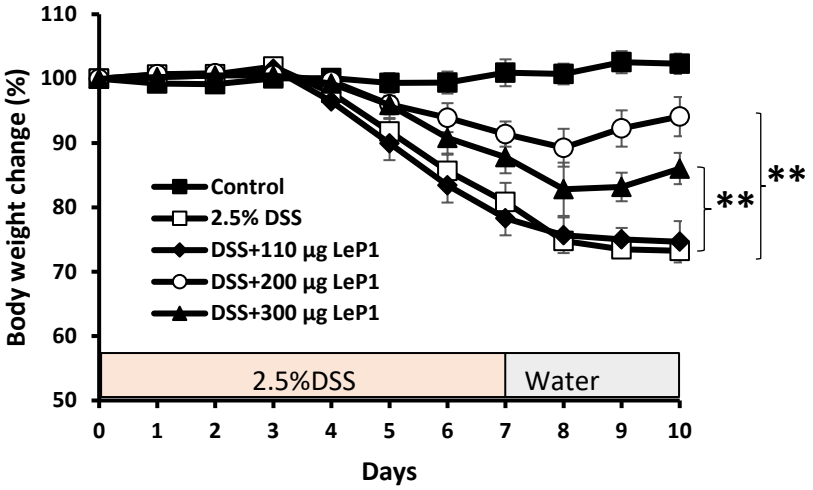

**
*
**
ns
Colon length (cm)
0
1
2
3
4
5
6
7
8
2.5% DSS - + + +
500 µg Crude Polys - - + -
500 µg DP-Polys - - - +

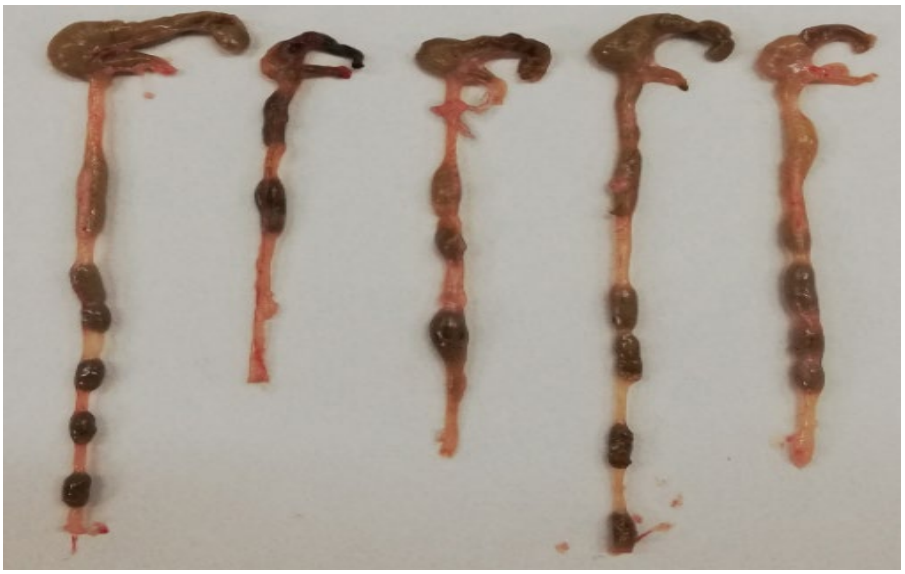
FIGURE 3

A



B

2.5% DSS	-	+	+	+	+
110 µg LeP1	-	-	+	-	-
200 µg LeP1	-	-	-	+	-
300 µg LeP1	-	-	-	-	+



C

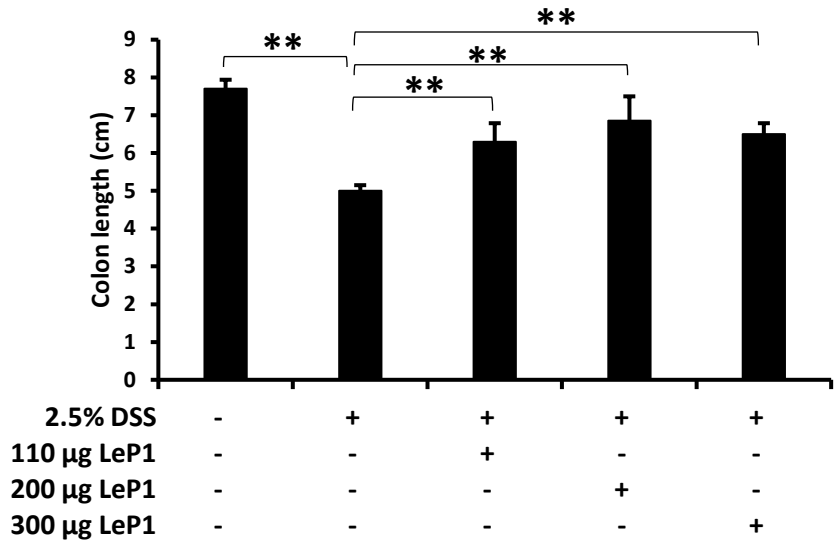
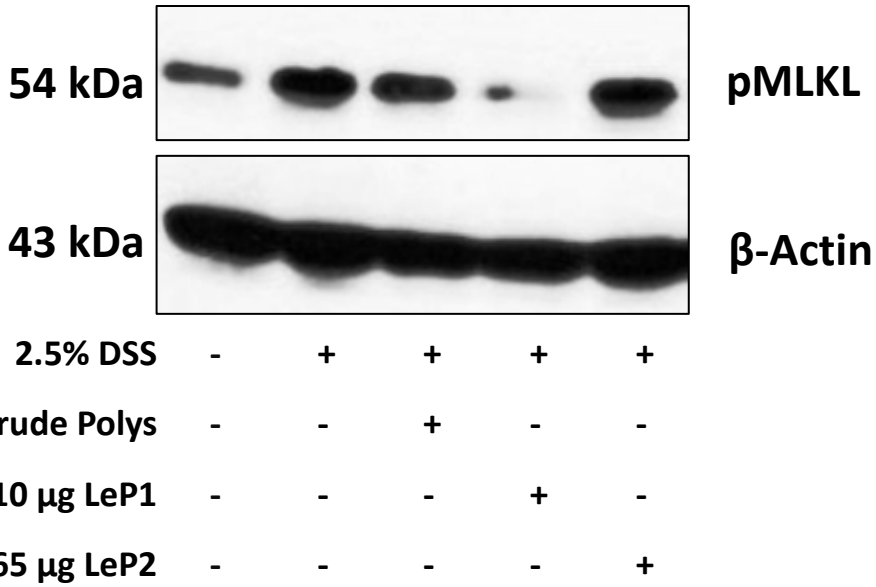
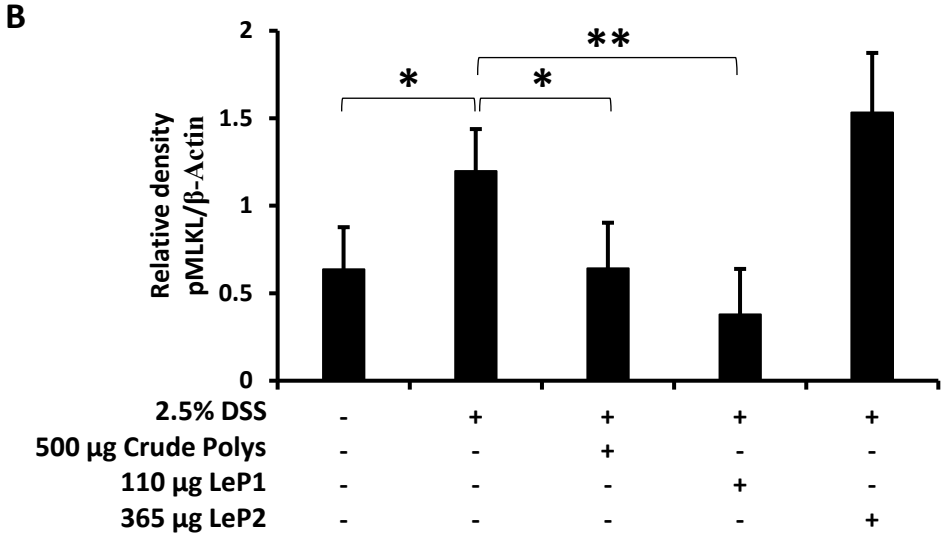

**
**
**
**
Colon length (cm)
9
8
7
6
5
4
3
2
1
0
2.5% DSS - + + + +
110 µg LeP1 - - + - -
200 µg LeP1 - - - + -
300 µg LeP1 - - - - +

FIGURE 4

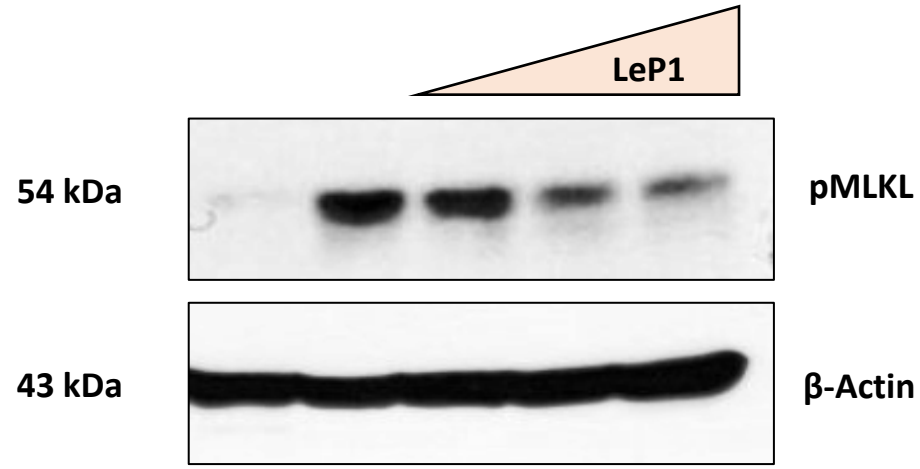
A


54 kDa
pMLKL
43 kDa
β-Actin
2.5% DSS - + + + +
500 µg Crude Polys - - + - -
110 µg LeP1 - - - + -
365 µg LeP2 - - - - +

B

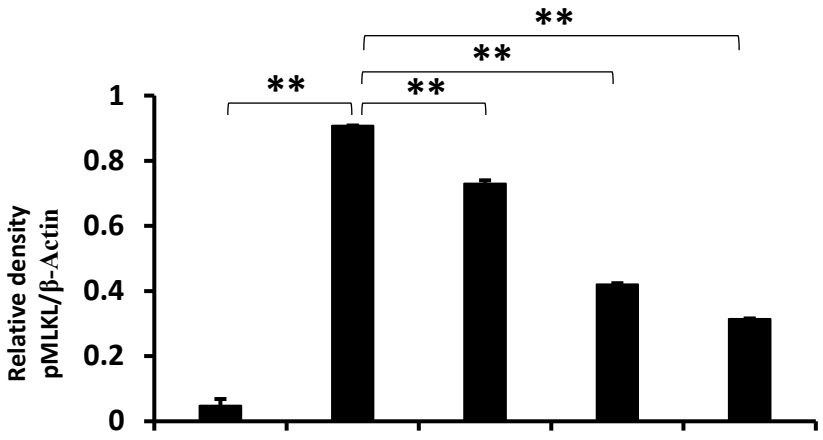

Relative density pMLKL/β-Actin
2
1.5
1
0.5
0
*
*
**
2.5% DSS - + + + +
500 µg Crude Polys - - + - -
110 µg LeP1 - - - + -
365 µg LeP2 - - - - +

C



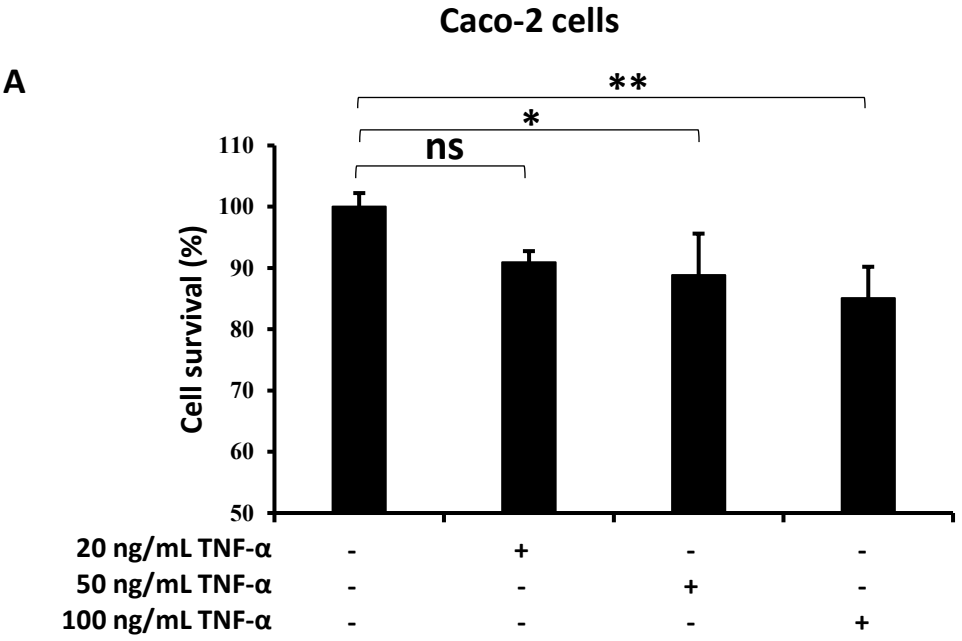
2.5% DSS	-	+	+	+	+
110 µg LeP1	-	-	+	-	-
200 µg LeP1	-	-	-	+	-
300 µg LeP1	-	-	-	-	+

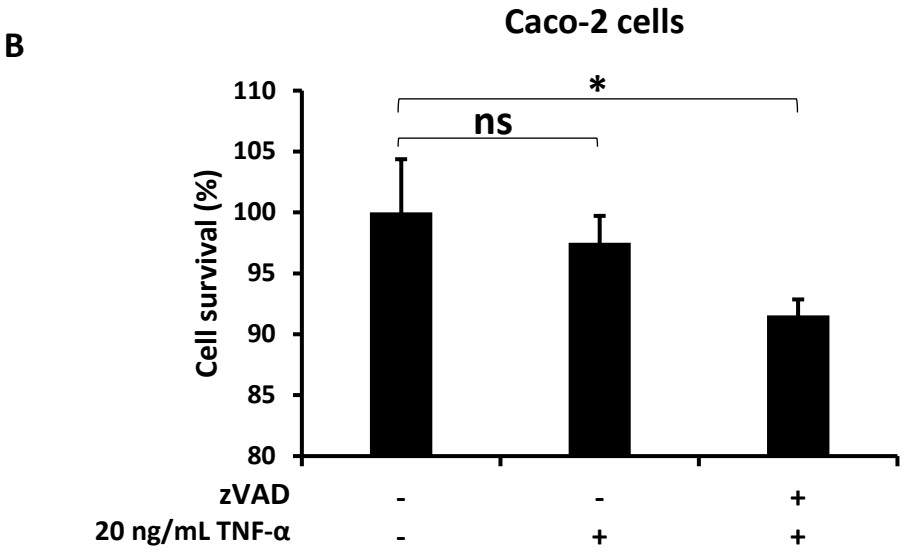
D



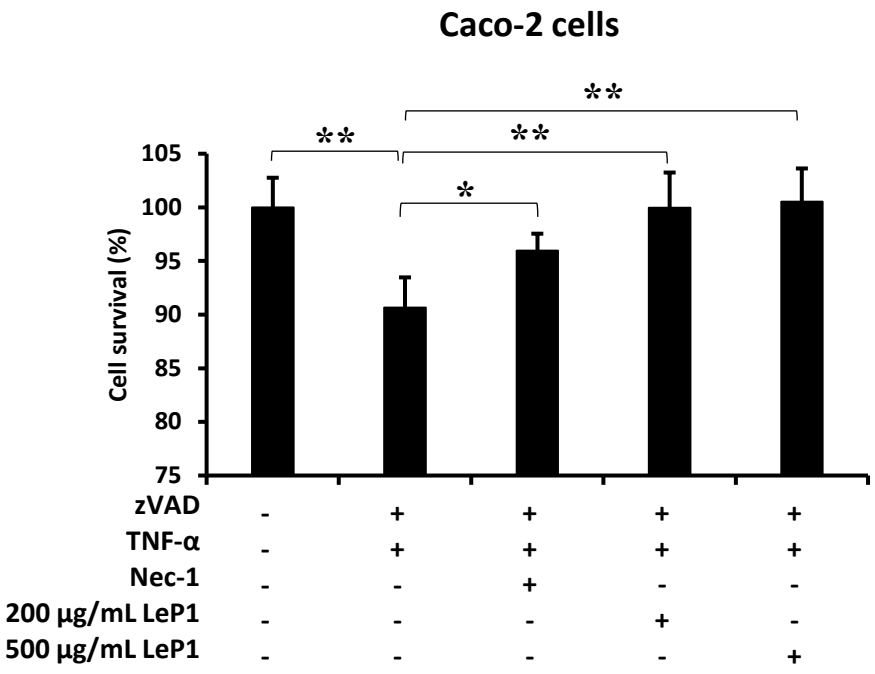
2.5% DSS	-	+	+	+	+
110 µg LeP1	-	-	+	-	-
200 µg LeP1	-	-	-	+	-
300 µg LeP1	-	-	-	-	+

FIGURE 5

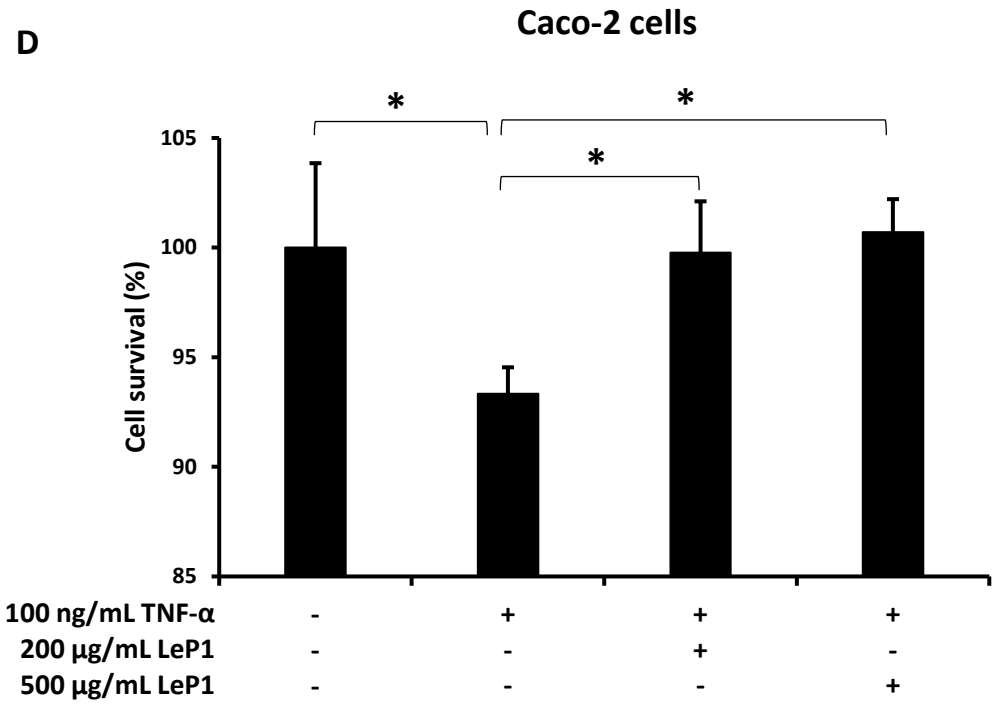

A
Caco-2 cells
**
*
ns
110
100
90
80
70
60
50
Cell survival (%)
20 ng/mL TNF-α - + - -
50 ng/mL TNF-α - - + -
100 ng/mL TNF-α - - - +


B
Caco-2 cells
*
ns
110
105
100
95
90
85
80
Cell survival (%)
zVAD - - +
20 ng/mL TNF-α - + +

C


Caco-2 cells
**
**
**
*
105
100
95
90
85
80
75
Cell survival (%)
zVAD - + + + +
TNF-α - + + + +
Nec-1 - - + - -
200 µg/mL LeP1 - - - + -
500 µg/mL LeP1 - - - - +

D


Caco-2 cells
Cell survival (%)
105
100
95
90
85
*
*
*
100 ng/mL TNF-α - + + +
200 µg/mL LeP1 - - + -
500 µg/mL LeP1 - - - +