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## Research Article

# A novel herbal formulation consisting of red ginseng extract and *Epimedium koreanum* Nakai-attenuated dextran sulfate sodium-induced colitis in mice

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## ABSTRACT

**Background:** Ulcerative colitis (UC) is a commonly encountered large intestine disease in the contemporary world that terminates into colorectal cancer; therefore, the timely treatment of UC is of major concern. *Panax ginseng* Meyer is an extensively consumed herbal commodity in South East Asian countries, especially Korea. It exhibits a wide range of biologically beneficial qualities for almost head-to-toe ailments in the body. *Epimedium koreanum* Nakai (EKN) is also a widely used traditional Korean herbal medicine used for treating infertility, rheumatism, and cardiovascular diseases.

**Materials and methods:** Separately the anti-inflammatory activities of both red ginseng extracts (RGEs) and EKNs had been demonstrated in the past in various inflammatory models; however, we sought to unravel the anti-inflammatory activities of the combination of these two extracts in dextran sulfate sodium (DSS)-induced ulcerative colitis in mice model because the allopathic remedies for UC involve more side effects than benefits.

**Results:** Our results have shown that the combination of RGE + EKN synergistically alleviated the macroscopic lesions in DSS-induced colitic mice such as colon shortening, hematochezia, and weight loss. Moreover, it restored the histopathological lesions in mice and decreased the levels of pro-inflammatory mediators and cytokines through the repression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and nucleotide-binding domain (NOD)-like receptor protein 3 (NLRP-3) expression. *In vitro*, this combination also reduced the magnitude of nitric acid (NO), pro-inflammatory mediators and cytokine through NF-κB and mitogen-activated protein kinase (MAPK) pathways in RAW 264.7 mouse macrophage cells.

**Conclusion:** In the light of these findings, we can endorse this combination extract as a functional food for the prophylactic as well as therapeutic treatment of UC in humans together with allopathic remedies.

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## 1. Introduction

Ulcerative colitis (UC) is a debilitating bowel disorder that is identified by inflammation of the colonic mucosa, blood in the

stool, and severe relapsing diarrhea with frequent abdominal pain. Weight loss is also a very common feature of ulcerative colitis, and it occurs in 2–7/100,000 people in USA annually [1]. A combination of environmental and disrupted immune system is generally

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thought to be the triggers for UC. High fat diet and westernized culture are also the contributing factors for UC [2]. The current therapeutic regimes for UC are mainly steroidal and nonsteroidal anti-inflammatory drugs like 5-amino-salicylate (5-ASA) or mesalamine [3]. This drug is quite efficacious for treatment of UC; however, prolonged treatment with this drug makes the homeostatic system of the body immune, and after some time, this drug loses its efficacy. Therefore, scientists and herbalists are working on some supportive remedies from herbal origin for supplementation with the pharmacological drugs for the treatment and prevention of UC.

Ginseng (*Panax ginseng* Meyer) is a wonder herb used extensively for its promising effects on overall health. The root of ginseng is basically the major benefactor of this plant although its leaves, seeds and stem cannot be underrated. Ginseng is available in many forms for consumption in liquid or solid forms like capsules, jellies, candies, or fermented products [4,5]. Red ginseng extract (RGE) is being prepared by steaming and drying the fresh ginseng roots. There has been many studies carried in past for the elucidation of the health enhancing effects of RGE like in various models of inflammation, in various types of cancers and as an anti-oxidant [6–10]. Furthermore the main constituents in the RGE are the major players for the beneficial properties of this extract. Since there is a lot of data already available on the effects of RGE on inflammation, we geared to check the effects of RGE in combination with another herbal plant widely used in Korea i.e., *Epimedium koreanum* Nakai.

*Epimedium Koreanum* Nakai is a perennial, evergreen flowering plant belonging to Berberidaceae family commonly noted for its beneficial effects in promoting fertility, amnesia, lumbago, auto-immunity, cardiovascular diseases, anti-oxidant and anticancerous effects [11–16]. It has also been reported separately for its immune modulating effects [17]. However, till now it has not been reported for its anticolitis effects. When two extracts are combined, they pose a stronger effect on the disease condition instead to when they are treated alone. Keeping this in mind, we checked the combined effects of RGE and EKN on DSS induced UC in mice. Our results have clearly demonstrated that the combination therapy synergistically attenuated UC lesion *in vivo* in mice and *in vitro* in RAW 264.7 cells.

## 2. Materials and methods

### 2.1. Chemicals and reagents

The chemicals and reagents used for cell culture were same as according to our previous study [18]. Dextran sulfate sodium (DSS), sulfasalazine, lipopolysaccharide (LPS) (*Escherichia coli* O55:B5) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma-Aldrich (St. Louis MO, USA). Specific antibodies used against phospho- and/or total form of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38, IKK  $\alpha/\beta$ , I $\kappa$ B, NF $\kappa$ B p65, inducible nitric oxide synthase (Inos), cyclooxygenase-2 (COX-2), nucleotide-binding domain (NOD)-like receptor protein 3 (NLRP3),  $\beta$ -actin and anti rabbit HRP-linked secondary antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Monoclonal antibodies (moAbs) against CD3e, CD4, CD8, CD25, CD69, CD19 and fluorochrome-labeled moAbs and isotype control IgGs were purchased from BD Biosciences (San Diego, CA, USA). All other reagents and chemicals were obtained from Sigma-Aldrich.

### 2.2. Sample preparation

EKN was prepared by boiling the dried leaves of the plant with 70% ethanol. Thereafter, the boiled mixture was filtered, cooled and

**Table 1**

Single compounds present in the RGE + EKN extract as determined by UPLC.

	Compounds	Content (mg/g)
A	Ginsenoside Rg1	3.00 ± 0.033
B	Epimedin	0.33 ± 0.010
C	Icariin	0.58 ± 0.012
D	Ginsenoside Rg2	3.26 ± 0.085
E	Ginsenoside Rb1	10.51 ± 0.048
F	Ginsenoside Rc	5.37 ± 0.074
G	Ginsenoside Rb2	4.51 ± 0.053
H	Ginsenoside Rb3	0.32 ± 0.067
I	Ginsenoside Rd	3.47 ± 0.111
J	Icariside II	0.07 ± 0.001
K	Ginsenoside Rg3	4.04 ± 0.049
L	Ginsenoside Rk1	4.04 ± 0.051

condensed and a powdered form was prepared by lyophilization. The extract was later analyzed for its individual components by Ultra Performance Liquid chromatography (UPLC). It consisted of a Mobile phase with (A) Water and (B) Acetonitrile with the flow rate of 0.5 mL/min, injection volume of 5  $\mu$ L, temperature of column oven was 40°C and the columns were from Phenomenex Luna Omega, 1.6  $\mu$ m, 2.1  $\times$  100 mm (CA USA). The UV detection wavelength was 205nm, and the sample quantity was 10 mg/mL with methanol. The Standards were 500  $\mu$ g/mL of ginsenosides and 50  $\mu$ g/mL of epimedin, icariin, icariside II with methanol. Ginsenosides were purchased from Chemface (Hubei, PRC) while Epimedin, icariin, and icariside II were purchased from Biopurify (Chengdu, PRC). Table 2 shows the relative quantities of different compounds present in the EKN extract while Fig. 1 shows the ultra performance liquid chromatography (UPLC) peaks of components present in the EKN extract.

### 2.3. Cell culture

The murine macrophage cell line, RAW 264.7, obtained from American Type culture collection was used for the experiment as described in our previous study [18].

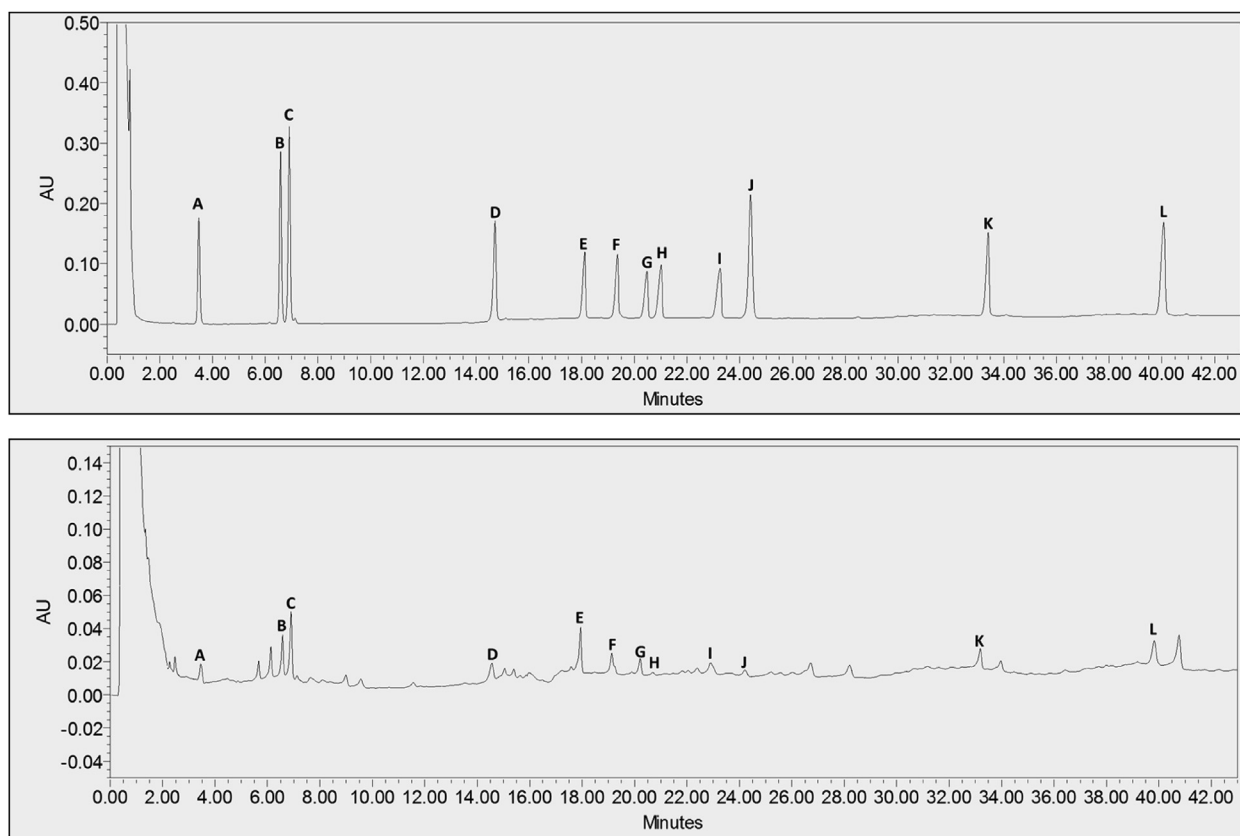
### 2.4. Nitric oxide assay

Griess method was used for determination of nitric oxide (NO). RAW 264.7 mouse macrophage cells were seeded in a 96-well plate and incubated with or without lipopolysaccharide (LPS) (0.1  $\mu$ g/mL)

**Table 2**

Primer Sequences for PCR.

Gene	Primer	Oligonucleotide sequence (5'-3')
GAPDH	F	5'-CAATGATACGGCTACAGCAAC-3'
	R	5'-AGGGAGATGCTCAGTGTGG-3'
iNOS	F	5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'
	R	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'
COX-2	F	5'-TCTCAGCACCCACCCGCTCA-3'
	R	5'-GCCCGTAGACCTGCTCGA-3'
IL-1 $\beta$	F	5'-CAGGGTGGGTGTGCCCTCTTTC-3'
	R	5'-TGCTTCCAACCTTTGACCTGGGC-3'
TNF- $\alpha$	F	5'-TTGACCTCAGCGCTGAGTTG-3'
	R	5'-CCTGTAGCCACGTCGTAGC-3'
IL-6	F	5'-GTACTCCAGAAGACCAAGAGG-3'
	R	5'-TGCTGGTGACAACCCAGGCC-3'
IL-5	F	5'-GAAGTGTGGCAGGAGAGAC-3'
	R	5'-GCACAGTTTTGTGGGGTTTT-3'
IL-13	F	5'-AGCATGGTATGGAGTGGGA-3'
	R	5'-TTGCAATTGGAGATGTTGGT-3'
NLRP3	F	5'-TGCTTTCACCTGCTATCAAGCCCT-3'
	R	5'-ACAAGCCTTGTCTCAGACCCTA-3'



**Fig. 1.** UPLC peaks for single compounds present in REG + EKN extract. A: Ginsenoside Rg1, B: Epimedien, C: Icariin, D: Ginsenoside Rg2, E: Ginsenoside Rb1, F: Ginsenoside Rc, G: Ginsenoside Rb2, H: Ginsenoside Rb3, I: Ginsenoside Rd, J: Icariside II, K: Ginsenoside Rg3, L: Ginsenoside Rk1.

in the absence or presence of RGE + EKN at indicated concentrations for 18 h. The later steps were according to our previously reported study [18]. Nitrite production from the plasma of DSS-induced colitis mice were measured using a NO colorimetric assay kit according to manufacturer's instructions (ab65328- Abcam, Seoul, Korea).

### 2.5. Cell viability assay

Cytotoxic effects of samples were measured by cell viability assay using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reagent according to our previously reported protocol [18].

### 2.6. Dextran sulfate sodium-induced colitis model and ginseng mixture treatment regimens

Male C57BL/6 mice (6–8 weeks old; 18–20 g) were obtained from Charles River (Orient Biotechnology, Gyeonggi-do, South Korea) and housed in a specific-pathogen-free barrier facility at  $21 \pm 2^\circ\text{C}$  with a relative humidity of  $60 \pm 10\%$  under a 12-h light and dark cycle. Feed and water were provided *ad libitum*. All animal care and experimental procedures were performed in accordance with internationally accepted guidelines on the use of laboratory animals (IACUC), and the protocols were approved by the Animal Care Committee of the College of Veterinary Medicine, Kyungpook National University, Daegu, Republic of Korea. Mice were divided into 6 groups ( $n = 6$  in each group): (1) control; (2) an amount of 3% DSS; (3) positive control, sulfasalazine (75 mg/kg) + DSS; (4) RGE (300 mg/kg) + DSS; (5) EKN (20 mg/kg) + DSS; and (6) mixture of

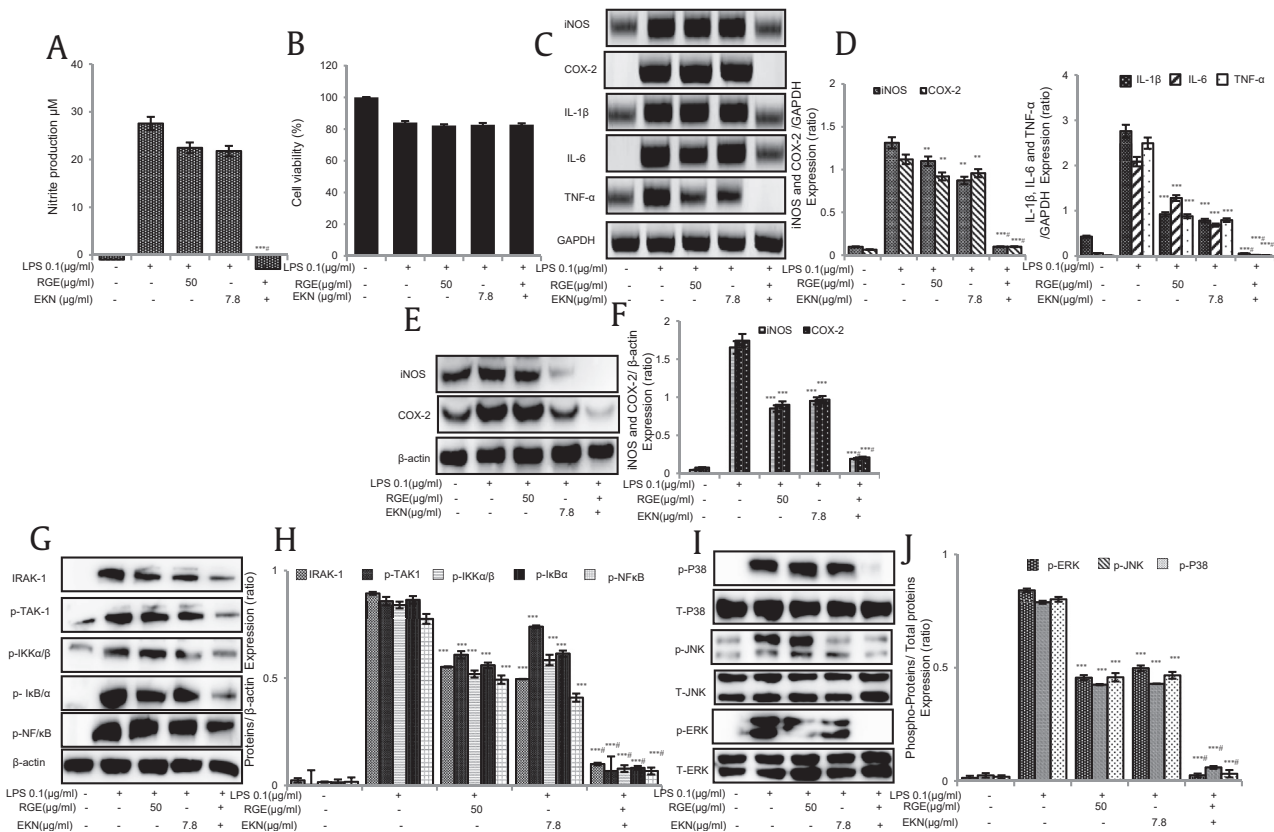
RGE + EKN (150 + 10mg/kg) + DSS. After the acclimatization period, all groups except group 1 were administered 3% DSS in drinking water *ad libitum* for 7 days. On the same day, oral administration of sulfasalazine, RGE, EKN and the mixture was initiated. On day 7, all mice were euthanized and blood, colon and spleen tissues collected for further experiments.

### 2.7. RNA extraction and polymerase chain reaction

RAW 264.7 cells were pretreated with or without RGE + EKN at the indicated concentrations for 30 min and then stimulated with LPS (0.1  $\mu\text{g}/\text{mL}$ ) for 18 h. RNA was collected using TRIZOL® reagent following the manufacturer's instructions. RNA from the colon tissue of DSS-induced colitis mice was also collected using TRIZOL® reagent and the performed subsequent steps are described further [18]. The polymerase chain reaction (PCR) primer sequences are given in Table 1.

### 2.8. Western blot analysis

RAW 264.7 cells were treated with RGE + EKN or left untreated in the presence or absence of LPS (0.1  $\mu\text{g}/\text{mL}$ ). Proteins from cells and from the colon tissue of DSS-induced colitis mice were collected using Cytosolic and nuclear proteins commercially available kit (NE-PER® Nuclear and cytosolic extraction reagents) (Thermo fisher Scientific, Seoul, Korea). The preceding steps were performed according to our previous study [18].



**Fig. 2. Inhibition of Nitric Oxide (NO) expression of pro-inflammatory mediators and cytokines by the nuclear factor  $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein kinase (MAPK) pathways RGE + EKN.** RAW 264.7 cells were pre-incubated with RGE + EKN for 30 min and then stimulated with LPS for 18 h. Cell supernatant was then mixed with equal amounts of Griess reagent and the NO production measured (A) Effects of RGE + EKN on cell viability were measured by an MTT assay (B). (C-D) RT-PCR, and (E-F) western blot (G-H) nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway, and (I-J) MAPK pathway. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and  $\beta$ -actin were used as internal control.\*\*\* $p < 0.001$ , \*\* $p < 0.05$  and \* $p < 0.01$  is considered significant compared to LPS and # $p < 0.01$  compared to inhibition by single red ginseng extract (RGE) and *Epimedium koreanum Nakai* (EKN). Values in bar graphs represent mean  $\pm$  SD of three independent experiments.

## 2.9. Gross examination of colon and disease activity index

After formulating the treatment regimen presented above, the mice were sacrificed, and the colon was removed; its length was then measured and plotted on a graph. Weight measurement was performed every day for 7 days and mice macroscopically observed for their general condition, consistency of stool, and presence of blood in stools. Thereafter, disease activity index (DAI), a combined score of weight loss, stool consistency, and presence of blood in stools, was determined according to Sann et al [19].

## 2.10. Enzyme linked immunosorbent assay for cytokines

The protein levels of IL-5, IL-13, IL-1 $\beta$  and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) in the plasma of DSS-induced colitis mice were measured using Mouse IL-5, Mouse IL-13, Mouse IL-1 $\beta$  and Mouse TNF- $\alpha$  Quantikine enzyme linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA), respectively, according to the manufacturers' protocols. All samples were analyzed in triplicates and normalized to the total protein content of the sample, expressed as pg/mg protein.

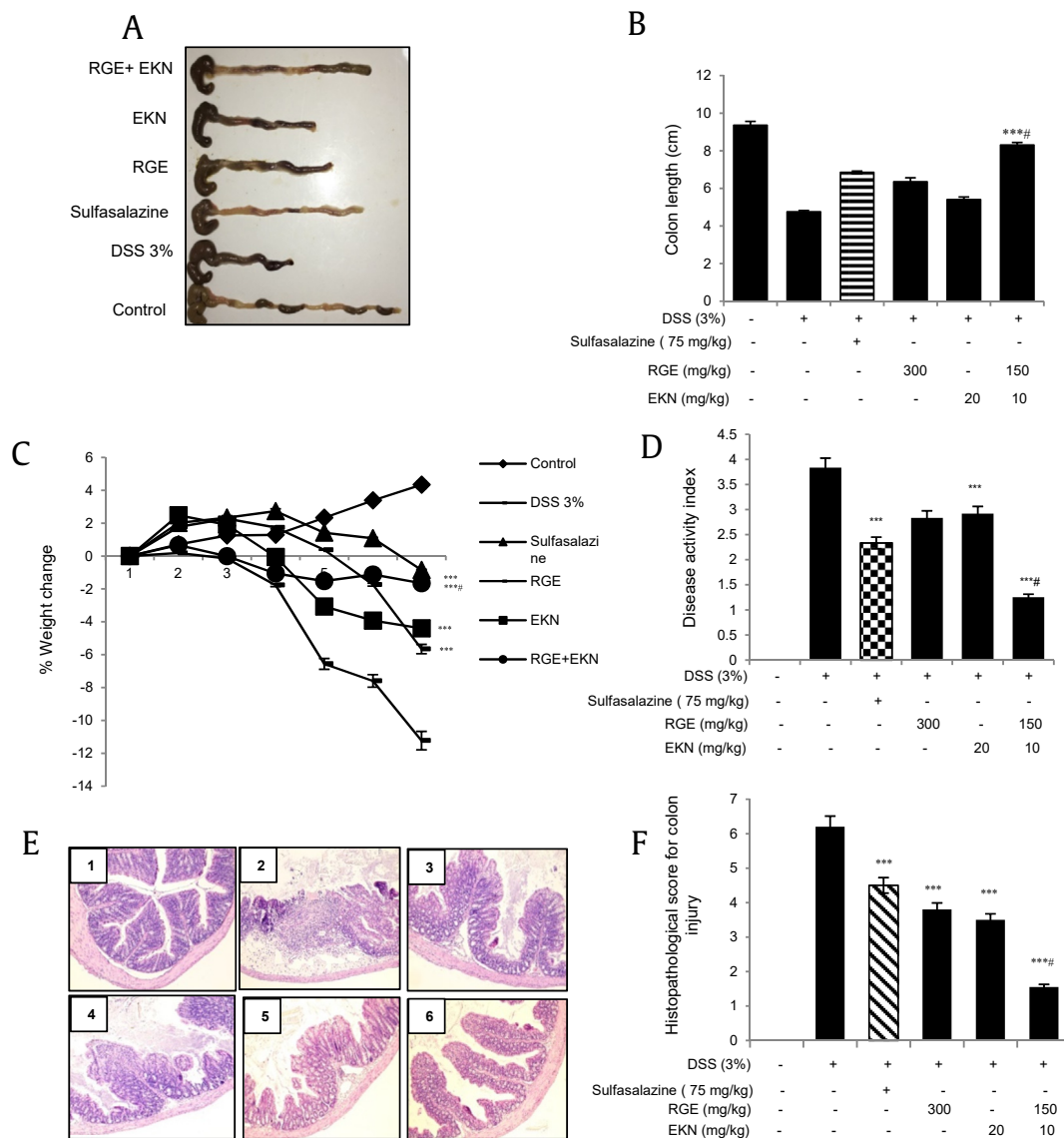
## 2.11. Hematoxylin and eosin staining

After euthanasia, colon tissues were collected in 10% Neutral buffered formalin and the tissues were processed for basic hematoxylin and eosin (H&E) staining, using established protocols [20].

## 2.12. Fluorescent antibody cell sorting

The spleens from mice were slightly pulverized using a Becton Dickinson (BD) syringe plunger and passed through a cell strainer (pore size, 70  $\mu$ m) into Dulbecco's phosphate-buffered saline (DPBS) and centrifuged at 1250 rpm for 5 min at 20–25 $^{\circ}$ C. The supernatant was then aspirated and 3 mL of ammonium-chloride-potassium (ACK) buffer added to lyse red blood cells (RBCs). After 1 min, 10 mL of DPBS was added to quench the lysis and the solution centrifuged at 1800 rpm for 5 min at 20–25 $^{\circ}$ C. The supernatant was again aspirated and 3 mL of fluorescent antibody cell sorting (FACS) buffer (DPBS+2% fetal bovine serum (FBS)) added, and the splenocytes suspended in the buffer. splenocyte counting was performed using a hemocytometer (Neubauer-improved C-Chip disposable hemocytometer, NanoEnTek, MA, USA), and for cell counting,  $1 \times 10^5$  cells were placed in each round bottom FACS tube with the volume adjusted to 1 mL using FACS buffer. The tubes were again centrifuged at 1750 rpm for 5 min at 20–25 $^{\circ}$ C. After centrifugation, the supernatant was aspirated, and an antibody cocktail comprising of fluorescein isothiocyanate (FITC), phycoerythrin (PE) and PE-Cy labeled antibodies was added into the FACS tubes; these tubes were placed at 4 $^{\circ}$ C for 30 min. A 750  $\mu$ L volume of FACS buffer was added to the tubes and centrifuged at 1750 rpm for 5 min at 20–25 $^{\circ}$ C. Finally, after centrifugation, the supernatant was aspirated and 500  $\mu$ L of 2% bovine serum albumin (BSA) in DPBS added to the tubes and FACS performed using BD FACSArialIII (BD Biosciences, San, Jose, USA).





**Fig. 3. Revival of colon length, DAI and histopathological lesions by RGE + EKN treatment.** (A) Gross examination of colon length (B) Quantification of colon length. (C) % Change in the body weight of mice. (D) Disease activity index (DAI). (E) H&E staining of colon tissues (1) Control, (2) dextran sulfate sodium (DSS), (3) Sulfasalazine + DSS, (4) RGE + DSS, (5) EKN + DSS and (6) RGE + EKN + DSS. Scale bar = 100  $\mu$ m. (F) Histopathological score for colon damage. \*\*\* $p < 0.001$  considered significant compared to DSS group and # $p < 0.01$  compared to inhibition by single RGE and EKN.

2.13. Statistical analysis

Data are presented as mean  $\pm$  SD. One way ANOVA and Dunnett's test were applied to statistically evaluate the data. Statistical analyses with \*\*\* $p < 0.001$  and \*\* $p < 0.05$  and \* $p < 0.01$  were considered significant compared to LPS and DSS group; # $p < 0.01$  when compared to RGE and EKN alone.

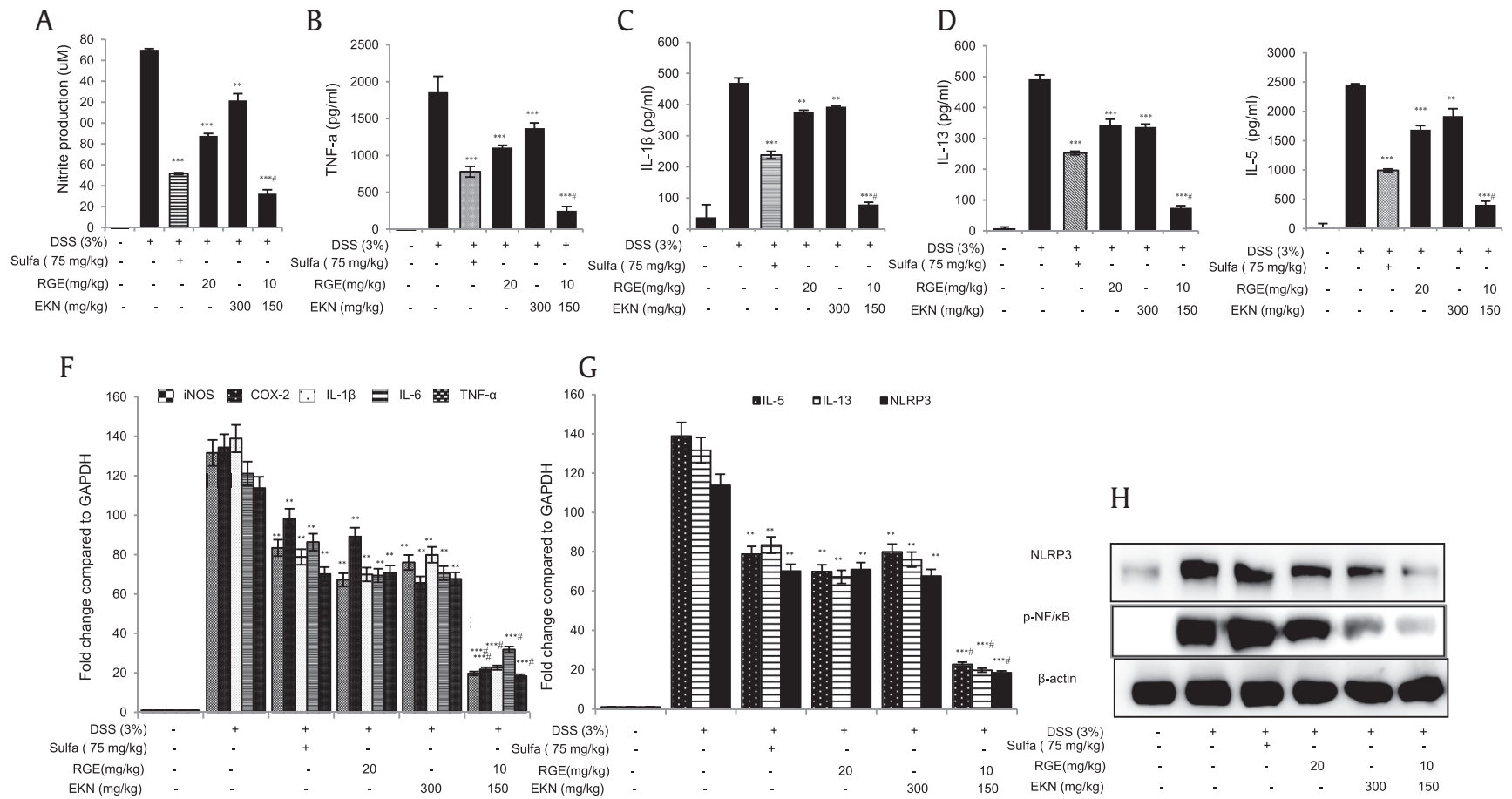
3. Results

3.1. RGE + EKN synergistically attenuated LPS-induced inflammation and the expression of pro-inflammatory mediators and cytokines via NF- $\kappa$ B and mitogen-activated protein kinase pathways

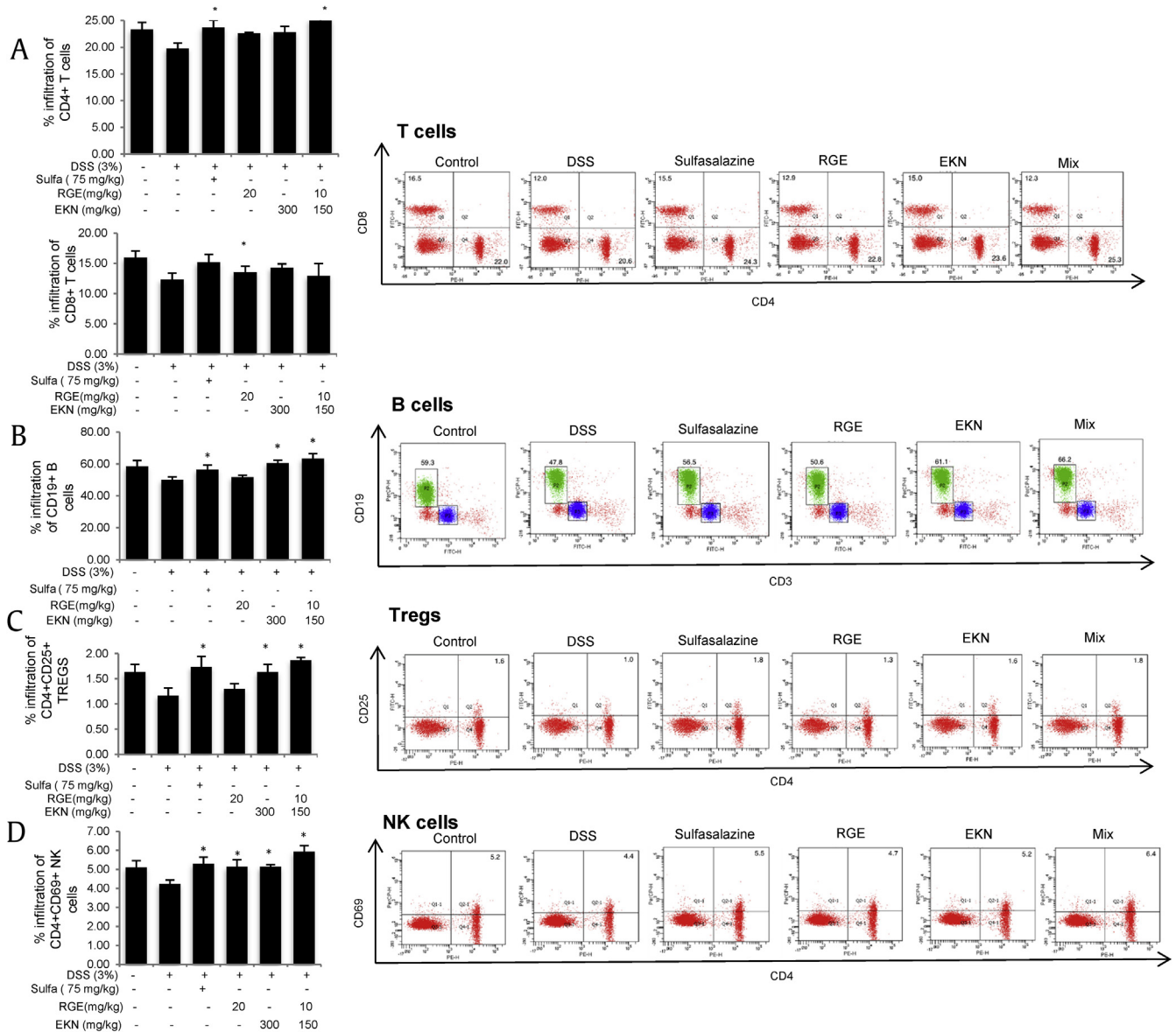
The production of nitric oxide (NO) is the basic defensive mechanism of cell towards foreign invasion. Once NO is released, a series of pro-inflammatory mediators and cytokines are also

activated that if uncontrolled can lead to neighboring cell death. In our study, we found that RGE + EKN extract synergistically attenuated the levels of NO in RAW 264.7 cells without cytotoxicity, as shown in Fig. 2A and B. Moreover, it also diminished the levels of pro-inflammatory mediators (i.e., inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2)) and pro-inflammatory cytokines (IL-1 $\beta$ , IL-6 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )) both at transcriptional and at translational levels as depicted in Fig. 2C–F.

NF- $\kappa$ B pathway is considered to be the Holy Grail for the extracts or compounds that possess anti-inflammatory activity. It is activated when LPS attaches to its Toll-like receptor 4 (TLR4) receptor on the cell membrane and thereafter a series of downstream factors like TAK1, IKK $\alpha/\beta$  and I $\kappa$ B $\alpha$  are activated that translocate the NF- $\kappa$ B from cytoplasm to nucleus. As shown in Fig. 2G and H, RGE + EKN had separately as well as synergistically in combination had inhibited the phosphorylation for NF- $\kappa$ B pathway. Mitogen-activated protein kinase pathway is another stress-activated pathway



**Fig. 4. RGE + EKN suppressed the expression of pro-inflammatory mediators and cytokines via nucleotide-binding domain (NOD)-like receptor protein 3 (NLRP3) and NF- $\kappa$ B inhibition in plasma of DSS mice.** (A) NO expression, (B) TNF- $\alpha$  expression, (C) IL-1 $\beta$  expression, (D) IL-13 expression, and (E) IL-5 expression. (F-G) Real-Time PCR for inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), IL-1 $\beta$ , IL-6, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), IL-5, IL-13, and NLRP3. (H) Western blot for NLRP3 and NF- $\kappa$ B.  $\beta$ -actin was used as an internal control. Images are representative of three independent experiments. Values in bar graphs represent mean  $\pm$  SD of three independent experiments. \*\*\* $p$  < 0.001 and \*\* $p$  < 0.05 were considered significant compared to DSS group and # $p$  < 0.01 compared to inhibition by single RGE and EKN.



**Fig. 5.** Effects of RGE + EKN on immune cell subtypes from spleen tissue. (A) Absolute number of CD4<sup>+</sup>CD8<sup>+</sup> cells from spleen tissue for the Sulfasalazine group and RGE + EKN group when compared to the DSS group at  $*p < 0.01$  by fluorescent antibody cell sorting (FACS) analysis. (B) Absolute number of CD3<sup>+</sup>CD19<sup>+</sup> cells from spleen tissue. (C) Absolute number of CD4<sup>+</sup>CD25<sup>+</sup> cells from spleen tissue for the RGE + EKN group when compared to the DSS-group at  $*p < 0.01$ . (D) Absolute number of CD4<sup>+</sup>CD69<sup>+</sup> cells from spleen tissue for the RGE + EKN group when compared to the DSS group at  $*p < 0.01$ . Values in bar graphs represent mean  $\pm$  SD of four independent experiments.

that also consists of transcriptional factors (P38, JNK, and ERK). However, according to Fig. 2I and J, the mixture of RGE + EKN strongly inhibited the transcriptional factors of this pathway as well. These results show that the inflammatory signal transduction of RGE + EKN takes place by both NF- $\kappa$ B and mitogen-activated protein kinase (MAPK) pathways.

### 3.2. Alleviation of macroscopic and microscopic lesions by mixture of RGE + EKN

The common macroscopic lesions that are associated with DSS-induced UC are dramatic weight loss, colon shortening, and blood in the stool. These all three measures are collectively called as disease activity index (DAI). As shown in Fig. 3, RGE + EKN separately and synergistically in combination reduced the DAI score over all. Moreover, the colon damages in the nature of histological

damage (i.e., disruption of epithelial cell integrity), thickening of mucosal wall, and infiltration of inflammatory cells that occurred as a result of DSS were also recovered to almost normal levels by the RGE + EKN treatment. The histopathological lesions in the colon tissue were graded according to the scoring system given by Geoboes et al [21] as shown in Fig. 3F.

### 3.3. RGE + EKN reduced the plasma and tissue levels of pro-inflammatory mediators and cytokines

In order to make a connection between the *in vitro* results with the *in vivo* experiments, we harvested the plasma from DSS-induced colitic mice and checked the levels of pro-inflammatory mediator (NO) Fig. 4A, and pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, IL-5, IL-13, and TNF- $\alpha$ ), Fig. 4B–E. Much to our expectations, RGE + EKN synergistically inhibited the levels of these cytokines in

the plasma of mice thus rescuing them from DSS-induced damage. In line with our *in vitro* results, RGE + EKN potently suppressed the expression of these factors via NF- $\kappa$ B and nucleotide-binding domain (NOD)-like receptor protein 3 (NLRP3) as shown in Fig. 4F–H. Here we checked the NLRP3 expression because there are many studies in past that have elucidated the connection between colitis and inflammasome activation.

#### 3.4. RGE + EKN increased the immune cell subtypes in spleen tissue

There is a direct connection between the suppression of immune system with colitis occurrence. In fact one of the pathophysiological factors for the occurrence of colitis is an imbalance in the immune system. Keeping this in mind, we checked the immune cell subtypes in the spleen tissue of the DSS-induced colitic mice. As shown in Fig. 5A, RGE + EKN increased the levels of CD4<sup>+</sup> cells which were comparable to positive control sulfasalazine group. Moreover, the mixture group also increased the levels of CD19<sup>+</sup> B cells as shown in Fig. 5B. Furthermore there was also a significant increase in T regulatory (Treg) cells (CD4<sup>+</sup>CD25<sup>+</sup>) as well as Natural killer (NK, CD4<sup>+</sup>CD69<sup>+</sup>) cells as can be seen from Fig. 5C and D. These results suggest that the mixture of RGE + EKN not only alleviates the inflammation but also enhances the immune system of the animal to fight against infectious agents.

#### 4. Discussion

In our study we have used DSS-induced colitis model as it is the most widely used reference model in research to study the effects of different drugs, extracts, single compounds, or for simple mechanistic studies on colitis [22]. Many allopathic remedies are devised for the treatment of UC which includes steroidal anti-inflammatory drugs (SAIDs), nonsteroidal anti-inflammatory drugs (NSAIDs), targeted antibody blocking for cytokines primarily TNF- $\alpha$  and many other drugs [23]. A major factor that is associated with UC is the “Relapse”. Even if with the use of allopathic drugs, UC can be alleviated to the maximum level, there is 90% chances of relapse [24]. There are also many studies done in past that have elucidated the effectiveness of herbal extracts in preventing the relapse of UC [25–27].

Our main objective was to devise a combinational herbal remedy for the treatment of UC. It is because of the fact that when some extracts are mixed in some defined proportions, their active compounds give a rather more synergistic effect than the additive effect. We have shown that the RGE which is already extensively reported in past for its anti-inflammatory effects [28],

when mixed with EKN that is wonder extract as an antiviral agent [29], showed remarkable anticolitis effects.

Besides the already known NF- $\kappa$ B, MAPK and pro-inflammatory mediators and cytokines that are activated by both LPS and DSS [30–32], we have shown how the mixture of RGE + EKN had inhibitory effects on the inflammasome marker, NLRP3.

Ulcerative colitis is clinically and histologically marked with erosion of colonic mucosa, distortion of cryptic architecture, cryptic shortening, lymphocytosis, mucin depletion and Paneth cell metaplasia [33,34]. This is also accompanied by severe weight loss, and bloody diarrhea. In our results, the mixture as well as the single treatment of RGE + EKN markedly recovered the mice from the above mentioned microscopic and well as the macroscopic lesions of colon that had occurred as a result of DSS induced colitis.

NLRP3 is the top prioritized member of the NOD-like receptor (NLR) family which interacts with an adaptor protein (apoptosis-associated speck like protein (ASC)). Upon the stimulation with exogenous stimuli like LPS, or other toxins or environmental stressors, NLRP3, ASC, and procaspase 1 culminate to form a single

complex called the NLRP3 inflammasome which in turn causes the cleavage of procaspase 1; which terminates into the maturation of IL-1 $\beta$  and IL-18 [35,36]. There are many reports on NLRP3 that shows its involvement in the DSS induced colitis and that it is inhibited by natural/herbal compounds/extracts. For example, Sun et al showed that Wogonoside protected the mice against DSS induced colitis by inhibition of NF- $\kappa$ B and NLRP3 inflammasome pathway [37]. Similarly, Liu et al demonstrated that MALT1 inhibitors prevented the onset of DSS induced colitis by suppressing NF- $\kappa$ B pathway and NLRP3 inflammasome activation [30]. Furthermore, He et al reported that Alpinetin attenuated DSS induced colitis by depressing the TLR4 and NLRP3 signaling pathways [38]. In the light the previous literature, we therefore have explored the expression of NLRP3 inflammasome in our study.

Our one of the major findings in this study was that the mixture of RGE + EKN increased the numbers of immune cell subtypes (T-helper cells (CD4<sup>+</sup>, B cells CD19<sup>+</sup>, Tregs CD4<sup>+</sup>CD25<sup>+</sup> and NK cells CD4<sup>+</sup>CD69<sup>+</sup>) in the spleen tissue of DSS induced colitic mice. We had assessed the level of these cells in regard to the extensive past literature on the connection between the immunity and colitis. Immune response is initiated when T-helper cells (CD4<sup>+</sup>) or Cytotoxic T lymphocyte (CTL) (CD8<sup>+</sup>) cells recognize an antigen, mostly of bacterial/endotoxin origin [39]. Dereliction of the immune system to modulate these T cell responses can lead to the activation of macrophages, neutrophils, and CD8<sup>+</sup> cells. CD4 and CD8 cells naturally differentiate into Th1 and Th2 subpopulations of T cells; Th1 cell subtypes are important for the secretion of IL-1, IL-2, IL-12, IL-18, TNF- $\alpha$  and interferon-gamma (IFN $\gamma$ ). Elevation of these cytokines is primarily responsible for the clinical manifestations of ulcerative colitis (UC) and Crohn's disease (CD); however, UC is found to exhibit biasness towards the Th2 cell cytokine release (i.e., IL-4, IL-5 and IL-10). Furthermore, many studies have shown that Th2 cytokine release confers protection in DSS-induced colitis mice [40,41]. Onho et al have also demonstrated that nanoparticle of curcumin alleviated the experimental colitis by modifying the gut microbiota and elevation of Tregs [42]. Moreover it has also been reported by Zhang et al that Tregs depletion exacerbates DSS-induced colitis [43]. In our study we also found that NK cells activity was increased by RGE + EKN treatment. This result is in line with some previous findings where treatment of animals with natural compounds/extracts increased the activity of NK cells to counteract with colitis (For example Pan et al reported that black Raspberries enhanced the NK cell infiltration into colon and suppressed the incidence of colorectal cancer [44], Sauberman et al reported that activation of NK cells by alpha-galactosylceramide provides protection against colitis in mice [45]. And, similarly, Sagami et al demonstrated that choline deficiency caused natural killer (NK) cell deficiency that elevated murine colitis [46].

#### 5. Conclusion

In conclusion, to the best of our knowledge, this is the first study to demonstrate the effects of RGE, EKN and the combined mixture on DSS-induced colitis in mice. RGE + EKN synergistically inhibited the expression of NO, iNOS, COX-2, IL-1 $\beta$ , IL-6, IL-5, IL-13, and TNF- $\alpha$  in the macrophage cells, plasma and colon tissue of the DSS-induced colitis mice. Moreover, these attenuating effects were signaled via NF- $\kappa$ B and MAPK, and by NLRP3 suppression. The RGE + EKN group experienced synergistic recovery of the macroscopic parameters (DAI, colon length, and weight loss) and microscopic lesions in their colons. RGE + EKN also upregulated the CD4<sup>+</sup>, CD19<sup>+</sup>, Tregs and NK cells in mice, thereby exhibiting itself as potent anti-inflammatory and immune boosting extract for colitis.



This extract can be taken as a prophylactic/therapeutic supplement at times of suspension/presence of UC provided the further experiments guaranteed in humans.

### Data availability

All data generated or analyzed during this study are included in this article.

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### Declaration of Competing Interest

All the authors have no competing interests to declare.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jgr.2020.02.003>.

### References

- Loftus Jr EV, Silverstein MD, Sandborn WJ, Tremaine WJ, Harmsen WS, Zinsmeister AR. Ulcerative colitis in Olmsted County, Minnesota, 1940–1993: incidence, prevalence, and survival. *Gut* 2000;46:336–43.
- Hanauer SB. Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities. *Inflamm Bowel Dis* 2006;12(Suppl 1):S3–9.
- Bantel H, Berg C, Vieth M, Stolte M, Kruijs W, Schulze-Osthoff K. Mesalazine inhibits activation of transcription factor NF-kappaB in inflamed mucosa of patients with ulcerative colitis. *Am J Gastroenterol* 2000;95:3452–7.
- Kim JH. Pharmacological and medical applications of Panax ginseng and ginsenosides: a review for use in cardiovascular diseases. *J Ginseng Res* 2018;42:264–9.
- Lu JM, Yao Q, Chen C. Ginseng compounds: an update on their molecular mechanisms and medical applications. *Curr Vasc Pharmacol* 2009;7:293–302.
- Lee SM, Bae BS, Park HW, Ahn NG, Cho BG, Cho YL, Kwak YS. Characterization of Korean red ginseng (*Panax ginseng* Meyer): history, preparation method, and chemical composition. *J Ginseng Res* 2015;39:384–91.
- Park TY, Hong M, Sung H, Kim S, Suk KT. Effect of Korean Red Ginseng in chronic liver disease. *J Ginseng Res* 2017;41:450–5.
- Yoo DG, Kim MC, Park MK, Song JM, Quan FS, Park KM, Cho YK, Kang SM. Protective effect of Korean red ginseng extract on the infections by H1N1 and H3N2 influenza viruses in mice. *J Med Food* 2012;15:855–62.
- Yun TK, Lee YS, Lee YH, Kim SI, Yun HY. Anticarcinogenic effect of Panax ginseng C.A. Meyer and identification of active compounds. *J Korean Med Sci* 2001;16(Suppl):S6–18.
- Kim JY, Park JY, Kang HJ, Kim OY, Lee JH. Beneficial effects of Korean red ginseng on lymphocyte DNA damage, antioxidant enzyme activity, and LDL oxidation in healthy participants: a randomized, double-blind, placebo-controlled trial. *Nutr J* 2012;11:47.
- Ma H, He X, Yang Y, Li M, Hao D, Jia Z. The genus *Epimedium*: an ethnopharmacological and phytochemical review. *J Ethnopharmacol* 2011;134:519–41.
- Wang T, Zhang JC, Chen Y, Huang F, Yang MS, Xiao PG. Comparison of anti-oxidative and antitumor activities of six flavonoids from *Epimedium koreanum*. *Zhongguo Zhong Yao Za Zhi* 2007;32:715–8.
- Zhang W, Chen H, Wang Z, Lan G, Zhang L. Comparative studies on antioxidant activities of extracts and fractions from the leaves and stem of *Epimedium koreanum* Nakai. *J Food Sci Technol* 2013;50:1122–9.
- Jin Q, Lee C, Lee JW, Yeon ET, Lee D, Han SB, Hong JT, Kim Y, Lee MK, Hwang BY. 2-Phenoxychromones and prenylflavonoids from *Epimedium koreanum* and their inhibitory effects on LPS-induced nitric oxide and interleukin-1 $\beta$  production. *J Nat Prod* 2014;77:1724–8.
- Peng X-L, Shi J-X, Liao H-M. Effects of the extract of *epimedium* Koreanum Nakai on immune organs and reproductive organs in female Gong Chickens. *J Jishou Univ (Nat Sci Ed)* 2005;3:24.
- Jiang J, Song J, Jia X-b. Phytochemistry and ethnopharmacology of *Epimedium L.* species. *Chin Herb Med* 2015;7:204–22.
- Wang C, Feng L, Su J, Cui L, Dan L, Yan J, Ding C, Tan X, Jia X. Polysaccharides from *Epimedium koreanum* Nakai with immunomodulatory activity and inhibitory effect on tumor growth in LLC-bearing mice. *J Ethnopharmacol* 2017;207:8–18.
- Saba E, Jeong D, Irfan M, Lee YY, Park SJ, Park CK, Rhee MH. Anti-inflammatory activity of rg3-enriched Korean red ginseng extract in murine model of sepsis. *Evid Based Complement Alternat Med* 2018;2018:6874692.
- Sann H, Erichsen J, Hessmann M, Pahl A, Hoffmeyer A. Efficacy of drugs used in the treatment of IBD and combinations thereof in acute DSS-induced colitis in mice. *Life Sci* 2013;92:708–18.
- Fischer AH, Jacobson KA, Rose J, Zeller R. Hematoxylin and eosin staining of tissue and cell sections. *CSH Protoc* 2008;2008. pdb prot4986.
- Geboes K, Riddell R, Öst A, Jensfelt B, Persson T, Löfberg R. A reproducible grading scale for histological assessment of inflammation in ulcerative colitis. *Gut* 2000;47:404–9.
- Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (DSS)-induced colitis in mice. *Curr Protoc Immunol* 2014;104. Unit 15 25.
- Meier J, Sturm A. Current treatment of ulcerative colitis. *World J Gastroenterol* 2011;17:3204–12.
- Fell JM, Muhammed R, Spray C, Crook K, Russell RK, group Blw. Management of ulcerative colitis. *Arch Dis Child* 2016;101:469–74.
- Han KH, Park JM, Jeong M, Han YM, Go EJ, Park J, Kim H, Han JG, Kwon O, Hahm KB. Heme oxygenase-1 induction and anti-inflammatory actions of *atractylodes macrocephala* and *Taraxacum herba* extracts prevented colitis and was more effective than sulfasalazine in preventing relapse. *Gut Liver* 2017;11:655–66.
- Kim KJ, Park JM, Lee JS, Kim YS, Kangwan N, Han YM, Kang EA, An JM, Park YK, Hahm KB. Oligonol prevented the relapse of dextran sulfate sodium-ulcerative colitis through enhancing NRF2-mediated antioxidative defense mechanism. *J Physiol Pharmacol* 2018;69.
- Li YH, Zhang M, Xiao HT, Fu HB, Ho A, Lin CY, Huang Y, Lin G, Bian ZX. Addition of berberine to 5-aminosalicylic acid for treatment of dextran sulfate sodium-induced chronic colitis in C57BL/6 mice. *PLoS One* 2015;10:e0144101.
- Baek KS, Yi YS, Son YJ, Yoo S, Sung NY, Kim Y, Hong S, Aravinthan A, Kim JH, Cho JY. In vitro and in vivo anti-inflammatory activities of Korean Red Ginseng-derived components. *J Ginseng Res* 2016;40:437–44.
- Cho WK, Weeratunga P, Lee BH, Park JS, Kim CJ, Ma JY, Lee JS. *Epimedium koreanum* Nakai displays broad spectrum of antiviral activity in vitro and in vivo by inducing cellular antiviral state. *Viruses* 2015;7:352–77.
- Liu W, Guo W, Hang N, Yang Y, Wu X, Shen Y, Cao J, Sun Y, Xu Q. MALT1 inhibitors prevent the development of DSS-induced experimental colitis in mice via inhibiting NF-kappaB and NLRP3 inflammasome activation. *Oncotarget* 2016;7:30536–49.
- Yin J, Wu M, Duan J, Liu G, Cui Z, Zheng J, Chen S, Ren W, Deng J, Tan X, et al. Pyrrolidine Dithiocarbamate inhibits NF-KappaB activation and upregulates the expression of Gpx1, Gpx4, occludin, and ZO-1 in DSS-induced colitis. *Appl Biochem Biotechnol* 2015;177:1716–28.
- You BH, Chae HS, Song J, Ko HW, Chin YW, Choi YH. alpha-Mangostin ameliorates dextran sulfate sodium-induced colitis through inhibition of NF-kappaB and MAPK pathways. *Int Immunopharmacol* 2017;49:212–21.
- Kornbluth A, Sachar DB. Practice parameters committee of the American College of G. Ulcerative colitis practice guidelines in adults: American College of gastroenterology, Practice parameters committee. *Am J Gastroenterol* 2010;105:501–23. quiz 524.
- Magro F, Langner C, Driessen A, Ensari A, Geboes K, Mantzaris GJ, Villanacci V, Becheanu G, Borralho Nunes P, Cathomas G, et al. European consensus on the histopathology of inflammatory bowel disease. *J Crohns Colitis* 2013;7:827–51.
- Abderrazak A, Syrovets T, Couchie D, El Hadri K, Friguet B, Simmet T, Rouis M. NLRP3 inflammasome: from a danger signal sensor to a regulatory node of oxidative stress and inflammatory diseases. *Redox Biol* 2015;4:296–307.
- Sutterwala FS, Haasken S, Cassel SL. Mechanism of NLRP3 inflammasome activation. *Ann N Y Acad Sci* 2014;1319:82–95.
- Sun Y, Zhao Y, Yao J, Zhao L, Wu Z, Wang Y, Pan D, Miao H, Guo Q, Lu N. Wogonoside protects against dextran sulfate sodium-induced experimental colitis in mice by inhibiting NF-kappaB and NLRP3 inflammasome activation. *Biochem Pharmacol* 2015;94:142–54.
- He X, Wei Z, Wang J, Kou J, Liu W, Fu Y, Yang Z. Alpinetin attenuates inflammatory responses by suppressing TLR4 and NLRP3 signaling pathways in DSS-induced acute colitis. *Sci Rep* 2016;6:28370.
- Schuler T, Qin Z, Ibe S, Noben-Trauth N, Blankenstein T. T helper cell type 1-associated and cytotoxic T lymphocyte-mediated tumor immunity is impaired in interleukin 4-deficient mice. *J Exp Med* 1999;189:803–10.
- Alex P, Zachos NC, Nguyen T, Gonzales L, Chen TE, Conklin LS, Centola M, Li X. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. *Inflamm Bowel Dis* 2009;15:341–52.

- [41] Dieleman LA, Palmen MJ, Akol H, Bloemena E, Pena AS, Meuwissen SG, Van Rees EP. Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. *Clin Exp Immunol* 1998;114:385–91.
- [42] Ohno M, Nishida A, Sugitani Y, Nishino K, Inatomi O, Sugimoto M, Kawahara M, Andoh A. Nanoparticle curcumin ameliorates experimental colitis via modulation of gut microbiota and induction of regulatory T cells. *PLoS One* 2017;12:e0185999.
- [43] Zhang HL, Zheng YJ, Pan YD, Xie C, Sun H, Zhang YH, Yuan MY, Song BL, Chen JF. Regulatory T-cell depletion in the gut caused by integrin beta7 deficiency exacerbates DSS colitis by evoking aberrant innate immunity. *Mucosal Immunol* 2016;9:391–400.
- [44] Pan P, Kang S, Wang Y, Liu K, Oshima K, Huang YW, Zhang J, Yearsley M, Yu J, Wang LS. Black Raspberries enhance natural killer cell infiltration into the colon and suppress the Progression of colorectal cancer. *Front Immunol* 2017;8:997.
- [45] Saubermann LJ, Beck P, De Jong YP, Pitman RS, Ryan MS, Kim HS, Exley M, Snapper S, Balk SP, Hagen SJ, et al. Activation of natural killer T cells by alpha-galactosylceramide in the presence of CD1d provides protection against colitis in mice. *Gastroenterology* 2000;119:119–28.
- [46] Sagami S, Ueno Y, Tanaka S, Fujita A, Niitsu H, Hayashi R, Hyogo H, Hinoi T, Kitadai Y, Chayama K. Choline deficiency causes colonic type II natural killer T (NKT) cell loss and alleviates murine colitis under type I NKT cell deficiency. *PLoS One* 2017;12:e0169681.