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ORIGINAL RESEARCH

Zanthoxylum bungeanum-Derived Nanobiotics for Effective Against Ulcerative Colitis in Mouse Model

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Introduction: Growing research is devoted to the development of plant-derived products as new therapeutic drugs to reduce side effects. Plant-derived exosome-like nanoparticles (ELNs) have shown promising potential in the treatment of colitis.

Methods: As a proof of concept, the efficacy of ELNs from edible *Zanthoxylum bungeanum* (ZbELNs) in protecting macrophages from inflammation was determined by in vitro experiments. Moreover, we assess the therapeutic effect of ZbELNs to colitis in a mouse model.

Results: ZbELNs were found to have an ideal particle size (160.0 nm) and contain a large number of lipids, some functional proteins or metabolites, and many small RNA molecules. The in vitro experiment results revealed that ZbELN pretreatment increased cell vitality and decreased the levels of pro-inflammatory cytokines. Furthermore, the in vivo experiments indicated that oral administration of ZbELNs can significantly reduce disease activity index, increase colon length, and inhibit colon wall thickening, thereby alleviating acute colitis in dextran sulfate sodium-induced model mice. In addition, ZbELN treatment can reduce the degree of histological damage in the colon and suppress pro-inflammatory cytokines levels in mice serum. Notably, miRNA-1 and miRNA-21 in ZbELNs showed similar therapeutic effects on macrophage inflammation.

Conclusion: These findings suggest that ZbELNs are a novel natural nanomedicine with promising therapeutic potential for the treatment of colonic diseases.

Keywords: exosome-like nanoparticles, colitis, inflammation, *Zanthoxylum bungeanum*, disease activity index

Introduction

Inflammatory bowel disease (IBD) primarily includes ulcerative colitis and Crohn's disease. The condition affects millions of people worldwide and places a heavy burden on healthcare systems.[1] Ulcerative colitis and Crohn's disease are characterized by symptoms such as diarrhea, abdominal pain, rectal bleeding, and weight loss.[2] Notably, the pathology of IBD involves a large number of pro-inflammatory cytokines and reactive oxygen species, which may lead to impaired intestinal barrier function and intestinal microbiota imbalance.[3–5] These complications may increase the risk of colon cancer.[6] Conventional treatments for IBD include systemic glucocorticoids and 5-aminosalicylic acid to reduce inflammation.[7] Despite their therapeutic effects, these drugs also have strong toxic side effects on healthy organs, such as allergic reactions and nausea, which limit their clinical application.[8]

In recent years, a growing number of studies have investigated the efficacy of exosome therapeutics targeting colitis tissue and colon tumors. As nanoscale bioactive substances of natural food sources, plant exosomes have good biocompatibility and oral safety and can cross biological barriers, reduce metabolic responses, and have potential therapeutic effects on IBD. Studies have shown that plants act on mammalian cells (especially intestinal macrophages) through exosome-like nanoparticles; notably, exosomes of different plants exert different biological effects.[9] For example, exosome-like nanoparticles derived from grapes and grapefruits target intestinal cells and can protect mice

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from colitis induced by dextran sulfate sodium (DSS).[10,11] A previous study found that hydrophobic curcumin can be transferred to colon tumors and normal colon tissues through plant exosomes, and exosomes can be used as carriers for cell treatment of carcinogenic miRNA.[12]

Zanthoxylum bungeanum (Zb) refers to the fruit of various kinds of Sichuan pepper in the Rutaceae family, which is widely used in Chinese cuisine for its spicy flavor and medicinal properties. Fresh Zb has a very high essential oil content of up to 11% and is described as having a fresh, spicy, floral, cool, and green aroma. Modern pharmacological studies have shown that Zb has analgesic, anti-inflammatory, hepatoprotective, antioxidant, anti-tumor, hypoglycemic, anti-Alzheimer's disease, and mosquito-repelling properties.[13–15] At present, the effect and mechanism of *Zb* on IBD remain unclear.

Various animal models of colonic inflammation have been established, exhibiting similar pathological features to IBD. Animal models of colitis can be induced by certain sensitive strains to the need to give specific concentrations of chemicals, such as DSS. At present, the most widely used IBD model is the DSS-induced intestinal inflammation model.[16] DSS administration in drinking water can reproduce acute or chronic colitis depending on the application; notably, animals given DSS showed signs of weight loss, loose stools or diarrhea, and even rectal bleeding.[17,18] The extent of inflammatory response in the colon can be assessed by clinical symptoms or histological changes. Clinical symptoms can be assessed using the disease activity index (DAI), which correlates with weight loss, stool hardness, and bleeding degree.[19] In addition, disease severity is associated with the expression levels of pro-inflammatory cytokines such as interleukin (IL) −6, IL-1β, and tumor necrosis factor (TNF)–α.[20]

In the present study, oral *Zb*-derived exosome-like nanoparticles (ZbELNs) were hypothesized to exert therapeutic effects on IBD, and the anti-inflammatory function of ZbELNs was analyzed in vivo and in vitro. Moreover, the therapeutic effect of ZbELNs on IBD symptoms, such as disease activity index, the expression levels of pro-inflammatory cytokines, colonic phenotype, and histopathology, were analyzed.

Materials and Methods

The Isolation of ZbELNs

Fresh fruit from the *Zb* tree was collected from Hanyuan County, Sichuan Province, China in August. The collected plant materials were identified by Professor Yuntong Ma from Chengdu University of Traditional Chinese Medicine and stored in the Herbarium of Institute of Botany, Chinese Academy of Sciences (PE 02021640). The fruits were washed with water, placed in cold phosphate buffer saline (PBS, pH 7.4), and homogenized in a blender. ZbELNs were isolated by differential centrifugation according to our previous study.[21] The diameter and concentration of ZbELNs were determined by nanoparticle tracking analysis. The resultant ZbELNs were stored at −80°C.

The Compositional Analysis of ZbELNs

For ZbELN lipid composition analysis, the total lipids of ZbELNs were extracted using a methanol/methyl tert-butyl ether (1:3, v/v) mixed solvent. Moreover, the lipidomic analysis of ZbELNs was performed by liquid chromatography with tandem mass-spectrometry (LC-MS) (Applied Biosystems, Foster City, USA).

For proteomics analysis, total protein was isolated from ZbELNs and analyzed by LC-MS. The proteomic analysis was performed by Duolaimi biotechnology Technology Co., Ltd (Wuhan, China).

For the microRNA (miRNA) sequencing, total miRNA was extracted from ZbELNs using the miRcute miRNA Isolation Kit (TianGen, Beijing, China), and 10 ng per sample miRNA was used as input for library preparation with TruseqTM Small RNA sample prep Kit from Illumina (San Diego, CA). Using the Illumina NovaSeq 6000 sequencer to sequence the library. Library construction and sequencing were completed by Majorbio (Shanghai, China).

Cellular and Intestinal Uptake Analysis of ZbELNs

For internalization of ZbELNs into cells, cells were exposed to 1×10^{10} particles mL^{-1} DiI (AAT Bioquest, USA) stained ZbELNs for 24 h, and the cells were fixed with 4% paraformaldehyde (Wako, Japan). Nuclei and cytoskeleton were then stained by 4',6'-diamidino-2-phenylindole (DAPI) (Beyotime, Shanghai, China) and Phalloidin-FITC (1:100, Abcam,

UK), respectively. Finally, a fluorescence microscope (Leica, Wetzlar, Germany) was utilized to analyze the cellular uptake.

Furthermore, ZbELN uptake by gastrointestinal tissue was determined by an in vivo experiment. Healthy mice were orally administered with DiR-loaded ZbELNs (1×10^{11} particles/kg) for 2 h, and the mice were imaged by the IVIS Lumina LT Series III system (PerkinElmer, Hopkinton, USA). Finally, the mice were sacrificed and their gastrointestinal tissues were imaged.

The in vitro Anti-Inflammatory Activity of ZbELNs

Differentiation of the human THP-1 monocytes (Procell, Wuhan, China) into macrophages was induced by 100 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich, USA) treatment for 24 h. THP-1 macrophages were seeded in 96-well plates at a density of 3×10^4 cells/well and incubated with different concentrations of ZbELNs. After incubation for 24 h, cells were stimulated with 2 μg/mL lipopolysaccharide (LPS; Sigma-Aldrich, USA) for 24 h to induce inflammation. Subsequently, cell vitality was tested by using a Cell Counting Kit-8 (Beyotime, Beijing, China), and the expression of inflammation-related cytokine genes was analyzed using reverse transcription-quantitative PCR (RT-qPCR).

The in vivo Studies Using Mouse Model

Male C57BL/6J mice weighing 18–22g were purchased from Dossy Experimental Animal Ltd. (Chengdu, China) and acclimatized for 10 days. The mice were kept under a 12 h dark/light cycle at 25 °C. The animal diet ingredients are listed in Supplementary Table 1. For experiments, 32 mice were randomly divided into four groups, including the negative control, positive control, CELNs, and RELNs groups (n = 8 per group). Mice in the CELNs and RELNs groups were administered ZbELNs (1×10^{11} particles/kg/day) by gavage for 17 days. Between days 8 and 14, 2% (w/v) DSS (36000–50000 Da) (MP Biochemicals, Santa Ana, USA) was added to the drinking water of the mice in the positive control, CELNs and RELNs groups.

To examine the toxicity effect of ZbELNs on normal mice. 24 healthy male C57BL/6J mice were randomly divided into four groups, including the control, CELNs, and RELNs groups (n = 8 per group). Mice in the CELNs and RELNs groups were administered ZbELNs (1×10^{11} particles/kg/day) by gavage for 7 days while the control group were given an equal volume of PBS. After oral administration of ZbELNs, the mice were anesthetized and subsequently euthanized. Serum samples and visceral organs (including the liver, heart, spleen, lung, kidney, and ileum) were harvested for function assessment, which involved the analysis of liver function, kidney function, myocardial enzyme spectrum levels, along with histological evaluation via hematoxylin and eosin (H&E) staining.

The Evaluation of Colitis

The parameters and scoring criteria for the DAI are shown in Supplementary Table 2.[19] Colon tissue samples were fixed in 4% paraformaldehyde fixation and paraffin sections (4 μm thickness), and stained with H&E staining. The histopathologic score of colon tissue was calculated by four parameters, as displayed in Supplementary Table 3.[22]

Enzyme-Linked Immunosorbent Assay of Pro-Inflammatory Factor Levels

The serum was extracted from the blood sample, and the levels of IL-6, IL-1β, and TNF-α were evaluated using enzyme-linked immunosorbent assay kits (Neobioscience, Shenzhen, China). The activity of liver function (glutamic pyruvic transaminase and aspartate aminotransferase), kidney function (creatinine and urea: serum urea), and myocardial enzyme spectrum (creatine kinase, creatine kinase isoenzyme, α-hydroxybutyrate dehydrogenase, and lactate dehydrogenase) in serum was measured by an automatic biochemical analyzer (Beckman, California, USA) following the manufacturer's methods.

RT-qPCR Assays

THP-1 macrophages were seeded in 6-well plates at a density of 5×10^5 cells/well and incubated with $1x10^8$/mL ZbELNs for 24 h. Thereafter, the cells were treated with or without 2 μg/mL LPS for another 24 h. Total RNA was extracted from the cells using Trizol (TianGen, Beijing, China), and 1 microgram of RNA was used for reverse transcription with M-MLV reverse transcriptase (Vazyme, Nanjing, China). The gene expression levels of inflammation-associated genes were analyzed using the

RT-qPCR Mix (Vazyme, Nanjing, China) in a Roche quantitative instrument (Rotkreuz, Switzerland). The following reaction conditions were set: 95°C for 1 min, followed by 40 cycles at 94°C for 15s and 56°C for 35s. The primers are shown in Supplementary Table 4.

The Metabolite Profiling Analysis in ZbELNs

The metabolites of ZbELNs were quantified by LC-MS and analyzed as described.[23,24] In addition, the ProteoWizard and Ropls software were used to construct principal components analysis (PCA) and heat maps. Significantly altered metabolites were defined as previously described.[25,26]

Statistical Analysis

All data were analyzed using IBM SPSS version 25.0 and OriginLab Corporation version 9.0, and the data were presented as means ± standard deviation (SD). One-way ANOVA was employed to compare the differences between groups, and a P value greater than 0.05 was considered not statistically significant.

Results

The Characterization of ZbELNs

To investigate ELNs in the fruit of *Zb*, ELNs from immature cyan fruit (CELNs) and ELNs from mature red fruit (RELNs) were extracted by differential centrifugation (Figure 1A). The transmission electron microscope images revealed that CELNs and RELNs have a membrane-enclosed vesicle-like structure and vary in size between 100–300 nm (Figure 1B). The diameter and concentration of CELNs and RELNs were determined by nanoparticle tracking analysis. The average diameter of CELNs and RELNs was 164 and 162 nm, respectively (Figure 1C). These results are consistent with the transmission electron microscope results, and the concentration of CELNs and RELNs isolated from 10 g of fruit was 1.1×10^{11} and 7×10^{10} particles/mL, respectively.

The lipidomic results indicated that ZbELNs (there was no significant difference in content between RELNs and CELNs) were enriched with triacylglycerol (TG), diacylglycerol (DG), free fatty acid (FFA), phosphatidylethanolamine (PE), phytoceramide (Cert), phosphatidylglycerol (PG), digalactosyldiacylglycerol (DGDG), and phosphatidylcholine (PC) (Figure 1D and Supplementary Table 5). These are common lipid components in ELNs. Based on the present liposome delivery system, these lipids in ZbELNs greatly promote the formation of nanoparticles and maintain its spherical structure.

Next, protein composition analysis revealed the presence of some membrane proteins and cytosolic proteins in ZbELNs, such as lipid-transfer protein, proton antiporter, ATP synthase subunit, and ribosomal subunit protein (Figure 1E and Supplementary Table 6). These membrane-associated proteins and cytosolic proteins maintain the nanoparticles-like properties of ZbELNs.[27]

Intestinal and Cellular Uptake of ZbELNs

The accumulation of orally ingested ELNs in the intestine represents a promising therapeutic option in the treatment of IBD.[12] The intestinal accumulation of oral DiR-labeled CELNs and RELNs in healthy mice were determined. Notably, the fluorescence intensity of CELNs and RELNs in the digestive tract, especially in colon tissue, was significantly enhanced compared with the control group (Figure 2A–D).

Furthermore, the uptake of CELNs and RELNs by human cells was assessed. THP-1 macrophage cells were treated with DiI-labeled CELNs and RELNs. The cell cytoskeleton was stained with phalloidin-FITC (green), and nuclei were stained with DAPI (blue). Microscope analysis revealed that the CELNs and RELNs were widely distributed throughout the cell cytosol and surrounding the nucleus, implying that both CELNs and RELNs can be absorbed by human cells (Figure 2E).

In vitro Anti-Inflammatory Activity of ZbELNs

In order to investigate the regulatory role of ZbELNs on macrophages after ingestion, the effect of ZbELNs on THP-1 proliferation was evaluated. As shown in Supplementary Figure 1, low concentration of ZbELNs ($<10^8$ particles/mL)

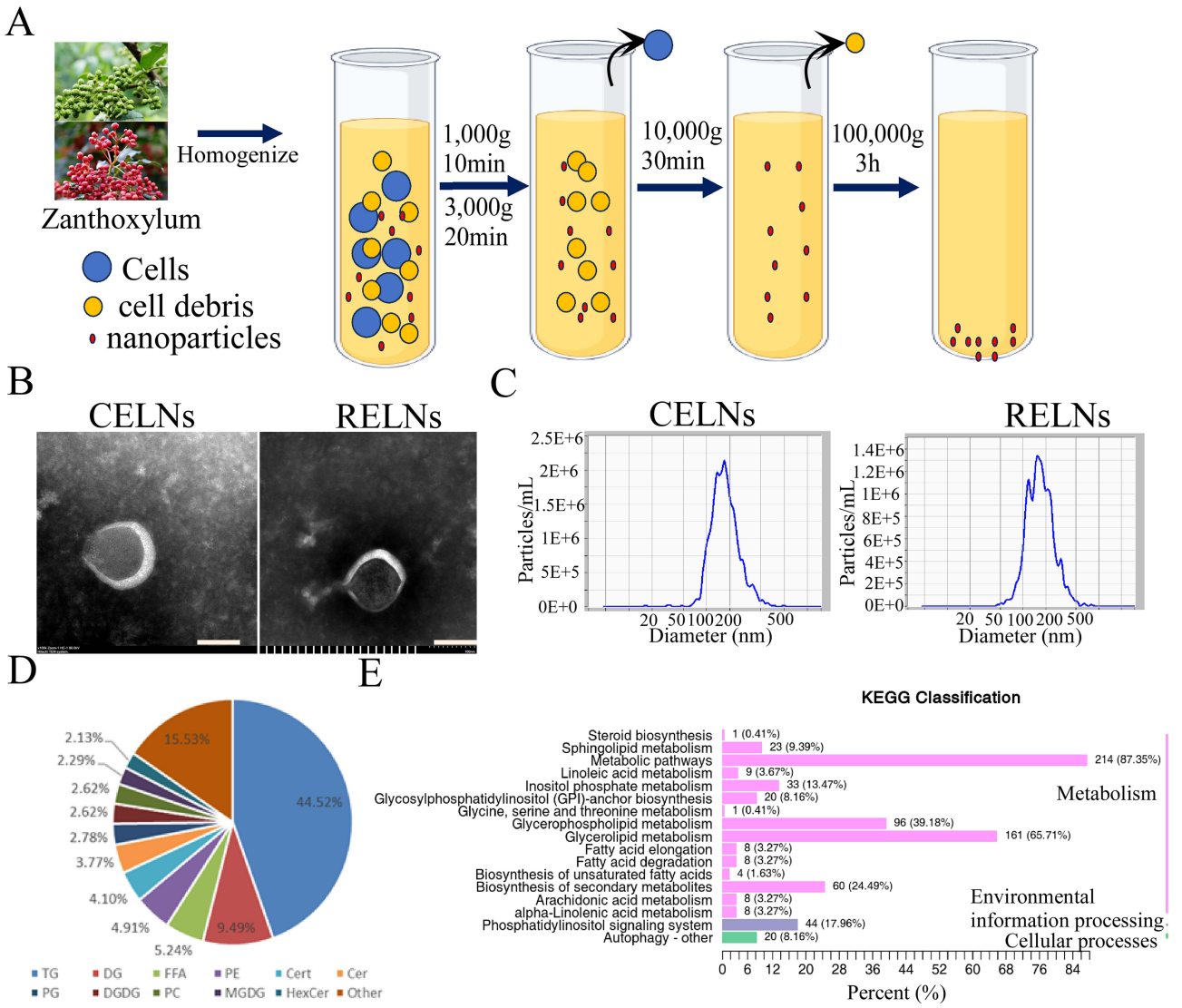

Figure 1 Isolation and characterization of *Zanthoxylum bungeanum*-derived exosome-like nanoparticles (ZbELNs). (**A**) The isolation scheme of ZbELNs. (**B**) ZbELNs were examined by transmission electron microscopy. Magnification: 120000X. Scale bar = 100 nm. (**C**) The particle size of ZbELNs was analyzed by nanoparticle tracking analysis (NTA). The sample (ZbELNs) was diluted 2000 times of the original sample for NTA detection, and the NTA concentration was the total number obtained by statistics on the number of particles under different nanoparticle sizes. (**D**) The lipid profiles of ZbELNs under positive and negative mode. The lipid composition of ZbELNs was determined by using a triple quadrupole mass spectrometer. The data are as percent ages of total signal for the molecular species determined after normalization of the signals in internal standards of the same lipid class. (**E**) The Kyoto Encyclopedia of Genes and Genomes (KEGG) classification of the proteins contained in ZbELNs.
Abbreviations: TG, triacylglycerol; DG, diacylglycerol; FFA, free fatty acid; PE, phosphatidylethanolamine; Cert, phytoceramide; Cer, ceramide; PG, phosphatidylglycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; MGDG, monogalactosyldiacylglycerol; HexCer, hexosylceramide.

exerted no significant influence or toxic effect on macrophage proliferation, while the IC_{50} values of CELNs and RELNs were $4.56*10^8$ and $5.31*10^8$ particles/mL, respectively.

Then, we evaluated the anti-inflammatory activity of ZbELNs in macrophages. The THP-1 cells pre-treated with ZbELNs showed restored features compared with the positive control group; the cell vitality increased with the increase in concentration of ZbELNs ($<10^8$ particles/mL) (Figure 3A and B). These findings suggest that ZbELNs can partially counteract the LPS-induced decrease in cell viability. Based on the results of the concentration gradient test, ZbELNs with a concentration of 1×10^8 particles/mL were used as the effective drug concentration for the cell test (the cell viability was maintained at about 85% and cytotoxicity was minimal).

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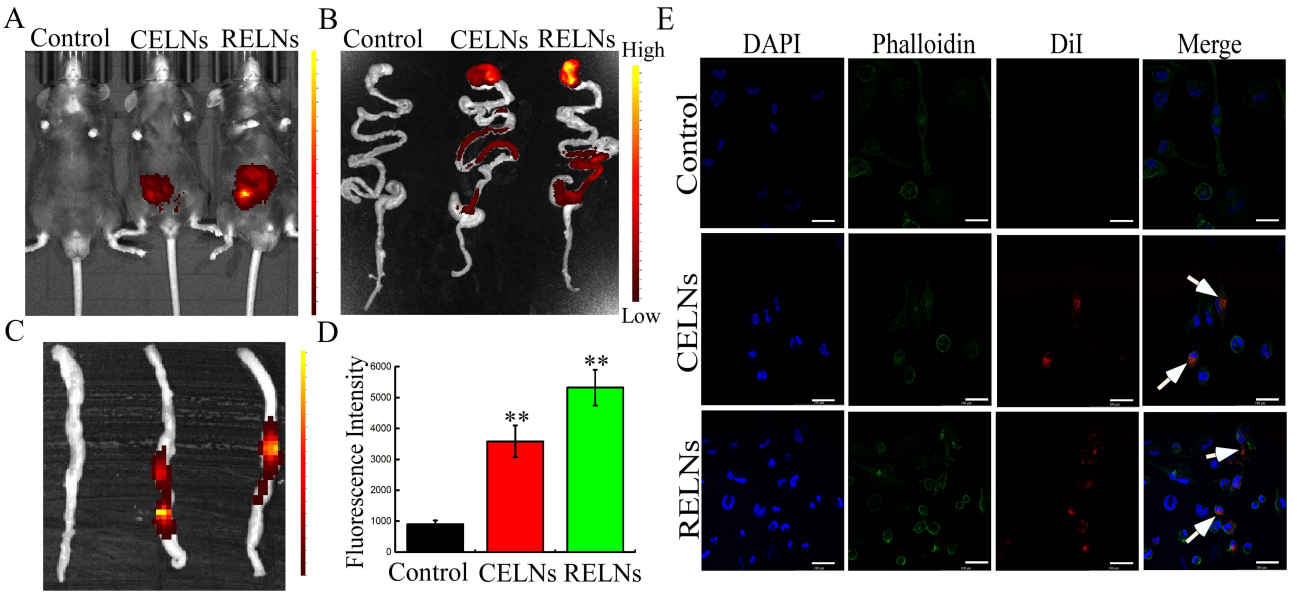

Figure 2 In vivo distribution and in vitro cell internalization of DiR-labeled ZbELNs. (**A**) Whole body imaging of the mice (healthy, LNVs treated and SNVs treated) at 2 h after oral administration of DiR-labeled ZbELNs (1×10^{11} particles/kg) by IVIS Spectrum Series imaging system. (**B**) The distribution of DiR-labeled ZbELNs in the gastrointestinal tract captured by the IVIS system at 2 h after gavage. (**C**) The distribution of DiR-labeled ZbELNs in the distal colons captured by the IVIS system at 2 h after gavage. (**D**) The relative fluorescence intensity in (**C**) was quantified using Image J software. Data are shown as the mean ± SD (n=3). **$p < 0.01$ compared to the control group. (**E**). Representative fluorescence images of cellular uptake of DiI-labeled ZbELNs in macrophages. Cells incubated with DiI-CELNs or DiI-RELNs and then labeled with DAPI and phalloidin-FITC. DiI staining indicates lipophilic staining that marks the ZbELNs. Phalloidin-FITC staining indicates actin filaments. The merged images show the colocalization of the ZbELNs (red) with the cell cytoskeleton (green) and the cell nuclei (blue). Representative uptake of ZbELNs are indicated by white arrows. Magnification: 200X. Scale bars = 20 μm.

In addition, the expression of inflammation cytokines IL-6, IL-1β, IL-8, and TNF-α was analyzed,[28] revealing that ZbELNs could significantly reduce the expression of these cytokines in the macrophage inflammation model (Figure 3C–F). The results suggested that ZbELNs could protect macrophages from inflammation.

Retardation of IBD Progression by ZbELNs

The therapeutic effects of ZbELNs on IBD was examined. Acute intestinal inflammation in mice was induced by 1.5%, 2.0% and 2.5% dextran sodium sulfate (DSS), establishing the mouse IBD model (the modeling process lasted for 7 days and the drug was discontinued). As shown in Supplementary Figure 2, 2.0% and 2.5% DSS treatments significantly reduced body weight and increased DAI from day 6 onwards. Meanwhile, 2.0% and 2.5% DSS treatment significantly reduced the colon length of mice, while 1.5% DSS treatment showed no significant effect on the colon phenotype of mice. On day 9, 2.5% of the DSS-treated mice had partially died, so 2.0% DSS for 7 days was used to establish the mouse IBD model in this project.

Figure 4A shows the scheme for the experimental investigation. The ZbELN treatment group was found to be dramatically higher than body weight as a positive control (Figure 4B). DAI can use to assess the severity of IBD. Compared with the negative control group, the weight of mice in the model group was significantly reduced, with more severe loose stools and blood in stools, resulting in a significant increase in DAI. However, the ZbELNs treatment group exhibited relieved loose stools and blood in stool compared to the model group, with a significant reduction in DAI (Figure 4C). Colon shortening is a key parameter in evaluating the severity of IBD. As shown in Figure 4D and E, the colon of the model group was dark brown due to colon injury and congestion, and the length was significantly shorter than that of the negative control group. In contrast, the ZbELNs treatment group showed a lesser degree of colon congestion and longer colon length. These findings indicate that ZbELNs can effectively prevent colon damage caused by DSS.

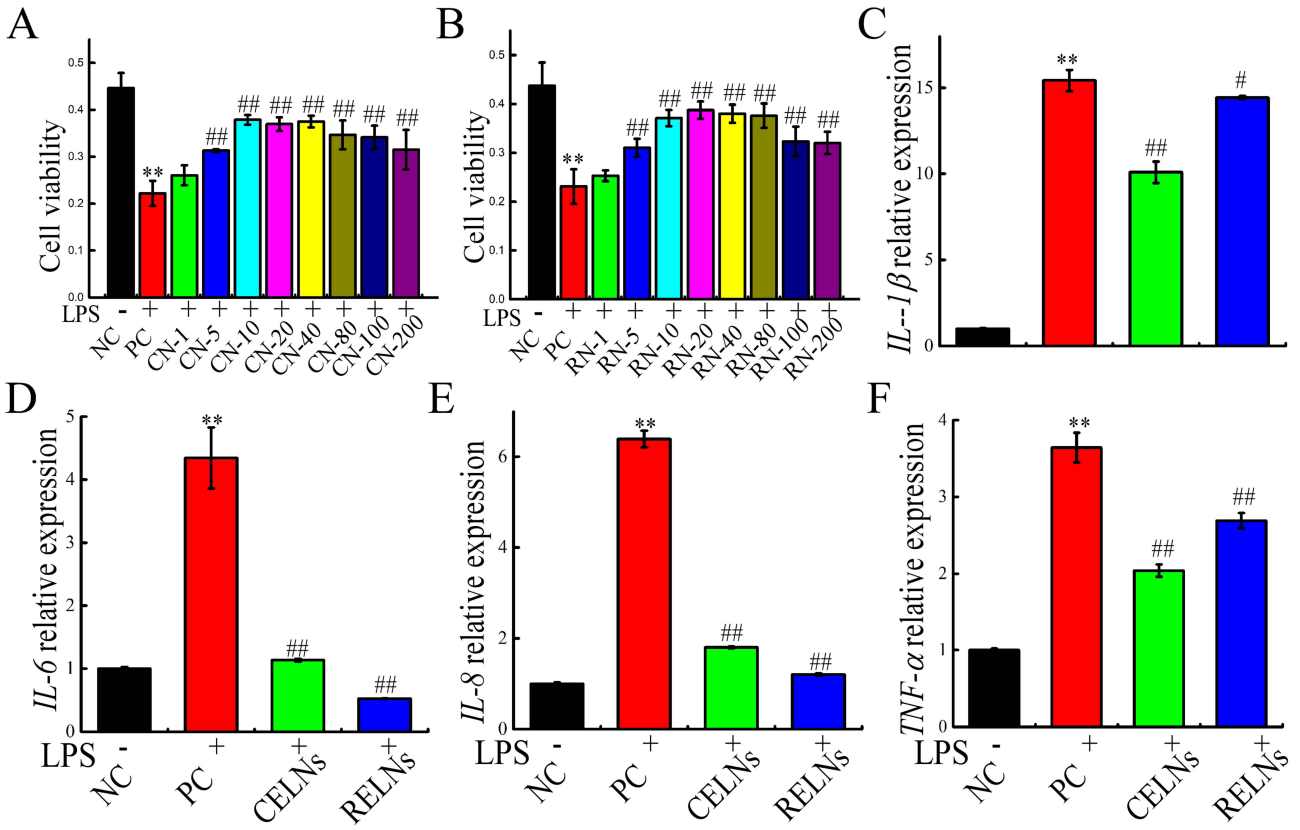

Figure 3 In vitro anti-inflammatory properties of ZbELNs. (**A** and **B**). The CELNs (**A**) and RELNs (**B**) dose experiments to the cell vitality of LPS stimulated macrophages. Different amounts of ELNs ($1x10^7/5x10^7/1x10^8/2x10^8/4x10^8/8x10^8/1x10^9/2x10^9$ particles/mL, represented by CN/RN-1/5/10/20/40/80/100/200) were added to macrophages for 48 h. The cell vitality was tested by Cell Counting Kit-8. (**C**–**F**) Result of inflammation-related cytokine IL-1β (**C**), IL-6 (**D**), IL-8 (**E**), and TNF-α (**F**) mRNA expression in LPS stimulated macrophages. Data were expressed as the mean ± SD (n=3). **p < 0.01 compared to the negative control group (NC); ##p < 0.01 and #p < 0.05 compared to the positive control group (PC).

Effects of ZbELNs on the Pathological Changes of Colon Tissue in IBD

As shown in Figure 5A, the H&E staining revealed severe mucosal damage and large inflammatory cell infiltration in the model group compared to the negative control group. However, ZbELN treatment improved the mucosal injury and inflammatory infiltrates in IBD mice. Further histological scoring of colon tissue showed that ZbELN treatment significantly decreased the score of colons (Figure 5B). To evaluate the anti-inflammatory effect of ZbELNs, the level of cytokines IL-6, TNF-α, and IL-1β in mouse serum was tested.[20] The results indicated significantly elevated levels of IL-6, TNF-α, and IL-1β in the model group compared to the negative control group (Figure 5C–E), while oral administration of ZbELNs significantly decreased secretion of these cytokines.

High production of pro-inflammatory cytokines can disrupt the integrity of the intestinal barrier.[3] Therefore, we examined whether ZbELNs restores the intestinal barrier by regulating the expression of genes (ZO-1, Occludin, and Claudin1) involved in intestinal barrier function. As shown in Supplementary Figure 3, ZO-1, Occludin, and Claudin1 mRNA expression levels in the colon from the IBD mice were dramatically lower than those control group. While treatment with ZbELNs strongly up-regulated these genes expression levels. These findings suggest that ZbELNs may effectively prevent and ameliorate intestinal barrier damage associated with IBD.

The Metabolite Profiling Analysis in ZbELNs

To uncover anti-inflammatory molecules in ZbELNs, the metabolite profile of ZbELNs was quantified by LC-MS metabolite profiling. A total of 182 metabolites were identified (Supplementary Table 7). The difference in metabolite accumulation between the CELNs and RELNs was analyzed, revealing several metabolites involved in anti-inflammatory activity, including 3-(2-hydroxyphenyl) propanoic acid, mesaconate, 6-hydroxyhexanoic acid, and D-ribose (Figure 6).

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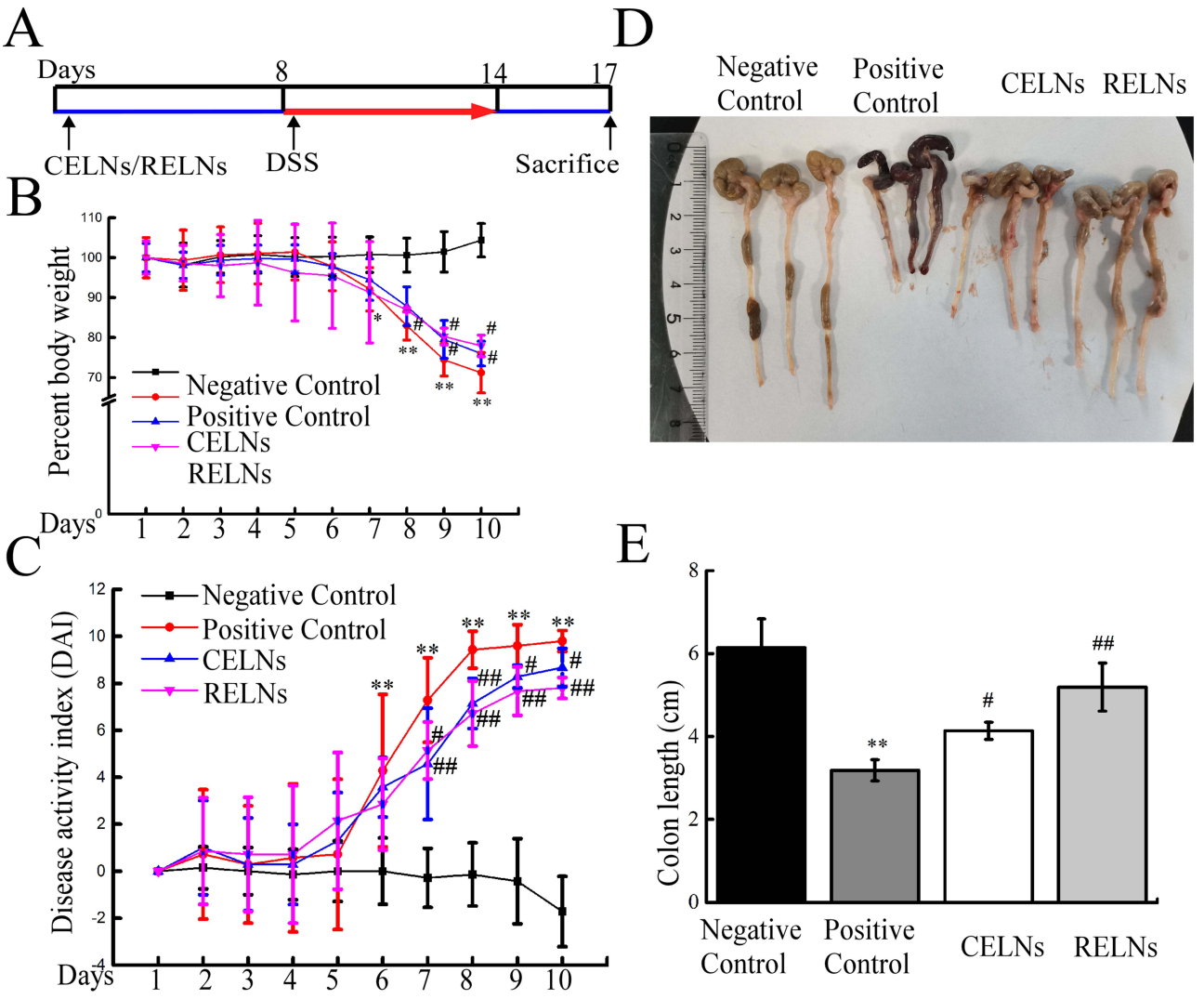

Figure 4 ZbELNs ameliorated the progression of DSS-induced mice colitis. (**A**) Experimental design to test the effects of ZbELNs on DSS-induced colitis in mice (n = 8/group). (**B**) Result of body weight on ZbELNs treatment in DSS-induced colitis. (**C**) Disease activity index (DAI) scores in each group. (**D**) Representative images of colon length in each group. (**E**) Statistical analysis of colon length in (**D**). Data were expressed as the mean ± SD (*n*=8). **p < 0.01 and *p < 0.05 compared to the negative control group; ##p < 0.01 and #p < 0.05 compared to the positive control group.

Notably, 3-(2-hydroxyphenyl) propanoic acid enhances the anti-ulcer effect by promoting blood circulation in the stomach.[29] Mesaconic acid can reduce the secretion of interleukin (IL)-6 and IL-12, thereby playing an immunomodulatory role in pro-inflammatory macrophages.[30] In addition, 6-hydroxyhexanoic acid can inhibit the production of pro-inflammatory cytokines in adipocytes and lipolysis, improving abnormal inflammation in obese mice.[31] D-ribose acts as a component of purines and pyrimidines to replenish cellular energy.[32]

miR-1 and miR-21 in ZbELNs Have Anti-Inflammatory Activity

The miRNAs in ELNs have received growing attention due to their important functional and therapeutic properties.[33,34] Among the most abundant miRNAs in RELNs and CELNs, miR-1 and miR-21 were selected for further research (Supplementary Table 8). These miRNA mimics were transfected into THP-1 cells (Supplementary Figure 4), followed by LPS-induced cell inflammation. Interestingly, miR-1 and miR-21 could dramatically increase the cell vitality (Figure 7A). Further research showed that the anti-inflammatory activity of miR-1 and miR-21 followed a dose-dependent relationship (Figure 7B and C). Collectively, our data indicated that miR-1 and miR-21 in ZbELNs protect cells from damage caused by inflammation.

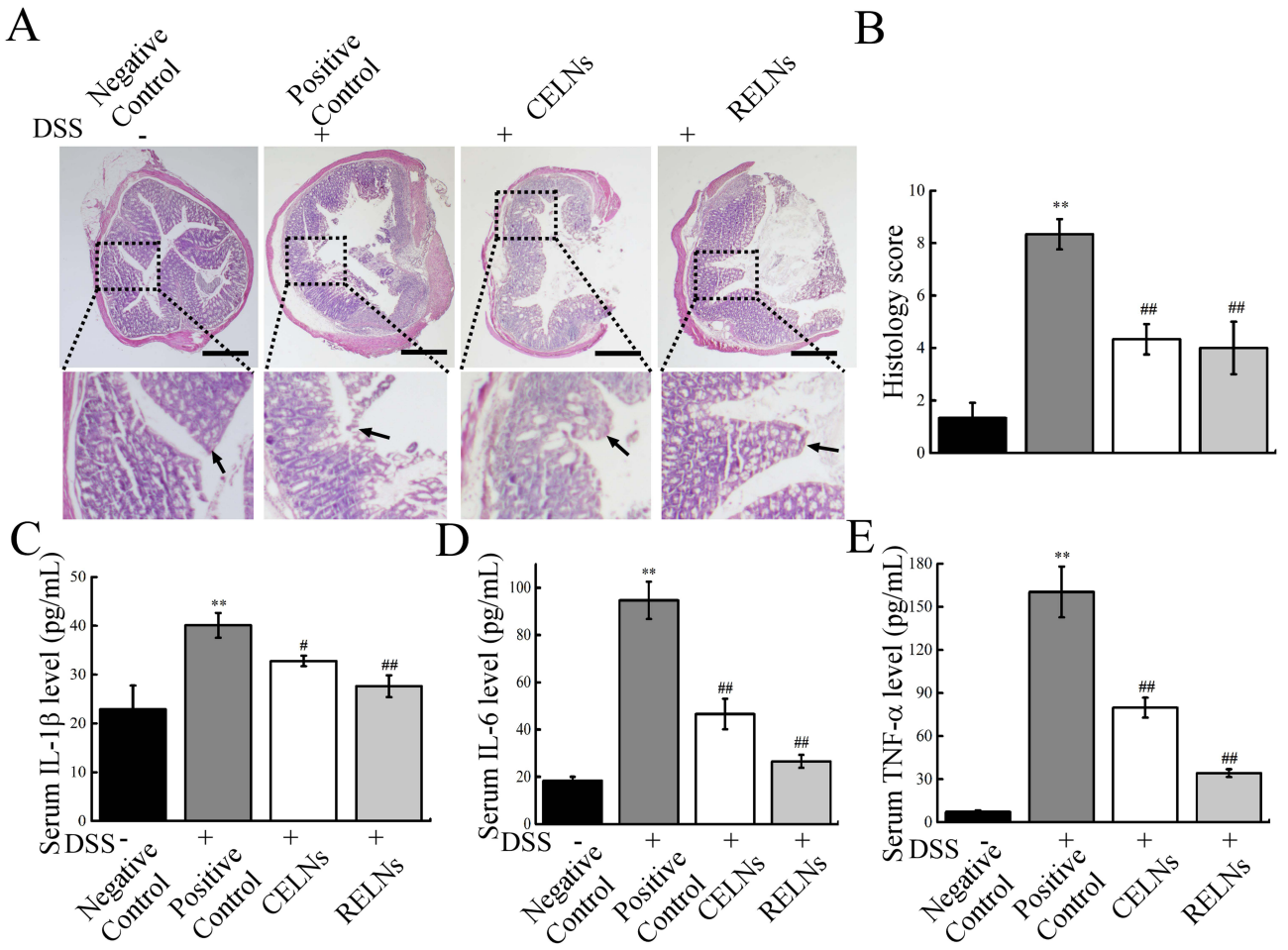

Figure 5 ZbELNs treatment improved mucosal inflammation of colitis tissues. (**A** and **B**). Representative colon histological sections stained with hematoxylin and eosin (H&E). The black arrows indicate areas of mucosal damage in the colon. Magnification: 100X. Scale bar = 200 μm. (**C**–**E**) Result of IL-1β, IL-6, and TNF-α in serum. Data were expressed as the mean ± SD (*n*=3). $^{**}p < 0.01$ compared to the negative control group; $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ compared to the positive control group.

Biosafety of ZbELNs in vivo

The safety profile of orally administered ZbELNs was assessed by collecting serum samples and vital organs from mice receiving ZbELNs (1×10^{11} particles/kg/day) for 7 days. Histological analysis of major organs using H&E staining revealed no apparent abnormalities or indications of organ damage in the ZbELN-treated group (Figure 8A). Additionally, levels of liver function, kidney function, and myocardial enzyme spectrum in the ZbELN-treated group were comparable to the control group (Figure 8B–D). Considering that ZbELNs are derived from the edible Zb, high biocompatibility is expected. Our comprehensive assessment offers experimental evidence supporting this assertion.

Discussion

IBD is a multifaceted disorder affecting the colon that impacts millions of individuals annually.[35] Nevertheless, the mechanisms underlying its pathogenesis remain largely elusive. DSS-induced colitis shares striking similarities in terms of clinical manifestations, histological features, site specificity, and cytokine profiles with human ulcerative colitis. Therefore, this model has been extensively employed in experiments investigating IBD.[19,36]

In recent years, a growing number of studies have explored the efficacy of plant extracts in IBD treatment and prevention. Notably, plant-derived ELNs have shown promising potential. The edible mulberry bark-derived ELNs can be absorbed by the intestinal tract and confer protection against IBD by promoting heat shock protein family A-mediated activation of the aryl-hydrocarbon receptor signaling pathway.[37] The garlic-derived ELNs have a therapeutic effect on IBD by downregulating pro-inflammatory cytokines and modulation of the intestinal microbiota.[38] Oral administration of

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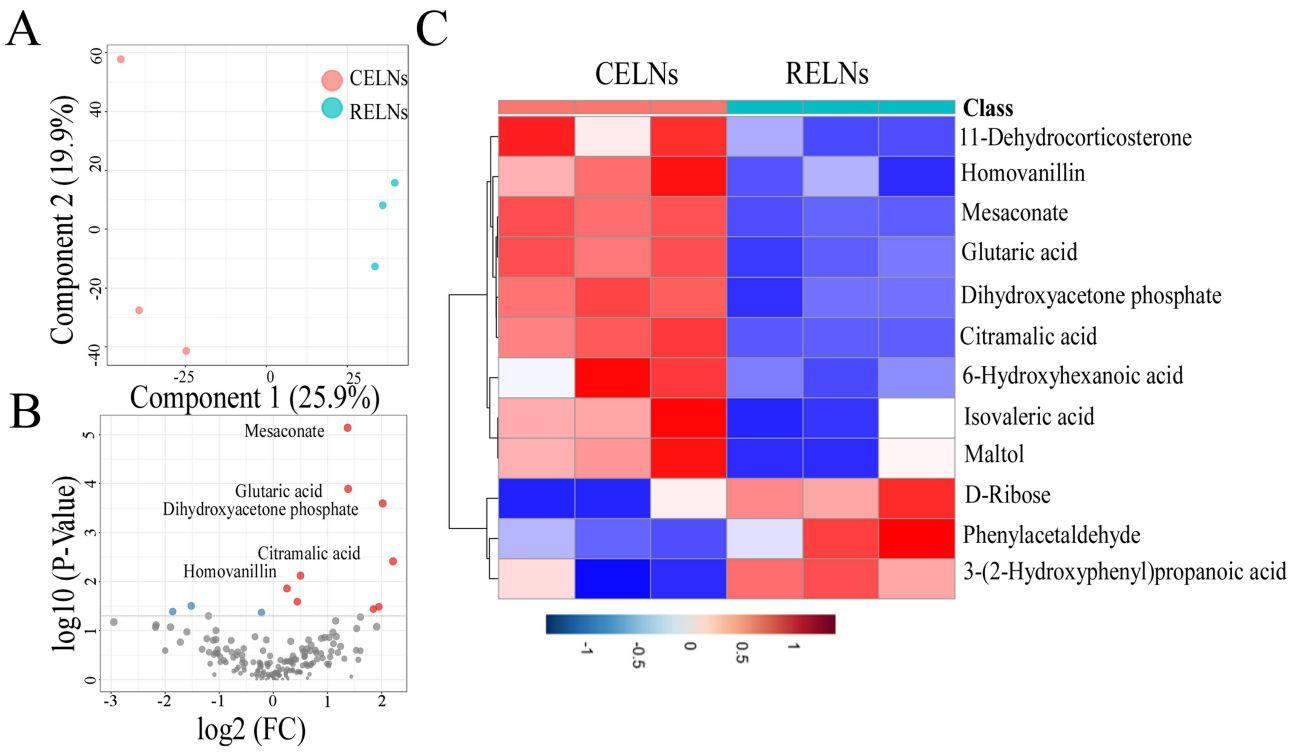

Figure 6 Metabolic profiling of CELNs and RELNs. (**A**) Principal components analysis of metabolite compositions of CELNs and RELNs. Percentage of total variance accounted for by each component is indicated. The abscissa represents the first principal component interpretation, and the ordinate represents the second principal component interpretation. Dots represent experimental samples, and colors represent different groups. The more clustered the samples within the group and the more dispersed the samples between the groups, the more reliable the results. (**B**) Volcano Plot of differential metabolites between CELNs and RELNs. Each point in the figure represents a metabolite, and the horizontal coordinate represents the logarithmic value of Log2 of the quantitative difference multiple of a metabolite in the two samples. The ordinate represents the p-value of -log10. The greater the absolute value of the horizontal coordinate, the greater the difference of the expression multiple of a metabolite between the two samples. The larger the ordinate value, the more significant the differentially expressed metabolites, and the more reliable the differentially expressed metabolites. In the figure, red dots represent up-regulated differentially expressed metabolites, blue dots represent down-regulated differentially expressed metabolites, and gray dots represent metabolites that were detected but did not meet filtering parameters. The first 5 metabolite names with the lowest P value are displayed by default. (**C**) Heatmap based on hierarchical clustering of metabolic composition. Values were represented as the Z-mean of the log2-transformed abundance (relative to internal standards) of each identified metabolite. Hierarchical clustering was conducted according to the Ward D2 method (see Experimental procedures).

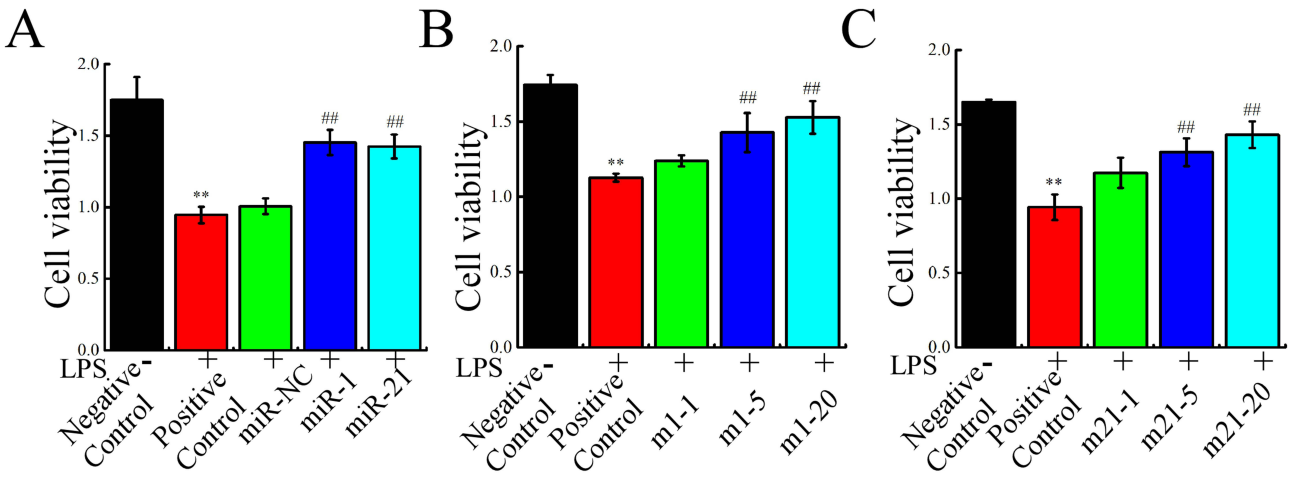

Figure 7 The miR-1 and miR-21 in ZbELNs were identified to protect macrophage from inflammation. (**A**) The miR-1 and miR-21 protect macrophage from inflammation. 20 nM of miRNA (miR) mimic or miRNA negative control (NC) were transfected. (**B**) The miR-1 dose-dependently increased the cell vitality of LPS stimulated THP-1 macrophages. (**C**) The miR-21 dose-dependently increased the cell vitality of LPS stimulated THP-1 macrophages. Different amounts of miR (1nM/5nM/20nM, represented by m1-1/5/20 or m21-1/5/20) were transfected into added macrophages for 4 h, then added 2 μg/mL LPS stimulated macrophages for 24 h. The cell vitality was tested by Cell Counting Kit-8. Error bar represents SD (n=3). **p < 0.01 compared to the negative control group; ##p < 0.01 compared to the positive control group.

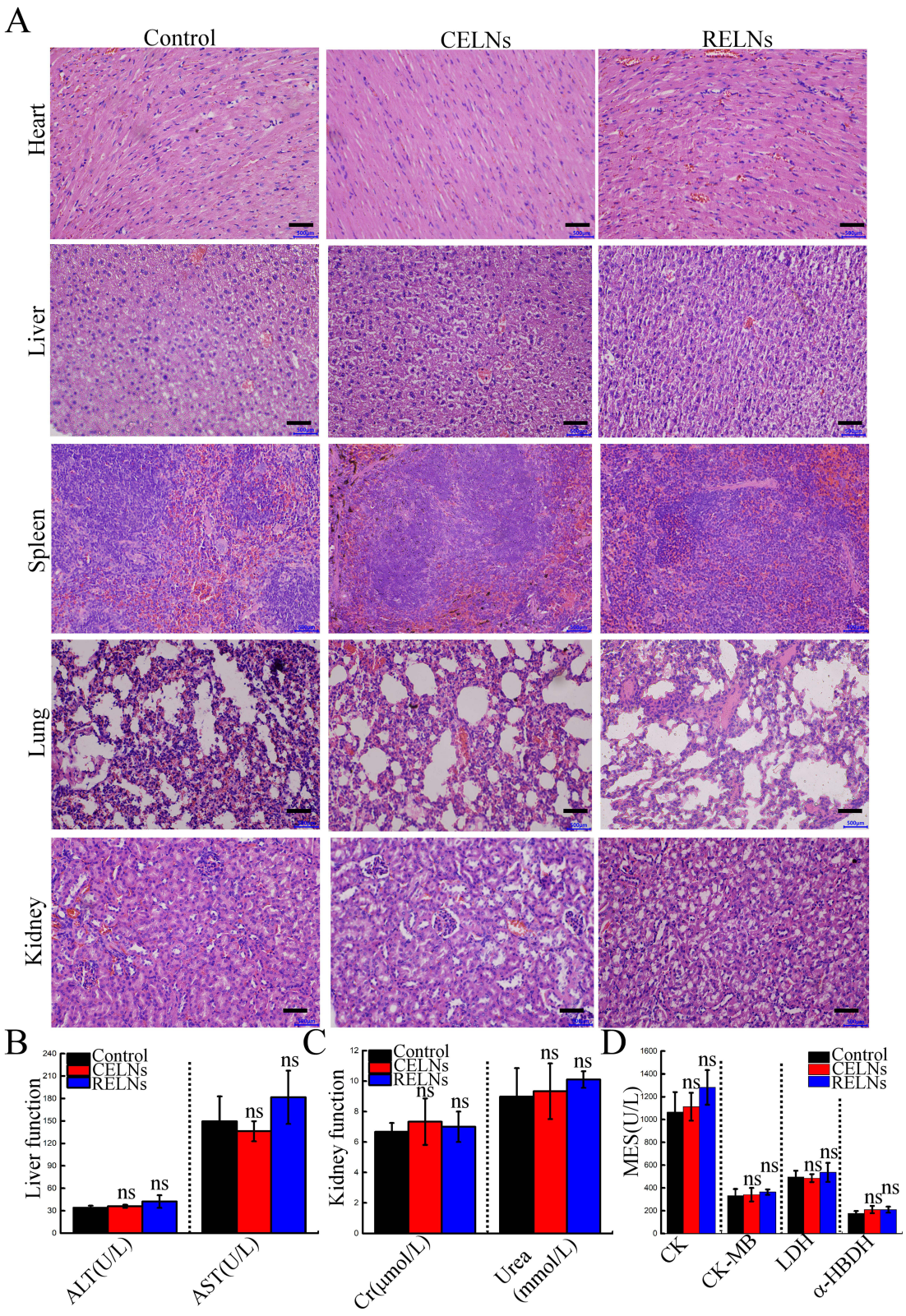

Figure 8 Biosafety of ZbELNs in mice. (**A**) H&E staining of vital organs (heart, liver, spleen, lung, and kidney) in control, CELNs, and RELNs group. Scale bar=500 μm. Magnification: 400X. (**B**–**D**) Expression of liver function (**B**), kidney function (**C**), myocardial enzyme spectrum (MES) (**D**) in the serum of normal mice. Error bar represents SD (n=3). $^{ns}p > 0.05$ compared to the control group.

Abbreviations: ALT, glutamic pyruvic transaminase; AST, aspartate aminotransferase; Cr, serum creatinine; Urea, serum urea; CK, creatine kinase; CK-MB, creatine kinase isoenzyme; LDH, lactate dehydrogenase; α-HBDH, α-hydroxybutyrate dehydrogenase.

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ELNs from turmeric effectively alleviated mice colitis by regulating the pro-inflammatory cytokine levels and antioxidant genes in mouse IBD models.[39] Zu et al also reported that natural tea-derived ELNs could treat IBD and colon cancer.[40]

Plant-derived ELNs target the colon, as evidenced by the accumulation of nanoparticles in the colon or colon stem cells after oral ingestion.[10,39,41] In the diseased colonic site, ELN accumulation can exert anti-inflammatory effects by inducing the proliferation of intestinal stem cells and restoring the intestinal barrier. For example, the expression of tight junction marker proteins such as occludin and ZO-1 was enhanced.[10,42]

Intestinal inflammation involves a complex interplay between innate and adaptive immune mechanisms.[43] Specifically, with cytokine imbalance is believed play a crucial role in the progression of IBD and immune pathogenesis.[44] Damage to the intestinal epithelium results in infiltration of inflammatory mediators into the intestinal barrier, leading to tissue damage. It has reported that key inflammatory cytokines such as IL-1β, IL-6, and TNF-α play important role in regulating innate immune responses and serve as markers for intestinal inflammation.[45,46] Previous research has demonstrated elevated levels of IL-1β, IL-6, and TNF-α in colitis.[47,48] In our present study, DSS treatment also increased these inflammatory cytokines levels in the serum. However, administration of ZbELNs markedly attenuated these pro-inflammatory cytokine levels. Consistent with previous findings from other studies, plant ELNs administration effectively prevented the elevation of serum IL-6, IL-1β and TNF-α levels.[38,39]

The massive production of cytokines can activate the NF-κB and MAPK pathways, as well as activate the JAK/STAT pathway to exert intracellular signaling. The activation of NF-κB can promote the synthesis of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α, thus playing an important role in the pathogenesis of IBD.[49] Furthermore, activation of the MAPK pathway leads to increased cell proliferation and macrophage differentiation, inducing the expression of TNF-α, IL −6, and IL-12.[50] The evolutionarily conserved JAK/STAT pathway is involved in a variety of cellular processes, including cell differentiation, inflammation, and apoptosis.[51] Moreover, plant ELNs exert anti-inflammatory effects by inhibiting inflammatory signaling pathways. Ginseng-derived ELNs exert anti-inflammatory activity by inhibiting NF-κB under inflammatory conditions.[52] Garlic-derived ELNs may protect the colon against DSS-induced injury by inhibiting the TLR4/MyD88/NF-κB signaling pathway.[38] Turmeric-derived ELNs plays a protective role in colitis by inhibiting the NF-κB pathway.[39] In our present study, administration of ZbELNs effectively prevented the elevation of serum IL-6, IL-1β, and TNF-α levels, which is consistent with the findings of other studies.[38,39] Whether ZbELNs also exert anti-inflammatory activity by inhibiting the NF-κB pathway requires further investigation.

Analysis of the lipid composition in ZbELNs showed that DGDG and MGDG accounted for 5.2% of total lipids. These lipids have a stabilizing effect on liposomes during freeze-thaw and freeze-drying.[53] During inflammation, galactose-type lectin is overexpressed on the surface of macrophages. Therefore, galactose is considered a key ligand for targeted drug delivery in macrophages.[54] Based on the proteomics analysis, NAD(P)H quinone oxidoreductase and superoxide dismutase were found in ZbELNs. Up-regulation of NAD(P)H quinone oxidoreductase-1 can enhance the M2 polarization and anti-inflammatory ability of macrophages.[55] Superoxide dismutase is a metal-containing antioxidant enzyme that reduces harmful free radical oxygen produced during metabolic cellular processes to oxygen and hydrogen peroxide.[56] The KEGG annotations revealed that the proteins found in ZbELNs were involved in the metabolic pathways and environmental information processing. Hence, the metabolite profile in ZbELNs was determined using LC-MS. The metabolite profile analysis detected the presence of metabolites involved in anti-inflammatory activity, including 3-(2-hydroxyphenyl) propanoic acid, mesaconate, and 6-hydroxyhexanoic acid. Notably, 6-hydroxycaproic acid can improve inflammation in obese mice by inhibiting pro-inflammatory cytokines.[31] Mesaconic acid can reduce the secretion of IL-6 and IL-12 by macrophages, thus achieving immunomodulatory effects.[30] These metabolites may exert anti-inflammatory effects through bacterial chemotaxis and microbial metabolism pathways based on the results of KEGG pathway enrichment analysis (Supplementary Figure 5 and Supplementary Table 9). Nonetheless, the exact mechanism underlying the anti-inflammatory effects of these metabolites through bacterial metabolic pathways needs further study.

Humans consuming natural edible plant juice or vegetable ingest miRNAs (encapsulated in ELNs for preservation from degradation), which traverse the gastrointestinal tract, are absorbed by small intestinal enterocytes, and ultimately transported to various organs.[57] For instance, miRNA-168a derived from rice was detected in mouse serum and liver, correlating with reduced low-density lipoprotein receptor levels.[58] Prior research indicates that microRNAs (miRNAs)

from ginger ELNs may suppress SARS-CoV-2 gene expression without eliciting adverse effects.[33] In our previous study, miR-44 from tea ELNs demonstrated potential inhibitory effects on liver fibrosis by reducing the protein expression of alpha-smooth muscle actin.[21] In this research, the highest abundance of miRNA-1 and miRNA-21 in ZbELNs were also found in mouse colon, and the accumulated miRNA abundance in RELNs treated group was significantly higher than that in CELNs treated group (Supplementary Figure 6), which was consistent with the miRNA sequencing results for RELNs and CELNs (Supplementary Table 8). Moreover, miRNA-1 and miRNA-21 significantly enhanced cell vitality in a macrophage inflammation model. Future investigations should clarify whether miRNAs from ZbELNs are absorbed by the intestine and provide any protection against intestinal inflammation.

However, the limitations of the present study should be acknowledged. The results of animal studies cannot be extrapolated to humans, and potential differences in microbiota should be addressed. In addition, differences are observed between colitis and other inflammatory diseases such as arthritis, pneumonia, hepatitis. Therefore, further research is required to elucidate these points.

Conclusion

Our work demonstrated that ZbELNs not only protect macrophages from inflammation with no toxic effect but also ameliorate DSS-induced colitis through by reducing the degree of histological damage in the colon and decreasing the expression of inflammatory factors.

Data Sharing Statement

The raw sequence of miRNA reported in our study have been deposited in China National Center for Bioinformation/ National Genomics Data Center (GSA number: CRA012608) that are accessible at https://ngdc.cncb.ac.cn/gsa.

Ethics Statement

All procedures in animal experiments were carried out following the guidelines of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Eighth Edition) and approved by the Ethics Committee of Southwest Jiaotong University (No. SWJTU-2503-NSFC (118)). The ethics committee undertook a thorough evaluation of the study's design, implementation process, and potential risks, adhering to internationally recognized ethical guidelines.

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Disclosure

The authors declare no competing interests in this work.

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