Differential relieving effects of shikonin and its derivatives on inflammation and mucosal barrier damage caused by ulcerative colitis

Hongwei Han [Equal first author, 1, 2], Wenxue Sun [Equal first author, 1, 2], Lu Feng [1, 2], Zhongling Wen [1, 2], Minkai Yang [1, 2], Yingying Ma [1, 2], Jiangyan Fu [1, 2], Xiaopeng Ma [1, 2], Xinhong Xu [1, 2], Zhaoyue Wang [1, 2], Tongming Yin [2], Xiao-Ming Wang [1, 2], Gui-Hua Lu [1, 3], Jin-Liang Qi [1, 2], Hongyan Lin [Corresp., 1, 2], Yonghua Yang [Corresp. 1, 2]

[1] State Key Laboratory of Pharmaceutical Biotechnology, Institute of Plant Molecular Biology, School of Life Sciences, Nanjing University, Nanjing, China

[2] Co-Innovation Center for Sustainable Forestry in Southern China, MOE Key Laboratory of Forest Genetics and Biotechnology, Nanjing Forestry University, Nanjing, China

[3] School of Life Sciences, Huaiyin Normal University, Huaian, China

Corresponding Authors: Hongyan Lin, Yonghua Yang
Email address: linhy@nju.edu.cn, yangyh@nju.edu.cn

Background: Ulcerative colitis (UC) is one of the most challenging human diseases. Natural shikonin (SK) and its derivatives (with have higher accumulation) isolated from the root of *Lithospermum erythrorhizon* have numerous beneficial effects, such as wound healing and anti-inflammatory activities. Some researchers have reported that hydroxynaphthoquinone mixture (HM) and SK attenuate the acute UC induced by dextran sulfate sodium (DSS). However, no existing study has systemically investigated the effectiveness of SK and other hydroxynaphthoquinone natural derivative monomers on UC.

Methods: In this study, mice were treated with SK and its derivatives (25 mg/kg) and mesalazine (200 mg/kg) after DSS administration daily for one week. Disease progression was monitored daily by observing the changes in clinical signs and body weight.

Results: Intragastric administration natural single naphthoquinone attenuated the malignant symptoms induced by DSS. SK or its derivatives remarkably suppressed the serum levels of pro-inflammatory cytokines while increasing the inflammatory cytokine interleukin (IL)-10 . Additionally, both SK and alkanin restrained the activities of cyclooxygenase-2 (COX-2), myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS) in serum and colonic tissues. SK and its derivatives inhibited the activation of nucleotide binding oligomerization domain-like receptors (NLRP3) inflammasome and NF-κB signaling pathway, thereby relieving the DSS-induced disruption of epithelial tight junction (TJ) in colonic tissues.

Conclusions: Our findings shed more lights on the pharmacological efficacy of SK and its derivatives in UC against inflammation and mucosal barrier damage.

1 **Differential Relieving effects of shikonin and its derivatives on**
2 **inflammation and mucosal barrier damage caused by ulcerative colitis**

4 Hongwei Han[1,2†], Wenxue Sun[1,2†], Lu Feng[1,2], Zhongling Wen[1,2], Minkai Yang[1,2], Yingying Ma[1,2],

5 Jiangyan Fu[1,2], Xiaopeng Ma[1,2], Xinhong Xu[1,2], Zhaoyue Wang[1,2], Tongming Yin[2], Xiaoming Wang[1,2],

6 Guihua Lu[1,3], Jinliang Qi[1,2], Hongyan Lin[1,2*], Yonghua Yang[1,2*]

8 [1]State Key Laboratory of Pharmaceutical Biotechnology, Institute of Plant Molecular Biology,

9 School of Life Sciences, Nanjing University, Nanjing, PR China

10 [2]Co-Innovation Center for Sustainable Forestry in Southern China, MOE Key Laboratory of

11 Forest Genetics and Biotechnology, Nanjing Forestry University, Nanjing, PR China

12 [3]School of Life Sciences, Huaiyin Normal University, Huaian, PR China

15 [†]These authors contributed equally to this work

16 *Corresponding authors

17 Contact information of main corresponding author:

18 Dr. Yonghua Yang,

19 State Key Laboratory of Pharmaceutical Biotechnology,

20 Nanjing University, Nanjing, PR China.

21 Tel/Fax: 86-25-89686305,

22 Email: yangyh@nju.edu.cn

24
25
26
27
28
29
30
31
32
33

34

Abstract

35

36 **Background:** Ulcerative colitis (UC) is one of the most challenging human diseases. Natural

37 shikonin (SK) and its derivatives (with have higher accumulation) isolated from the root of

38 *Lithospermum erythrorhizon* have numerous beneficial effects, such as wound healing and anti-

39 inflammatory activities. Some researchers have reported that hydroxynaphthoquinone mixture

40 (HM) and SK attenuate the acute UC induced by dextran sulfate sodium (DSS). However, no

41 existing study has systemically investigated the effectiveness of SK and other

42 hydroxynaphthoquinone natural derivative monomers on UC.

43 **Methods:** In this study, mice were treated with SK and its derivatives (25 mg/kg) and

44 mesalazine (200 mg/kg) after DSS administration daily for one week. Disease progression was

45 monitored daily by observing the changes in clinical signs and body weight.

46 **Results:** Intragastric administration natural single naphthoquinone attenuated the malignant

47 symptoms induced by DSS. SK or its derivatives remarkably suppressed the serum levels of pro-

48 inflammatory cytokines while increasing the anti-inflammatory cytokine interleukin (IL)-10.

49 Additionally, both SK and alkanin restrained the activities of cyclooxygenase-2 (COX-2),

50 myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS) in serum and colonic

51 tissues. SK and its derivatives inhibited the activation of nucleotide binding oligomerization

52 domain-like receptors (NLRP3) inflammasome and NF-κB signaling pathway, thereby relieving

53 the DSS-induced disruption of epithelial tight junction (TJ) in colonic tissues.

54 **Conclusions:** Our findings shed more lights on the pharmacological efficacy of SK and its

55 derivatives in UC against inflammation and mucosal barrier damage.

56
57
58

59

Introduction

61 Inflammatory bowel disease (IBD), which encompasses Crohn's disease (CD) and ulcerative

62 colitis (UC), is a kind of refractory and relapsing gastrointestinal inflammatory disease. IBD has

63 not only affected the living quality of patients but also increased the risk of colon cancer if not

64 treated in a timely manner (*Ungaro et al., 2017*; Stone et al., 2003). An increasing amount of

65 evidence indicates that the incidence of colorectal cancer in IBD patients is generally higher than

66 that in the general population (*Terzic et al., 2010*). Currently, traditional treatment strategies for

67 IBD can be classified as three categories, namely, aminosalicylic acid agents, glucocorticoids,

68 and immunosuppressive agents (*Huang et al., 2009*). Typically, 5-aminosalicylic acid remains

69 the preferred drug in treating IBD, which can directly treat enteritis through inhibiting

70 inflammatory reaction in the intestinal tract and exerting antibacterial function. Nonetheless, this

71 drug will be quickly absorbed by the small intestine if taken orally, leading to acute and chronic

72 renal injury. Moreover, the drug cannot reach the inflammatory site, which can hardly meet the

73 treatment goal (*Munk et al., 2004*). In addition, glucocorticoid is likely to develop dependence

74 after long-term application, and disease recurrence is common after drug withdrawal (*Katz et al.,*

75 *2004*). By contrast, immunosuppressant is suitable for patients with chronic enteritis, which can

76 inhibit lymphocyte proliferation, to achieve the therapeutic effect; nonetheless, it is associated

77 with strong toxic side effects (*Schroder et al., 2003)*. The emergence of anti-tumor necrosis

78 factor therapy has made a major advance in the treatment of patients with IBD for the past two

79 decades (*Khan et al., 2019*). However, despite the fact that this therapy is useful to over half of

80 IBD patients, a substantial proportion of patients are primary nonresponders or lose response

81 with long-term use (*Hanauer et al., 2002; Ma et al., 2014*). Moreover, this therapy causes some

82 disturbing safety issues encompassing the increased risk of infections and malignant tumor in

83 some groups (*Lichtenstein et al., 2012*). These factors are the reason for searching other safe,

84 mild, durable, and effective drugs to cure IBD.

85   The disease characteristics of IBD and shortcomings of current treatments forced us to turn

86 to the traditional Chinese medicine (TCM). *Lithospermum erythrorhizon* (*L. erythrorhizon*) is a

87 commonly used TCM in the Chinese pharmacopoeia of the People's Republic of China and is

88 typically abundant in shikonin (SK) derivatives, which have anti-tumor, anti-virus, and anti-

89 inflammatory activities (*Chen et al., 2019; Yoshida et al., 2017*). For example, Fan et al. reported

90 that the crude extract of *Arnebia euchroma* is effective on rats with experimental colitis (*Fan et

91 al., 2013*). Dozens of species of SK are available in the Boraginaceae plants, among which the

92 representative ones are SK, *β, β*-dimethylacryl-SK (*β, β*-dimethylacryl-SK), acetyl-SK (acetyl-

93 SK), 5,8-dihydroxy-1, 4-naphthoquinone (naphthoquinone), and alkanin (AK, the enantiomeric

94 of SK) with high contents (*Papageorgiou et al., 2006*). They belong to the naphthoquinones that

95 are collectively known as SKs, which typically possess the advantages of bright color, long-

96 lasting effect, and nontoxic side effects. Currently, the efficacy of each individual component of

97 the crude extract has not been studied in detail. The pathological changes in the DSS-induced

98 IBD model are similar to human UC. Thus, it is an ideal model that has been widely used to

99 study the mechanism of UC and for screening potential drugs (*Farooq et al., 2018*).

100   In the present work, we successfully established a murine IBD model by treating C57BL/6

101 mice with DSS and the effectiveness of some natural SKs with high contents in *L. erythrorhizon*

102 were further studied and compared for their efficacy in IBD treatment to explore the potential

103 mechanisms.

104

105
106

Materials & Methods

Chemicals, Regents and antibodies

Chemicals and Regents were described in Supplementary File 1. All of compounds were characterized by ^{13}C NMR, ^{1}H NMR in Supplementary File 2. All antibodies are described in Table S1. ECL Kit (#34077) was purchased from Thermo Scientific (USA). Haematoxylin-Eosin/HE Staining Kit (#20170804) was purchased from Solarbio Science & Technology Co. Ltd (Beijing, China).

Animals

A total of 66 health male C57BL/6 mice weighted 18-20 g (6-8 weeks old) were selected for in vivo experiment. All of them were purchased from Model Animal Research Center of Nanjing University (Nanjing, China), All animal experiments and welfare were treated in strict accordance with the relevant Guidelines for Care and Use of Laboratory Animals of Nanjing University and approved by the Laboratory Animal Ethics Committee of School of Life Sciences, Nanjing University (IACUC-1909002). The mice were group-housed in a laboratory with controlled conditions (22±2°C and 60%±5% humidity) under a 12 h light-dark cycle throughout the experiment. Animals were supplied with standard diet and sterile water ad libitum. All efforts were aimed at reducing the number and suffering of experimental animals while meeting the needs of the experiment.

DSS-induced colitis and design of drug treatment

The mice were randomly divided into eight groups (n = 6 per group) after one week of acclimatization, including the model (the dextran sulfate sodium; 3.5% (w/v) DSS), SK (6.25, 12.5, 25 mg/kg), AK (25 mg/kg), naphthoquinone (25 mg/kg), acetyl-SK (25 mg/kg), *β*, *β*-

131 dimethylacryl-SK (25 mg/kg), mesalazine (positive drug; 100, 200 mg/kg) and control group,

132 then all the groups mice except for control group were exposed to 3.5% DSS, which was

133 dissolved in drinking water. All drugs were dissolved in olive oil and were perfused 200 μL per

134 mice once daily at a predetermined dose by gavage which started at the same time as the DSS

135 treatment and continued one week. In the control and DSS-induced groups, the mice fed orally

136 with the same amount of olive oil. There was no significant difference in water intake among all

137 groups. Body weight and the disease activity index (DAI) were measured and recorded everyday

138 (*Farooq et al., 2018*). The DAI was calculated according to the standard listed in Table S2. After

139 one week of treatment, the entire mice monocular eyeball in each mouse was removed under

140 lightly anesthetized (carbon dioxide euthanasia) for blood collection. Then, all mice were

141 euthanized by cervical dislocation in the unconscious state. The colonic tissues were quickly and

142 safely removed and flushed with ice-cold PBS for pathological analysis. The same protocol was

143 carried out at least three times in independent experiments.

144 **Measurement of Cytokines, COX-2, MPO and iNOS in serum**

145 After drug treatment, mice were sacrificed at the end of the experiment. The whole bloods

146 samples were collected from peripheral blood after reperfusion. Afterwards, the blood was

147 allowed to clot by standing at room temperature for 30 min, and later the clot was removed by

148 centrifuging at 2000 g for 10 min in a refrigerated centrifuge. The resultant supernatant (serum)

149 was then immediately transferred into a clean polypropylene tube. Kits were used to assay the

150 level of various cytokines (IL-6, IL-1β, TNF-α and IL-10), COX-2, MPO and iNOS in the serum

151 of mice according to the product's guidelines (*Yang et al., 2020*).

152 **Haematoxylin & Eosin (H&E) staining**

153   Small sections of colonic tissue were fixed in 10% buffered formalin and embedded in paraffin.

154   Thereafter, the sections were stained with haematoxylin and eosin (H&E) and the colonic

155   mucosa was histologically evaluated by a pathologist in a blinded fashion, which was widely

156   used as evaluation criterion (*Fan et al., 2013*).

157   **Immunohistochemical analysis**

158   Immunohistochemistry was performed for Cyclooxygenase-2 (COX-2), NF-κB, NLRP3, the

159   assay was conducted as described in (*Xu et al., 2019*). Briefly, the tissues were incubated with

160   primary antibodies: anti-NF-κB p65 (1:100), anti-COX-2 (1:200) and anti-NLRP3 (1:100) at 4°C

161   for at least 12 h. Then, the sections were wash with PBS thrice and added with Alexa Fluor 488

162   labeled anti-mouse secondary antibody (Invitrogen, USA) at 25°C for 60 min. Signals were

163   developed with Hematoxylin and DAB (Dako, Agilent Technologies, USA). Sections were

164   examined using light microscopy and image analysis software (Image-pro plus 6.0; Media

165   Cybernetics, Inc., Rockville, MD, USA) was used. The positive cumulative optical density (IOD)

166   and the tissue pixel AREA (AREA) of each photo were measured, and thus average optical value

167   (AO) was calculated by using AO=IOD/AREA.

168   **Western blot analysis**

169   Colonic samples were lysed with RIPA lysis buffer which containing 1% PMSF at 1:100 dilution

170   on ice for 30 min. The insoluble components of cell lysates were removed by centrifugation

171   (10000 rpm at 4°C for 10 min) and the harvested protein concentrations were assessed by using

172   the BCA Protein Assay kit. Then the following immunoblot analysis was referred to the study by

173   Farooq et al (*Farooq et al., 2018*). Finally, the immunoblots were quantified by densitometry with

174   Image J Software (National Institutes of Health, Bethesda, Maryland, USA).

175   **Data and Statistical analysis**

176   Results were presented as mean±S.E.M. of three independent experiments, with six mice (n=6)

177   per group to ensure their reliability. Statistical comparisons between the treated and untreated

178   groups were performed using Student's *t*-test (two-tailed) using GraphPad PRISM5. Differences

179   with a *P*-value < 0.05 were considered statistically significant.

180

181   **Results**

182   **SK and its derivatives protected the colon from dextran sulfate sodium (DSS)-induced**

183   **damage in an acute ulcerative colitcs mouse model**

184   An acute DSS-induced ulcerative colitis (UC) mouse model was established to investigate the

185   effects of SK and its derivatives on colitis. Mice were given either sterile water or 3.5% DSS

186   dissolved in sterile water in the model. Six other groups received 3.5% DSS and were

187   administered SK (25 mg/kg), naphthoquinone (25 mg/kg), *β, β*-dimethylacryl-SK (25 mg/kg),

188   acetyl-SK (25 mg/kg), AK (25 mg/kg), and mesalazine (200 mg/kg) orally both at the beginning

189   of the experiment (n=6 mice per group). SK and its derivatives could significantly attenuate the

190   severity level of inflammation at the end of this experiment. DSS group mice suffered a

191   shortening of about 30% compared with normal mice in the case of the colorectum length. SK

192   and its derivatives significantly prevented this shortening (Fig. 1A-1B). Moreover, SK and its

193   derivatives-treated mice showed improvements in body weight and disease activity index, as

194   well as the alleviation of splenomegaly (Fig. 1C–1F). Therefore, SK and its derivatives

195   ameliorated the colon syndromes of DSS-induced damage in mice.

196   **SK and its derivatives ameliorated the DSS-induced histological damage**

197   The colon tissues of mice in each group were stained with H&E staining to observe the

198   pathological changes. The results (Fig. 2A) indicated that compared with other derivatives group,

199   inflammatory cells infiltration significantly decreased, the number of goblet cells decreased,

200   andcrypt abscesses were distinct reduced in SK and AK-treated group. Grading was performed in

201   a blinded manner by a pathologist and the inflamation score result was showed in Fig. 2B. In

202   brief, the administration of SK and AK markedly relieved the symptoms of DSS-induced IBD in

203   mice.

204   **SK and its derivatives relieved DSS-induced colonic epithelial TJ disruption in mice**

205   Zonula occludens 1 (ZO-1), vascular cell adhesion molecule-1 (VCAM-1), Occludin and

206   Claudin-1 proteins play an important role in epithelial TJ, which can protect mucosa epithelial

207   cells from breaching by the harmful substances, maintain cellular permeability and integrity, and

208   ensure homeostasis of internal environment. Therefore, the effect of SK and its derivatives on

209   epithelial TJ proteins were further investigated. These results suggested that the expression of

210   ZO-1, VCAM-1, Occludin, and Claudin-1 were remarkably reduced in the DSS group compared

211   with the control group. However, SK and its derivatives at 25 mg/kg can restore the expression

212   of these epithelial TJ proteins (Fig. 3A-3B).

213   **SK and its derivatives inhibit the activation of NLRP3 inflammasome in colonic tissues**

214   Many studies have revealed that NLRP3 affects several inflammatory disorders to a large extent

215   including colitis *(Lesuis et al., 2012)*. Fig. 4A-4B shows the significantly up-regulated expression

216   of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), caspase-1, and

217   IL-1β in DSS group compared with control group. However, changes of caspase-1 and IL-1β

218   were significantly reduced by SK and naphthoquinone. SK- and β,β-Dimethylacryl-SK-treament

219   groups evident reduced the level of ASC protein.

220   **SK and its derivatives limited the expression of pro-inflammatory cytokines**

221   When the circulating leukocytes are recruited into the colon, pro-inflammatory mediators are

222   released, including pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α. The levels of IL-

223   6, IL-1β, TNF-α and IL-10 were detected in the serum from 3.5% DSS-exposed mice given

224   either control diet or SK and its derivatives to determine whether SK and its derivatives

225   attenuated the release of pro-inflammatory cytokines, thereby protecting the intestinal mucosa.

226   Fig. 5A–5D showed that treatment with SK and its derivatives at 25 mg/kg significantly reduced

227   the levels of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) and increased the level of

228   anti-inflammatory cytokine (IL-10) in the serum. SK showed the best anti-inflammation effect

229   that was similar to mesalazine group at 200 mg/kg. Fig. 5E–5H showed that SK and mesalazine

230   (the positive control) reduced the levels of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α)

231   and increased the level of anti-inflammatory cytokine (IL-10) in a dose-dependent manner, and

232   SK treatment resulted in improved anti-inflammatory effect at a dose of 25 mg/kg. SK and its

233   derivatives may improve the severity of colon damage induced by DSS by inhibiting the

234   expression of pro-inflammatory cytokines.

235   **SK and its derivatives reduced the activities of MPO, COX-2 and iNOS in serum**

236   MPO is an enzyme that is largely present in neutrophils, and in monocytes and macrophages in

237   small concentrations (*Davies et al., 2020*) and the high levels of COX-2 (*Chen et al., 2016*) and

238   iNOS (*Pandurangan et al., 2014*) plays a central role in the initiation and propagation of the

239   inflammation. MPO, COX-2 and iNOS concentrations in all the mice groups are shown in Fig.

240   6A-6C. The MPO activity is an indicator of neutrophil infiltration in inflamed colon tissues.

241   Administration of DSS increased the activity of MPO as compared with normal mice. SK and

242   AK administration at 25 mg/kg markedly reduced the concentrations of MPO, COX-2 and iNOS

243   compared with DSS-induced mice.

244   **SK and its derivatives attenuate the production of COX-2, NF-κB and NLRP3 in colonic**

245   **tissues**

246   Immunohistochemical expressions of COX-2, NF-κB and NLRP3 in all the groups of mice are

247   shown in Fig. 7A-7C. DSS administration caused the increased production of COX-2, NF-κB

248   and NLRP3 in colonic tissues compared with the control group. Usage of SK and its derivatives

249   reduced the expressions of COX-2, NF-κB and NLRP3 significantly compared with mice

250   induced with DSS.

251   **SK and its derivatives regulate the COX-2 and iNOS expressions and the activation of NF-**

252   **κB in colonic tissues**

253   Transcription factors of the NF-κB family play a vital role in regulating genes, which can

254   influence the immune and inflammatory response (*Andujar et al., 2012*). Evidence has verified

255   that NF-κB plays an important role in IBD development. In this disease, NF-κB and STAT-3

256   activation is observed in inflamed colonic mucosa, leading to the production of COX-2 and

257   iNOS. The phosphorylation level of STAT-3, NF-κB p65 and IκB was detected to evaluate the

258   mechanism of SK. The outcome shown in Fig. 8A-8B suggested that the phosphorylation levels

259   of STAT-3, NF-κB p65 and IκBα were markedly upregulated in DSS-treated group compared

260   with the control group. These increases were markedly attenuated by treatment with DSS plus

261   SK and its derivatives at 25 mg/kg, thereby decreasing the generation of COX-2 and iNOS in the

262   colon homogenates (Fig. 8C-8D).

263   **Discussion**

264   *L. erythrorhizon* is a kind of TCM used for wound healing in ancient times. Its roots are typically

265   rich with a variety of bioactive substances, including natural naphthoquinones, which represent

266   an ongoing source of therapeutic agents (*Qiu et al., 2018*). Among the naphthoquinones isolated

267   from *L. erythrorhizon*, SK has attracted extensive interest due to its multiple biological activities,

268   such as anti-inflammatory *(Yoshida et al., 2017; Yang et al., 2014)*, wound healing *(Yao et al.,*

269   *2019*), anti-microbial (*Andujar et al., 2013*), anti-ulcer (*Lesuis et al., 2012*), anti-thrombus

270   (*Andrikopoulos et al., 2003*), and anti-cancer activities (*Lin et al., 2018*). Nearly 20 kinds of

271   natural SK derivatives have been identified.

272   The anti-inflammatory effects of natural naphthoquinones have been extensively

273   investigated. For instance, Fan et al. investigated the effectiveness of hydroxynaphthoquinone

274   mixture (HM) isolated from *A. euchroma* in rats with experimental colitis induced by 2, 4, 6-tri-

275   nitrobenzene sulfonic acid (TNBS) in 2013 (*Fan et al., 2013*). Their results suggested that HM

276   can remarkably attenuate the clinical and histopathological severity of the TNBS-induced colitis

277   in a dose-dependent manner. In addition, HM can also do the following: alleviate loss of body

278   weight, hematochezia, and inflammation; reduce the macroscopic damage score; and improve

279   the histological signs. Moreover, HM can evidently reduce the inflammatory infiltration, ulcer

280   area, and the severity of goblet cell depletion. Notably, HM, which is isolated from *A. euchroma*,

281   contains seven AK derivatives, including AK, deoxyalkannin, acetylalkannin, *β, β*-

282   dimethylacrylalkannin, α-methylalkannin, isovalerylalkannin, and *β*-acetoxyisovalerylalkannin.

283   Isabel et al. reported that the oral administration of SK can attenuate the DSS-induced acute UC

284   by blocking the activation of two major targets, NF-κB and STAT-3 (*Andujar et al., 2012).*

285   However, the effectiveness of the commonly used SK and its natural derivatives on acute colitis

286   has not been systematically investigated yet.

287   In the present study, five representative natural naphthoquinones, namely, naphthoquinone,

288   SK, acetyl-SK, *β, β*-dimethylacryl-SK and AK, were selected to treat DSS-induced colitis in

289   mice. The administration of these five natural naphthoquinones could ameliorate the clinical

290   severity of the wasting disease, prevent the shortening the length of colorectum, decrease the loss

291   of body weight, improve the appearance of feces, and prevent bloody excrements. Such

292   beneficial effects were further proved by histological evaluation, as evidenced by the markedly

293   reduced severity and extent of inflamed tissue damage and infiltration of inflammatory cells.

294   Typically, the relative expression of pro-inflammatory cytokines, including IL-6, IL-1β and

295   TNF-α in serum in colon tissues, suggested that these five natural naphthoquinones had

296   beneficial effects. MPO is a marker of neutrophil infiltration, which has been observed to be

297   activated in several experimental colitis models, including the DSS-induced colitis. Therefore,

298   MPO is an indicator to quantify intestinal inflammation and evaluate the severity of

299   inflammation. In addition, the pro-inflammatory synthases, including iNOS and COX-2, are

300   inflammatory mediators with vital functions during the pathogenesis of UC (*Itzkowitz et al.,*

301   *2006*). The five natural naphthoquinones could suppress MPO, iNOS and COX-2 activities. Such

302   results were consistent with those found in histological examination, in which the inflammatory

303   extent in colonic tissues was less than that in SK- and AK-treated animals and naphthoquinone-,

304   acetyl-SK- and *β, β*-dimethylacryl-SK-treated mice. Moreover, results of western blot had further

305   verified that SK can relieve the colitis in mice by inhibiting the activation of NF-κB and NLRP3

306   inflammasome and disruption of epithelial TJ proteins. Collectively, these aforementioned

307   results offer insights into the potential use of SK and its derivatives in treating IBD.

308
309   **Conclusions**
310
311   Our results suggested that intra-gastric administration of single compound like SK or its

312   derivatives contributed to attenuating the loss of body weight, shortening the colon length,

313   increasing the spleen weight and relieving colonic epithelial tight disruption in mice induced by

314   DSS. Through a number of related pathological experiments, we have explained the underlying

315   mechanism that SK and its derivatives could inhibit the activation of NLRP3 inflammasome and

316   NF-κB signaling pathway. These findings provide new evidence for the potential use of SK and

317   its derivatives to treat the inflammatory bowel disease (IBD).

318   **Additional Information and Declarations**

319

320   **Data Availability**

321   The following information was supplied regarding data availability:

322   The raw data are available in the Supplementary File 3. The raw data is showing H&E data, IHC

323   raw data and Western blot bands.

324

325   **References**

326

327   **Ungaro R, Mehandru S, Allen PB, Peyrin-Biroulet L, Colombel JF. 2017.** Ulcerative colitis.

328   *Lancet* **359:** 1756-1770 https://doi.org/ 10.1016/S0140-6736(16)32126-2.

329   **Stone MA, Mayberry JF, Baker R. 2003.** Prevalence and management of inflammatory bowel

330   disease: a cross-sectional study from central England. *European journal of gastroenterology &*

331   *hepatology* **15:**1275-1280 https://doi.org/ 10.1097/00042737-200312000-00004.

332   **Terzic J, Grivennikov S, Karin E, Karin M. 2010.** Inflammation and colon cancer.

333   *Gastroenterology* **138:** 2101-2114 https://doi.org/ 10.1053/j.gastro.2010.01.058.

334   **Huang LJ, Zhu Q, Lei M, Cao Q. 2009.** Current use of immunosuppressive agents in

335   inflammatory bowel disease patients in East China. *World journal of gastroenterology* **15:**3055-

336   3059 https://doi.org/10.3748/wjg.15.3055.

337   **Munk EM, Pedersen L, Floyd A, Norgard B, Rasmussen HH, Sorensen HT. 2004.**

338   Inflammatory bowel diseases, 5-aminosalicylic acid and sulfasalazine treatment and risk of acute

339   pancreatitis: a population-based case-control study. *The American journal of gastroenterology*

340   **99:**884-888 https://doi.org/ 10.1111/j.1572-0241.2004.04123.x.

341 **Katz JA. 2004.** Treatment of inflammatory bowel disease with corticosteroids. *Gastroenterology*

342 *clinics of North America* **33:**171-189 https://doi.org/ 10.1016/j.gtc.2004.02.002.

343 **Schroder O, Stein J. 2003.** Low dose methotrexate in inflammatory bowel disease: current

344 status and future directions. *The American journal of gastroenterology* **98:**530-537

345 https://doi.org/ 10.1016/S0002-9270(02)06027-6.

346 **Khan S, Rupniewska E, Neighbors M, Singer D, Chiarappa J, Obando C. 2019.** Real-world

347 evidence on adherence, persistence, switching and dose escalation with biologics in adult

348 inflammatory bowel disease in the United States: A systematic review. *Journal of clinical*

349 *pharmacy and therapeutics* **44:**495-507 https://doi.org/ 10.1111/jcpt.12830.

350 **Hanauer SB, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF,**

351 **Rachmilewitz D, Wolf DC, Olson A, Bao W, Rutgeerts P, Group AIS. 2002.** Maintenance

352 infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* **359:**1541-1549

353 https://doi.org/ 10.1016/S0140-6736(02)08512-4.

354 **Ma C, Huang V, Fedorak DK, Kroeker KI, Dieleman LA, Halloran BP, Fedorak RN. 2014.**

355 Crohn's disease outpatients treated with adalimumab have an earlier secondary loss of response

356 and requirement for dose escalation compared to infliximab: a real life cohort study. *Journal of*

357 *Crohn's & colitis* **8:**1454-1463 https://doi.org/ 10.1016/j.crohns.2014.05.007.

358 **Lichtenstein GR, Feagan BG, Cohen RD, Salzberg BA, Diamond RH, Price S, Langholff W,**

359 **Londhe A, Sandborn WJ. 2012.** Serious infection and mortality in patients with Crohn's

360 disease: more than 5 years of follow-up in the TREAT registry. *The American journal of*

361 *gastroenterology* **107:**1409-1422 https://doi.org/ 10.1038/ajg.2012.218.

362 **Chen Y, Chen ZY, Chen L, Zhang JY, Fu LY, Tao L, Zhang Y, Hu XX, Shen XC. 2019.**

363 Shikonin inhibits triple-negative breast cancer-cell metastasis by reversing the epithelial-to-

364   mesenchymal transition via glycogen synthase kinase 3 beta-regulated suppression of beta-

365   catenin signaling. *Biochemical pharmacology* **166:**33-45 https://doi.org/

366   10.1016/j.bcp.2019.05.001.

367   **Yoshida LS, Kakegawa T, Yuda Y, Takano-Ohmuro H. 2017.** Shikonin changes the

368   lipopolysaccharide-induced expression of inflammation-related genes in macrophages. *Journal*

369   *of natural medicines* **71:**723-734 https://doi.org/ 10.1016/j.bcp.2019.05.001.

370   **Fan HY, Zhang ZL, Liu K, Yang MY, Lv WH, Che X, Xu H, Song WW. 2013.** Effectiveness

371   of a hydroxynaphthoquinone fraction from Arnebia euchroma in rats with experimental colitis.

372   *World journal of gastroenterology* **19:**9318-9327 https://doi.org/ 10.3748/wjg.v19.i48.9318.

373   **Papageorgiou VP, Assimopoulou AN, Samanidou VF, Papadoyannis IN. 2006.** Analytical

374   methods for the determination of alkannins and shikonins. *Current Organic Chemistry* **10:**583-

375   622 https://doi.org/ 10.2174/138527206776055277.

376   **Farooq SM, Hou Y, Li H, O'Meara M, Wang Y, Li C, Wang JM. 2018.** Disruption of GPR35

377   Exacerbates Dextran Sulfate Sodium-Induced Colitis in Mice. *Digestive diseases and sciences*

378   **63:**2910-2922 https://doi.org/ 10.1007/s10620-018-5216-z.

379   **Yang L, Zhou RY, Tong Y, Chen PF, Shen Y, Miao S, Liu XQ. 2020.** Neuroprotection by

380   dihydrotestosterone in LPS-induced neuroinflammation. *Neurobiology of Disease* **140:** 104814-

381   104827. https://doi.org/10.1016/j.nbd.2020.104814.

382   **Xu M, Duan XY, Chen QY, Fan H, Hong ZC, Deng SJ, Nan Z, Wu H, Dong YL, Liu YJ,**

383   **Zhou CZ. 2019.** Effect of compound sophorae decoction on dextran sodium sulfate (DSS)-

384   induced colitis in mice by regulating Th17/Treg cell balance. *Biomedicine & Pharmacotherapy*

385   **109:**2396-2408 https://doi.org/ 10.1016/j.biopha.2018.11.087.

386   **Andujar I, Rios JL, Giner RM, Miguel Cerda J, Recio Mdel C. 2012.** Beneficial effect of

387   shikonin on experimental colitis induced by dextran sulfate sodium in BALB/c mice. *Evidence-*

388   *based complementary and alternative medicine : eCAM* **2012:**271606-271620 https://doi.org/

389   10.1155/2012/271606.

390   **Qiu HY, Wang PF, Lin HY, Tang CY, Zhu HL, Yang YH. 2018.** Naphthoquinones: A

391   continuing source for discovery of therapeutic antineoplastic agents. *Chemical biology & drug*

392   *design* **91:**681-690 https://doi.org/ 10.1111/cbdd.13141.

393   **Yang Y, Wang J, Yang Q, Wu S, Yang Z, Zhu H, Zheng M, Liu W, Wu W, He J, Chen Z.**

394   **2014.** Shikonin inhibits the lipopolysaccharide-induced release of HMGB1 in RAW264.7 cells

395   via IFN and NF-kappaB signaling pathways. *International immunopharmacology* **19:**81-87

396   https://doi.org/ 10.1016/j.intimp.2014.01.003.

397   **Yao CH, Chen KY, Chen YS, Li SJ, Huang CH. 2019.** Lithospermi radix extract-containing

398   bilayer nanofiber scaffold for promoting wound healing in a rat model. *Materials science &*

399   *engineering C, Materials for biological applications* **96:**850-858 https://doi.org/

400   10.1016/j.msec.2018.11.053.

401   **Andujar I, Rios JL, Giner RM, Recio MC. 2013.** Pharmacological Properties of Shikonin - A

402   Review of Literature since 2002. *Planta Medica* **79:**1685-1697 https://doi.org/ 10.1055/s-0033-

403   1350934.

404   **Lesuis N, Befrits R, Nyberg F, van Vollenhoven RF. 2012.** Gender and the treatment of

405   immune-mediated chronic inflammatory diseases: rheumatoid arthritis, inflammatory bowel

406   disease and psoriasis: an observational study. *BMC Medicine* **10:**82-90. https://doi.org/

407   10.1186/1741-7015-10-82.

408   **Davies MJ, Hawkins CL. 2020.** The Role of Myeloperoxidase in Biomolecule Modification,

409   Chronic Inflammation, and Disease. *Antioxid Redox Sign.* **32:** 957-981. 10.1089/ars.2020.8030.

410 **Chen L, Teng H, Fang T, Xiao J. 2016.** Agrimonolide from Agrimonia pilosa

411 suppresses inflammatory responses through down-regulation of COX-2/iNOS and

412 inactivation of NF-κB in lipopolysaccharide-stimulated macrophages. *Phytomedicine*

413 **23:**846–855. https://doi.org/10.1016/j.phymed.2016.03.016.

414 **Pandurangan AK, Kumar SA, Dharmalingam P, Ganapasam S. 2014.** Luteolin, a

415 bioflavonoid inhibits azoxymethane-induced colon carcinogenesis: involvement of iNOS and

416 COX-2. *Pharmacogn. Mag.* **10:** 306–310. https:// doi.org/10.4103/0973-1296.133285.

417 **Andrikopoulos NK, Kaliora AC, Assimopoulou AN, Papapeorgiou VP. 2003.** Biological

418 activity of some naturally occurring resins, gums and pigments against in vitro LDL oxidation.

419 *Phytotherapy Research* **17:**501-507 https://doi.org/ 10.1002/ptr.1185.

420 **Lin HY, Han HW, Sun WX, Yang YS, Tang CY, Lu GH, Qi JL, Wang XM, Yang YH. 2018.**

421 Design and characterization of alpha-lipoic acyl shikonin ester twin drugs as tubulin and PDK1

422 dual inhibitors. *European journal of medicinal chemistry* **144:**137-150 https://doi.org/

423 10.1016/j.ejmech.2017.12.019.

424 **Itzkowitz SH. 2006.** Molecular biology of dysplasia and cancer in inflammatory bowel disease.

425 *Gastroenterology clinics of North America* **35:**553-571 https://doi.org/ 10.1016/j.gtc.2006.07.002.

426

Figure 1

SK andits derivatives protect the colon from DSS-induced damage

(**A**) Macroscopic observation of colon length. (**B**) Bar graph showing colon length. **$p<0.01$ versus control group. (**C**) Pattern of daily weight changes. *$p<0.05$, **$p<0.01$ versus DSS-treated group. (**D**) Disease activity index of mice in each group. *$p<0.05$, **$p<0.01$ versus DSS-treated group. (**E**) Macroscopic observation of spleen size. (**F**) Bar graph showing spleen weight. Data are shown as mean ± S.E.M, *$p<0.05$ versus control group (n=6 per group).
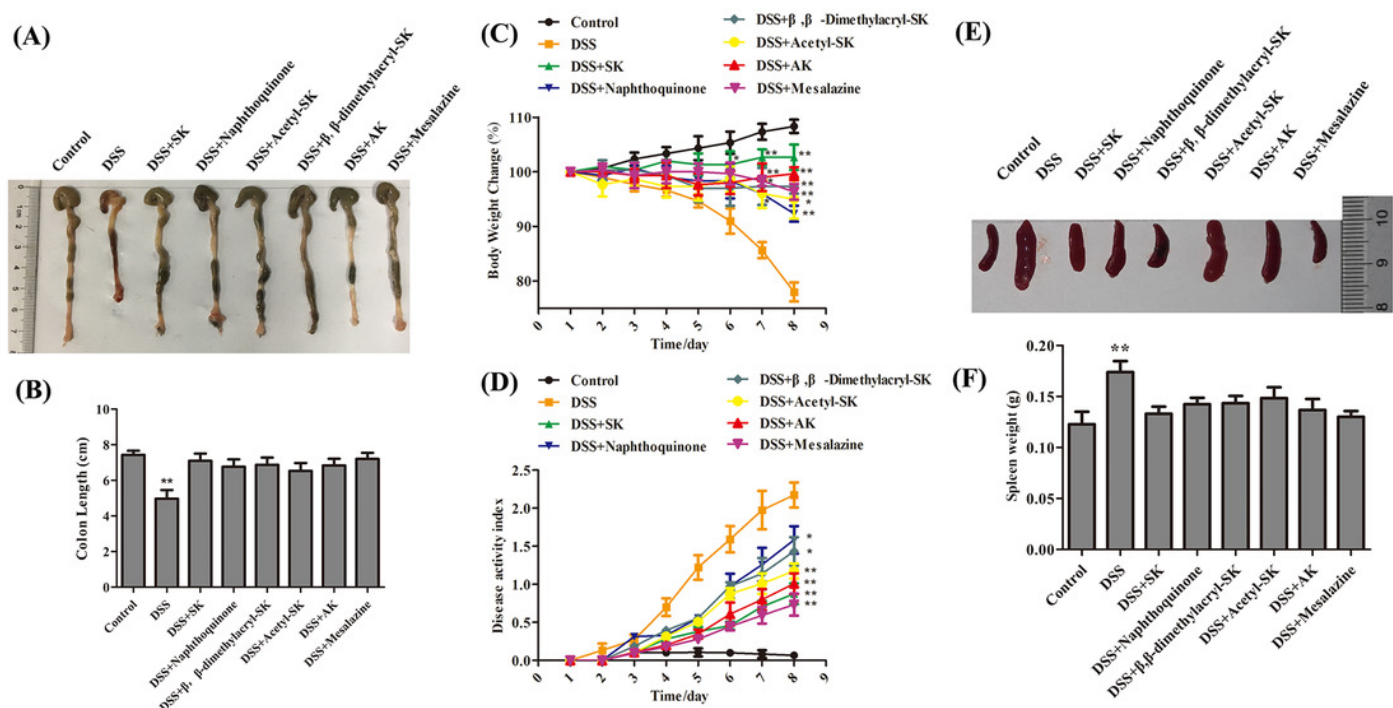
Figure 2

Colon histology and inflammation score of mice in each group (H&E staining 200× and 400×)

(**A**) The colon histology sections were stained with hematoxylin and eosin. The colon histology sections of DSS-treated group mice revealed sever pathology with observation of submucosal edema with extensive inflammatory cell infiltration. The frame indicates the region magnified in the bottom panel. (**B**) Inflammation scores of each group. Data are shown as mean ± S.E.M, *$p<0.05$, **$p<0.01$ versus control group; #$p<0.05$, ##$p<0.01$ versus DSS-treated group (n=6 per group).
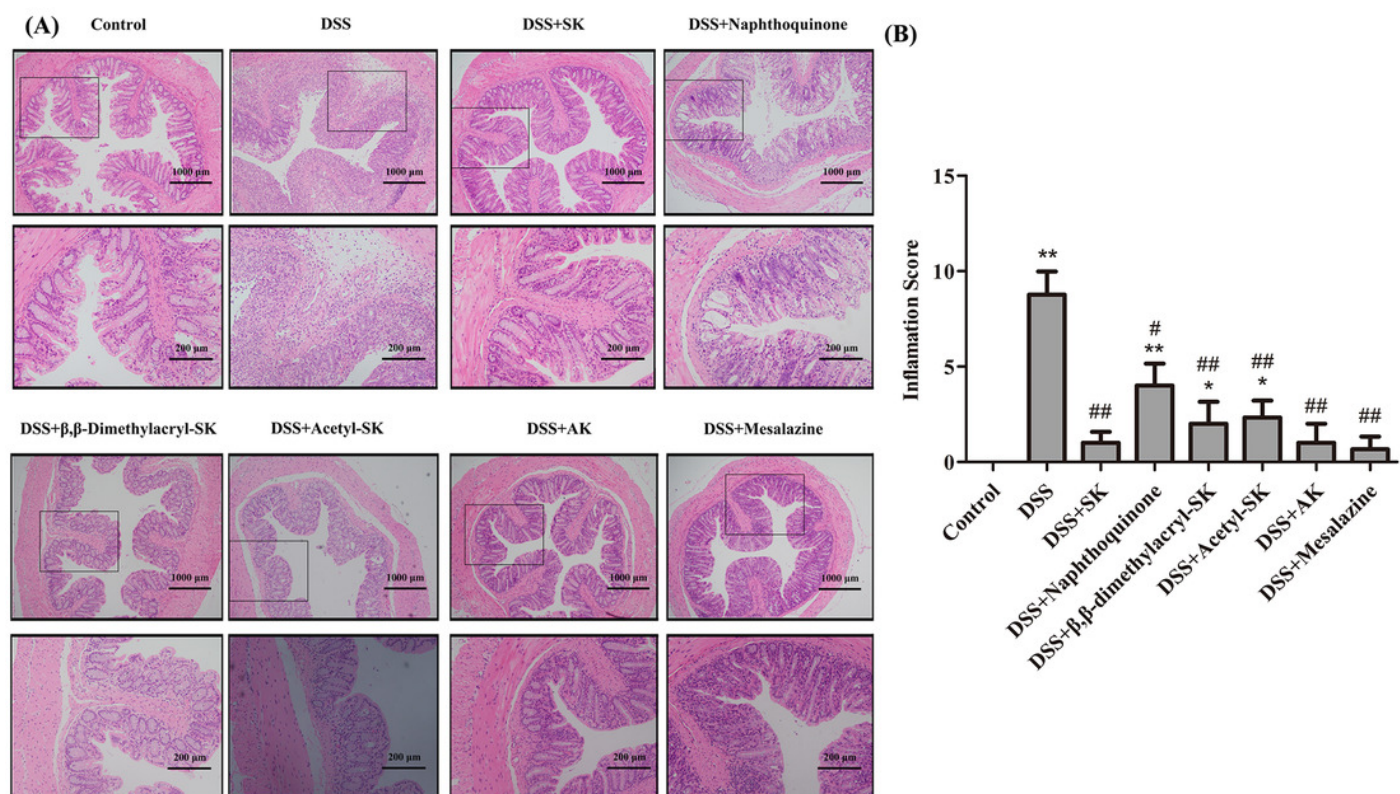
Figure 3

SK and its derivatives relieved DSS-induced colonic epithelial tight junction disruption in mice

(**A**) Protein expression of ZO-1, VCAM-1, Occludin and Claudin-1 in the colon tissues were analyzed by Western blot. (**B**) Relative protein expression ratios of were determined by densitometry and normalized to GAPDH. Each point represents the mean ± S.E.M. from three replicates (*$p<0.05$, **$p<0.01$ versus control group; #$p<0.05$, ##$p<0.01$ versus DSS-treated group).
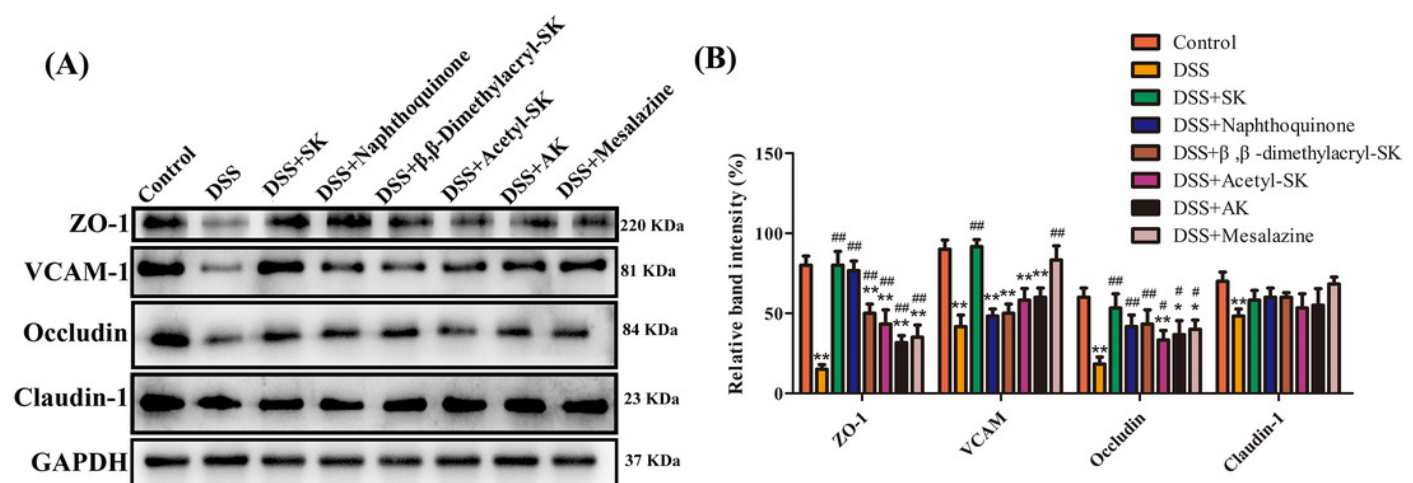
Figure 4

SK andits derivatives reduced the level of NLRP3 inflammasome activation in colonictissues

(**A**) Protein levels of NLRP3, ASC, caspase-1, and IL-1β were determined by Western blotting. β- a ctin was used as a control. (**B**) Relative protein expression ratio was determined by densitometry and normalized to β- a ctin. Each point represents the mean ± S.E.M. from three replicates (*$p<0.05$, **$p<0.01$ versus control group; #$p<0.05$, ##$p<0.01$ versus DSS-treated group).
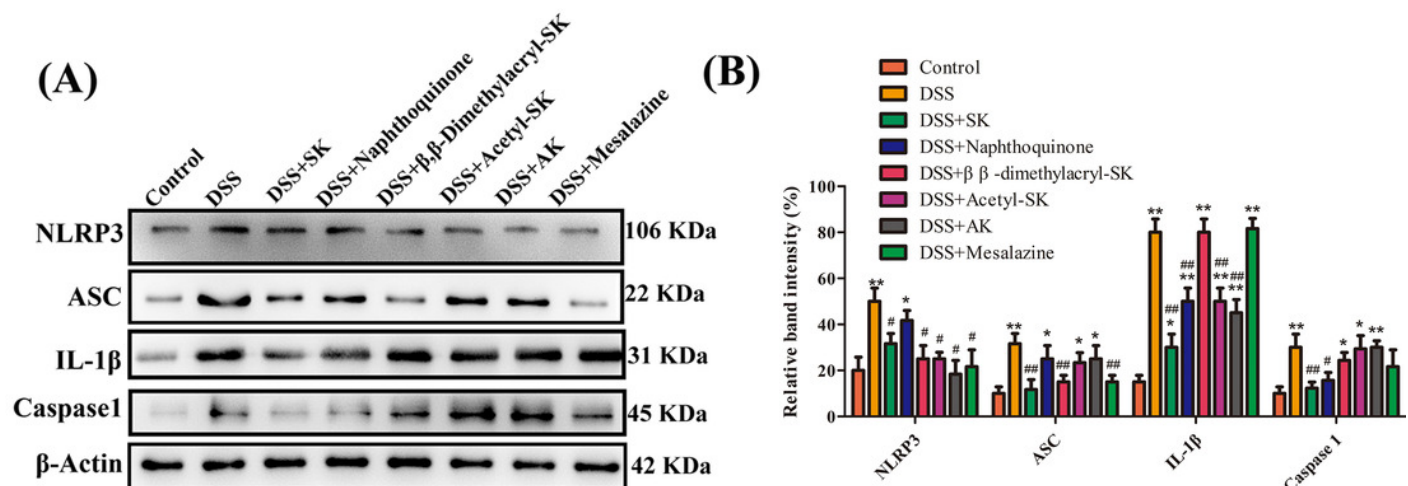
Figure 5

SK and its derivatives suppressed pro-inflammatory cytokines and promoted anti-inflammatory cytokine in serum

(**A-D**) The protein level of cytokines IL-6, IL-1β, TNF-α and IL-10 in serum were determined by ELISA (DSS: 3.5%; SK, Naphthoquinone, *β, β*-dimethylacryl-SK, Acetyl-SK, AK: 25 mg/kg; Mesalazine: 200 mg/kg). (**E-H**) Effects of different concentrations of SK on the levels of cytokines IL-6, IL-1β, TNF-α and IL-10. Data are shown as mean ± S.E.M, *$p<0.05$, **$p<0.01$ versus control group; #$p<0.05$, ##$p<0.01$ versus DSS-treated group (n=6 per group).
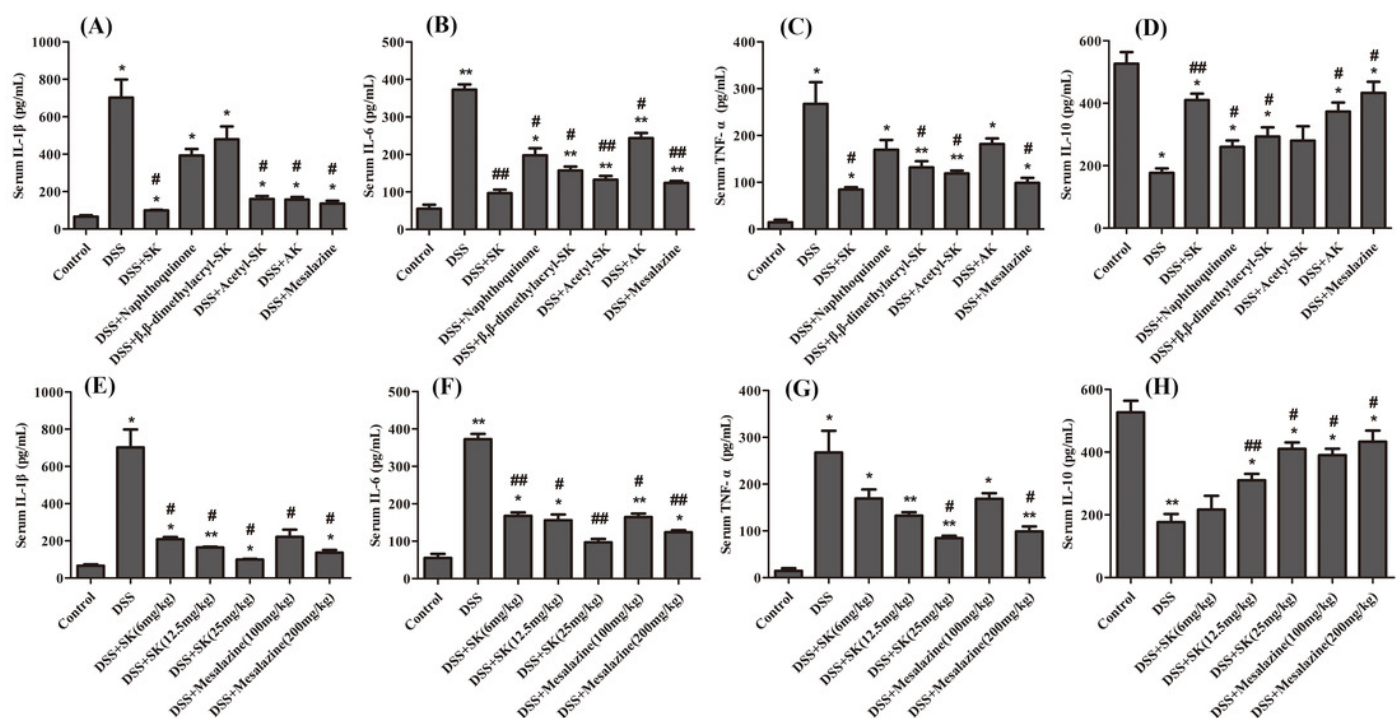
Figure 6

SK and its derivatives reduced the level of COX-2, MPO and iNOS in the serum of mice

The concentrations of COX-2 (**A**), MPO (**B**) and iNOS (**C**) were measured using an enzyme-linked immunosorbent assay kit. Each point represents the mean ± S.E.M. from three replicates (*p<0.05, **p<0.01 versus control group; #p<0.05, ##p<0.01 versus DSS-treated group).
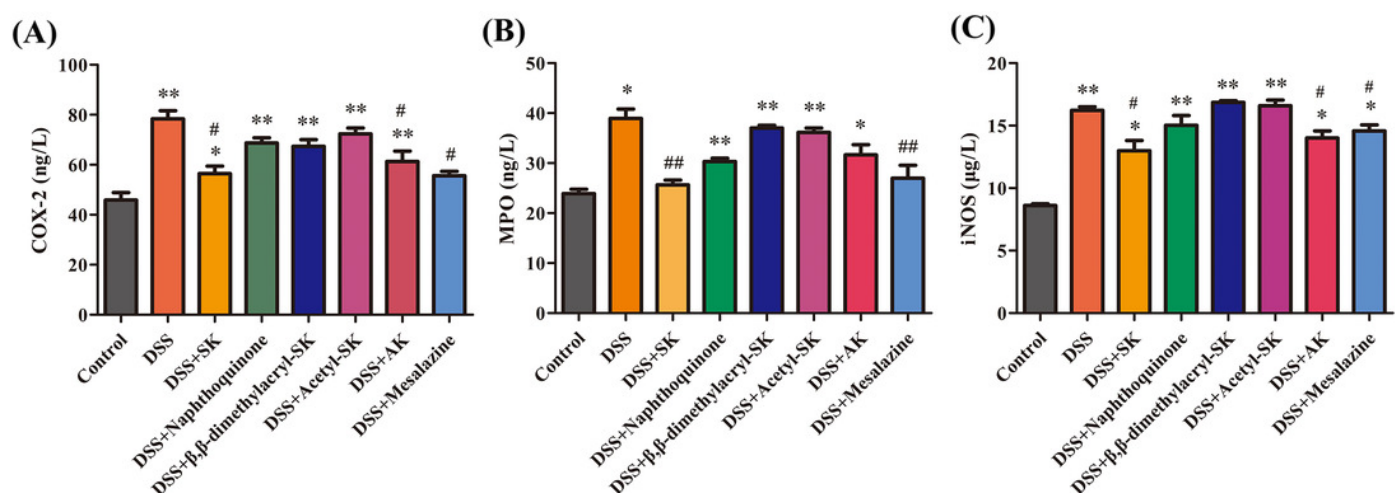
Figure 7

SK andits derivatives attenuate the expression of COX-2, NF-κB and NLRP3

(**A**) Immuno-histochemical analysis of COX-2 in each group. (**B**) Immuno-histochemical analysis of NF-κB in each group. (**C**) Immuno-histochemical analysis of NLRP3 in each group. The slides were incubated with primary antibody. After the secondary antibody incubation, the slides were developed with DAB and counter-stained with hematoxylin. The images were taken at 400×magnification. Data are presented as means ± S.E.M. from three replicates (*$p<0.05$, **$p<0.01$ versus control group; #$p<0.05$, ##$p<0.01$ versus DSS-treated group).
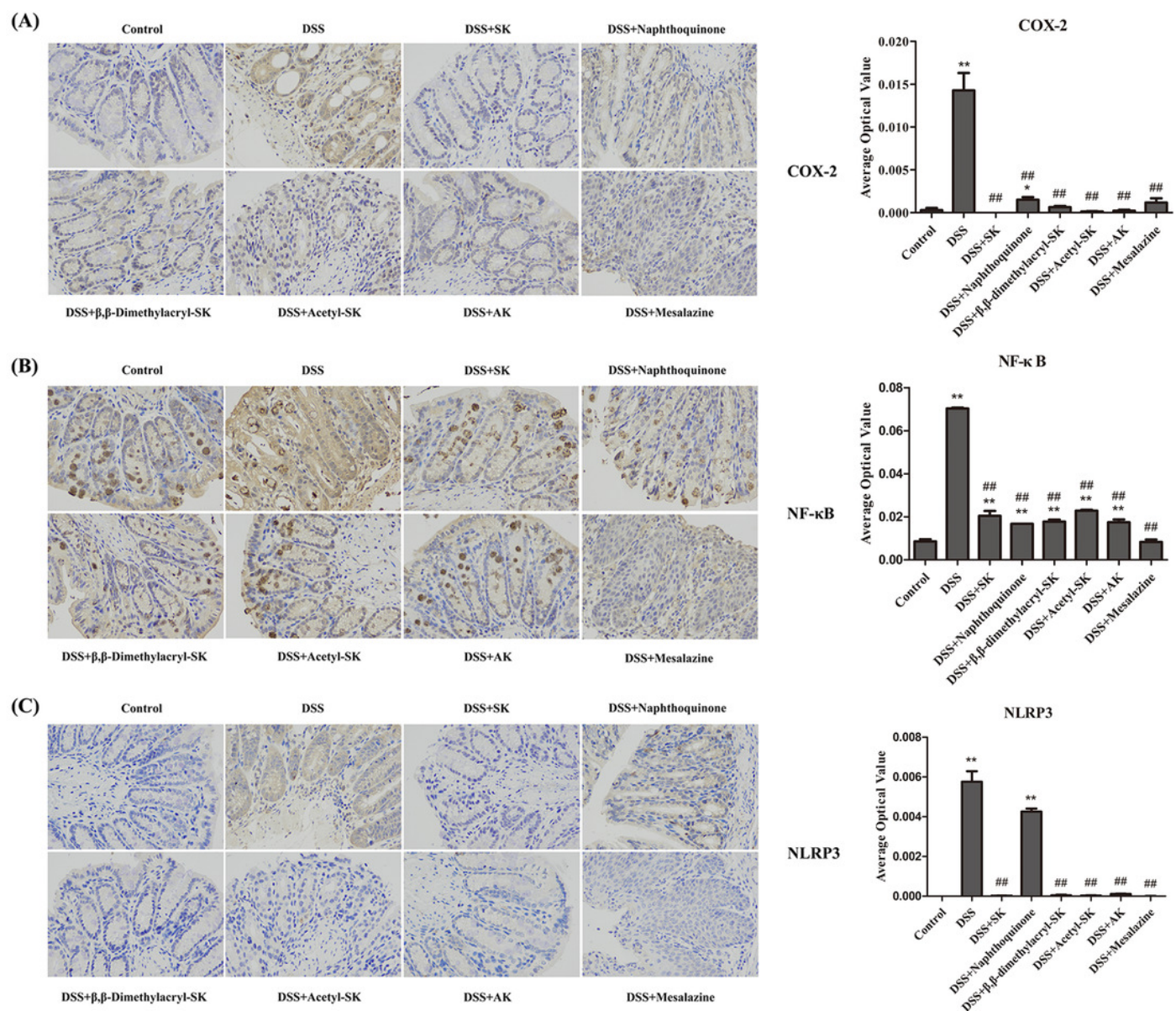
Figure 8

SK andits derivatives modulated COX-2, iNOS expression, and NF-κB signal pathway in UCmice

(A) Western blot analysis of COX-2 and iNOS levels in colonic tissues in each group. Data shown are representative of three independent experiments. (B) Relative protein

expression ratios of COX-2 and iNOS were determined by densitometry. (C) Western blot analysis of proteins expression level of NF-κB signal pathway. (D) Relative

protein expression ratio was determined by densitometry. Each point represents the mean ± S.E.M. from three replicates (*p<0.05, **p<0.01 versus control group;

#p<0.05, ##p<0.01 versus DSS-treated group).