

1           **Natural naphthoquinones isolated from *Lithospermum***  
2           ***erythrorhizon* suppress dextran sulfate sodium-induced murine**  
3           **experimental colitis**

4  
5   Wenxue Sun<sup>1,2†</sup>, Hongwei Han<sup>1,2†</sup>, Zhaoyue Wang<sup>1</sup>, Zhongling Wen<sup>1</sup>, Minkai Yang<sup>1</sup>,  
6   Yinsong Wang<sup>1</sup>, Jiangyan Fu<sup>1</sup>, Lu Feng<sup>1</sup>, Xinhong Xu<sup>1</sup>, Tongming Yin<sup>2</sup>, Xiaoming  
7   Wang<sup>1</sup>, Guihua Lu<sup>1,3\*</sup>, Jinliang Qi<sup>1,2\*</sup>, Hongyan Lin<sup>1,2\*</sup>, Yonghua Yang<sup>1,2\*</sup>.

8  
9   <sup>1</sup>*State Key Laboratory of Pharmaceutical Biotechnology, Institute of Plant Molecular*  
10   *Biology, School of Life Sciences, Nanjing University, Nanjing 210023, PR China*

11   <sup>2</sup>*Co-Innovation Center for Sustainable Forestry in Southern China, MOE Key*  
12   *Laboratory of Forest Genetics and Biotechnology, Nanjing Forestry University,*  
13   *Nanjing, 210037, PR China*

14   <sup>3</sup>*School of Life Sciences, Huaiyin Normal University, Huaian 223300, China*

15  
16   <sup>†</sup>These authors contributed equally to this work

17   \*Corresponding authors

18  
19   \*Corresponding author:

20   Yong-Hua Yang,

21   State Key Laboratory of Pharmaceutical Biotechnology,

22 Nanjing University, Nanjing 210023, PR China.

23 Tel/Fax: 86-25-89686305,

24 Email: yangyh@nju.edu.cn.

## Abstract

The purpose of this study was to explore the effects of natural shikonin and its derivatives on mice experimental colitis induced by dextran sulfate sodium, and to investigate the underlying mechanisms *in vivo*. Our results suggested that, intragastric administration of single compound like shikonin and its derivatives contributed to attenuating symptoms of malignant induced by DSS. Meanwhile, shikonin or its derivatives could also remarkably reduce the disease activity index and histopathological scores, suppress the levels of pro-inflammatory cytokines (including IL-6, IL-1 $\beta$  and TNF- $\alpha$ ), while increase that of inflammatory cytokine IL-10 in serum. Additionally, both shikonin and alkanin were found to restrain the levels of COX-2, MPO and iNOS in serum and colonic tissues. Moreover, western blotting results demonstrated that shikonin and its derivatives could inhibit the activation of the NLRP3 inflammasome and the NF- $\kappa$ B signaling pathway, relieve the DSS-induced disruption of colonic epithelial tight junction (TJ) in colonic tissues. Further, docking simulation had been performed to prove that shikonin and its derivatives could bind to the active sites of NLRP3 inflammasome and the NF- $\kappa$ B to generate an effective inflammatory effect. Taken together, our experimental data can provide some evidence for the potential use of shikonin and its derivatives to treat the inflammatory bowel disease (IBD).

**Keywords:** shikonin and its derivatives, colitis, pro-inflammatory cytokines, NF- $\kappa$ B , NLRP3 inflammasome

# **Introduction**

Inflammatory bowel disease (IBD), a kind of refractory and relapsing gastrointestinal inflammatory disease, has not only affected the living quality of patients, but increased the risk of colon cancer[1, 2]. Recently, an increasing amount of evidence indicates that the incidence of colorectal cancer (CRC) in IBD patients is generally much higher than that in general population[3, 4]. Currently, traditional treatment strategies for IBD can mainly be classified as three categories, namely, amino-salicylic acid agents, glucocorticoids and immunosuppressive agents[5]. Typically, 5-aminosalicylic acid remains the preferred drug to treat IBD, which can directly treat enteritis through inhibiting inflammatory reaction in the intestinal tract and exerting the antibacterial function. Nonetheless, it will be quickly absorbed by the small intestine if taken orally, leading to acute and chronic renal injury; besides, the drug can not reach the inflammatory site, which can hardly achieve the treatment goal[6]. In addition, glucocorticoid is likely to develop dependence after long-term application, and disease recurrence is common after drug withdrawal[7]. By contrast, immunosuppressant is suitable for patients with chronic enteritis, which can inhibit lymphocyte proliferation to achieve the therapeutic effect; nonetheless, it is associated with strong toxic side effects[8]. Fortunately, the emergence of anti-tumor necrosis factor (anti-TNF) therapy has made a major advance in the treatment of patients with IBD for the past two decades[9]. However, despite the therapy is useful in over half of IBD patients, a substantial proportion of patients are primary non-responders or lose

response with long term use[10, 11]. What's more, this therapy also exists some disturbing safety issues encompassing the increased risk of infections and malignant tumor in some groups[12, 13]. These factors pushed the need for finding some other safe, mild and durable effective drugs to cure IBD.

The disease characteristics of IBD and shortcomings of current treatments force us to turn to the traditional Chinese medicine (TCM). *Lithospermum erythrorhizon* is a commonly used TCM in the Chinese pharmacopoeia of the People's Republic of China. Typically, it is abundant in shikonin derivatives, which have anti-tumor, anti-virus, and anti-inflammatory activities[14-18]. Numerous studies have indicated that shikonin and some of its derivatives are the main active ingredients in *Lithospermum erythrorhizon*[19-21]. There are dozens of species of shikonin in the Boraginaceae plants, among which, the representative ones are shikonin,  $\beta$ ,  $\beta$ -dimethylacryl-shikonin, acetyl-shikonin, 5, 8-dihydroxy-1, 4-naphthoquinone, and alkanin (the enantiomeric of shikonin) with high contents[22, 23]. They belong to the naphthoquinones that are collectively known as shikonins. Typically, shikonin and its derivatives, which are isolated from the root of *Lithospermum erythrorhizon*, possess the advantages of bright color, long lasting effect, and non-toxic side effects. It has been reported that the crude extract of *Arnebia euchroma* is effective in rats with experimental colitis[24]. Whereas, the efficacy of each individual component of the crude extract has not been study detailly. In this work, we will further study and compare the effectiveness of some natural shikonins with high content in

90 *Lithospermum erythrorhizon* for IBD treatment and to probe the potential

91 mechanisms.

## Materials and Methods

### Chemicals, Regents and antibodies

Shikonin (SK, #B21682; CAS NO. 517-89-5; LOT: R10J8F39560), alkanin (AK, #B50783, CAS NO. 517-88-4; LOT: P05M7F14232), naphthoquinone (#AA18930, CAS NO. 475-38-7), acetyl-shikonin (acetyl-SK, #B21508, CAS NO. 24502-78-1; LOT: R21J7F16576) and  $\beta$ ,  $\beta$ -dimethylacryl-shikonin ( $\beta$ ,  $\beta$ -dimethylacryl-SK, #B24012, CAS NO. 24502-79-2; LOT: P12M7F11131) were ordered from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China). All of them were characterized by  $^{13}\text{C}$  NMR,  $^1\text{H}$  NMR, melting properties and MS analysis, which were exactly accord with the structure described (*SI Appendix*). DSS (white powder; CAS NO. 9011-18-1; LOT: Q6182; M.W. = 36000 – 50000 Da) was purchased from MP Biomedicals (Shanghai, China). Mesalazine (#A600043-0050; CAS NO. 89-57-6; LOT: B326BA1390) was purchased from Sigma-Aldrich (Shanghai, China). Murine TNF- $\alpha$  (#ab208348), IL-1 $\beta$  (#ab197742), IL-6 (#ab100712), IL-10 (#ab108870) Elisa assay kits were purchased from Abcam (Cambridge, England). COX-2 (#SBJ-M0847), iNOS (#SBJ-M0041) and MPO (#SBJ-M0329) Elisa assay kits were ordered from Nanjing SenBeiJia Biological Technology Co., Ltd., (Nanjing, China). Primers were purchased from Genscript (Nanjing, China). All molecular docking results were in strict accordance with operation specification of Discovery Studio 3.5. The docking calculation of mesalazine, shikonin and its derivatives was showed in [S1 Table](#).

PMSF (phenylmethanesulfonyl fluoride), RIPA lysis buffers (#P0013B) were

purchased from Beyotime Institute of Biotechnology (Haimen, China). BCA protein assay kit (#23227) was purchased from Pierce (Rockford, IL, USA). anti-iNOS (#E1A8233A), anti-NF- $\kappa$ B p65 (#10745-1-AP) and anti-GAPDH (#10494-1-AP) were purchased from Enogene (Nanjing, China). Goat anti-mouse IgG (H+L), HRP conjugate (#SA00001-1) and Goat anti-rabbit IgG (H+L), HRP conjugate (#SA00001-2) were purchased from Proteintech (Wuhan, China). anti-NLRP3 (#AF2155), anti-STAT3 (#AF1492) were purchased from Beyotime Biotechnology (Nanjing, China). anti-ZO-1 (#WL03419), anti-Occludin (#WL01996), anti-Claudin-1 (#WL03073), anti-ASC (#WL02462), anti-IL-1 $\beta$  (#WL02257), anti-COX-2 (WL01750), anti-p-STAT3 (#WLP2412), anti-IK $\beta$  (#WL00148), anti-p-IK $\beta$  (#WL02495) and anti-p-NF- $\kappa$ B p65 (Ser536) (#WL02169) were purchased from Wanlei Biotech Co. Ltd (Shenyang, China), anti-VCAM-1 (#GB11336), anti-Caspase1 (#GB11383) and anti- $\beta$ -actin (#GB11001) were purchased from Servicebio Technology Co. Ltd (Wuhan, China). anti-IK $\alpha$  (#2682) and anti-IK $\beta$  (#2678) were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). 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 48 health male C57BL/6 mice weighted 18-20 g were selected for *in vivo*

experiment. All of them were purchased from Model Animal Research Center of Nanjing University (Nanjing, China) and were randomly divided into eight groups for 1 week to let them adapt to their new environment, each consisting of six animals, including the model (the dextran sulfate sodium; 3.5% (w/v) DSS), SK (25 mg/kg), AK (25 mg/kg), naphthoquinone (25 mg/kg), acetyl-SK (25 mg/kg),  $\beta$ ,  $\beta$ -dimethylacryl-SK (25 mg/kg), mesalazine (positive drug; 200 mg/kg) and control group. All mice housed under a 12 hours light-dark cycle at  $22 \pm 2^\circ\text{C}$  and  $60\% \pm 5\%$  humidity. Animals were supplied with standard diet and sterile water ad libitum for 1 week, then all the groups mice except for control group were exposed to 3.5% DSS, which was dissolved in drinking water. The drugs were given orally once daily started at the same time as the DSS treatment and continued 1 week. 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 Animal Ethics Committee of the Ministry of Science and Technology of China (2006). All efforts were aimed at reducing the number and suffering of experimental animals while meeting the needs of the experiment.

## **Establishment of acute enteritis model and treatment with SK and its derivatives**

Acute colitis was induced by free intake of sterile water containing 3.5% DSS for one week. Another six groups (n=6 mice per group) of mice which also fed orally with 3.5% DSS in sterile water accompanied with the usage of SK, AK, naphthoquinone,

acetyl-SK,  $\beta$ ,  $\beta$ -dimethylacryl-SK and mesalazine (the positive control group) every day. All drugs were dissolved in olive oil and were perfused 200  $\mu$ L per mice at a predetermined dose by gavage. In the DSS group, the mice fed orally with the same amount of olive oil. In the blank control group, the mice received only sterile water and fed orally with the same amount of olive oil. There was no significant difference in water intake among all groups. Body weight and the disease activity index (DAI) were measured and recorded everyday[25]. The DAI was calculated according to the standard listed in [S2 Table](#). After one week of treatment, all the mice monocular eyeball was removed for blood collection under lightly anesthetized with ether. Then, the mice were euthanized by cervical dislocation. The colonic tissues were removed for pathological analysis. The same protocol was carried out at least three times in independent experiments.

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

The expressions of various cytokines (IL-6, IL-1 $\beta$ , TNF- $\alpha$  and IL-10), COX-2, MPO and iNOS in the serum of mice were detected by an enzyme-linked immunosorbent assay kit according to the product's guidelines.

#### **Haematoxylin & Eosin (HE) staining**

Small sections of colonic tissue were fixed in 10% buffered formalin and embedded in paraffin. Then, sections were stained with haematoxylin and eosin.

Histologic evolution of colonic mucosa was carried out in a blinded fashion by a pathologist which has been widely used as evaluation criterion[24].

### **Immunohistochemical analysis**

Immunohistochemistry was performed for Cyclooxygenase-2 (COX-2), NF- $\kappa$ B, NLRP3. The tissues were incubated with primary antibody at 4 °C for at least 12 h after deparaffinized and rehydrated. The Alexa Fluor 488 labeled anti-mouse secondary antibody (Invitrogen, USA) were treated 60 min at 25 °C. Signals were developed with Hematoxylin and DAB (Dako, Agilent Technologies, USA).

### **Western bolt analysis**

Colonic samples were lysed with RIPA lysis buffer which containing 1% PMSF. The harvested protein concentrations were assessed by using the BCA Protein Assay kit. 60-80  $\mu$ g of total protein per sample were loaded onto 10 % SDS PAGE and transferred onto PVDF membranes for immunoblot analysis[25]. The blots were detected by super ECL detection reagent (Yeasen Biotech Co., Ltd) according to the schemes of the manufacturer and then exposed to film by digital camera (Tanon 5200, Tanon, China). Finally, the immunoblots were quantified by densitometry with Image J Software (National Institutes of Health, Bethesda, Maryland, USA), and  $\beta$ -actin or GAPDH was used as loading control.

## **RNA isolation and quantitative real-time PCR analysis**

The extraction of colonic tissues total RNA was according to the manufacture instructions by using RNAprep pure Tissue Kit (TIANGEN BIOTECH, BeiJing, China). After that, the first step product was converted to cDNA using FastKing RT Kit (With gDNase) (TIANGEN BIOTECH, BeiJing, China). Then, the collected cDNA solution was used for qRT-PCR using Power SYBR® Green PCR Master Mix (Roche, Indianapolis, USA) according to the schemes of the manufacturer, which was carried out by the BioRad CFX96 Touch™ RealTime PCR Detection System (BioRad, CA). The sequences of each primer are listed in [S3 Table](#).

## **Docking simulation**

Molecular docking of the compounds binding to the three-dimensional X-ray structure of MPO (PDB code: 4C1M), NF-κB (PDB code: 4DN5), NLRP3 (PDB code: 6NPY), TNF-α (PDB code: 2AZ5), IL-1β (PDB code: 3O4O), IL-6 (PDB code: 5U98), IFNγ (PDB code: 3OQ3) and IL-10 (PDB code: 5T5W) were performed using Discovery Studio (version 3.5). SK, AK, naphthoquinone, acetyl-SK, β, β-dimethylacryl-SK and mesalazine were constructed, minimized and prepared. The RCSB Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>) provides the crystal structures of the protein complex download. All bound waters and ligands were removed from the protein. After the molecular docking, Discovery Studio (version 4.5) were used for analysis of types of interactions between the ligands and docked proteins.

219

## 220 **Data and Statistical analysis**

221 Results were presented as mean  $\pm$  S.E.M. of three independent experiments. Statistical  
 222 comparisons between the treated and untreated groups were performed using one-way  
 223 ANOVA using GraphPad PRISM5 (Graphpad Inc., La Jolla, USA).  $P < 0.05$  was  
 224 considered to be significant;  $P < 0.01$  were considered to be very significant.

## Results

### **SK and its derivatives protected the colon from DSS-induced damage in an acute ulcerative colitis mouse model**

To investigate the effects of SK and its derivatives on colitis, we established an acute DSS-induced ulcerative colitis mouse model. In the model, mice were given either sterile water or 3.5% DSS dissolved in sterile water. Six other groups received 3.5% DSS and were administered SK, naphthoquinone,  $\beta$ ,  $\beta$ -dimethylacryl-SK, acetyl-SK, AK, mesalazine orally both at the beginning of the experiment (n=6 mice per group). At the end of this experiments, SK and its derivatives could attenuate the severity level of inflammatory significantly. In the case of the colorectum length, DSS group mice suffered a shortening of about 30% when compared to normal mice. While SK and its derivatives significantly prevented this shortening (Fig 1A and 1B). Moreover, SK and its derivatives treated mice showed improvements in body weight, disease activity index (DAI) and alleviation of splenomegaly (Fig 1C-1F). SK and its derivatives thus ameliorated the colon syndromes of DSS-induced damage in mice.

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

Once the circulating leukocytes were recruits into the colon, it will lead to the release of pro-inflammatory mediators, including pro-inflammatory cytokines such as IL-6, IL-1 $\beta$ , TNF- $\alpha$  and pro-inflammatory synthases such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). To determine whether SK and its derivatives

attenuates the release of pro-inflammatory cytokines, therefore protecting the intestinal mucosa, we detected the levels of IL-6, IL-1 $\beta$ , TNF- $\alpha$  and IL-10 (anti-inflammatory cytokine) in the supernatant of cultured colons of 3.5% DSS-exposed mice given control diet or SK and its derivatives. As shown in Fig 2A-2D, treatment with SK and its derivatives at a dosage of 25 mg/kg, significantly reduced the levels of pro-inflammatory cytokines (IL-6, IL-1 $\beta$  and TNF- $\alpha$ ) and added the level of anti-inflammatory cytokine (IL-10) in the serum. Among them, SK showed the best anti-inflammation effect that was similar to mesalazine group at a dosage of 200 mg/kg. As shown in Fig 2E-2H, SK and mesalazine (the positive control) were found to reduce the levels of pro-inflammatory cytokines (IL-6, IL-1 $\beta$  and TNF- $\alpha$ ) and increased the level of anti-inflammatory cytokine (IL-10) in a dose-dependent manner and SK treatment resulted in better anti-inflammatory effect at a dose of 25 mg/kg. The above results suggest that SK and its derivatives may improve the severity of colon damage induced by DSS through inhibiting the expression of pro-inflammatory cytokines.

### **SK and its derivatives strengthened the prevention of colon from DSS-induced the histological damage**

The severity level of colon tissues was assessed by distortion of crypts, loss of goblet cells, infiltration of inflammatory cells, and severe mucosal damage which were observed by HE staining. The results shown in Fig 3A and 3B indicated that the

serious deterioration of the colons were markedly protected by SK and its derivatives.

In contrast, we found SK and AK showed better effects than other derivatives and the

protection of them are similar to that of mesalazine.

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

MPO is an enzyme which is present largely in neutrophils, as well as in monocytes

and macrophages in small concentrations. The concentrations of MPO, COX-2 and

iNOS in all the groups of mice are shown in [Fig 4](#). The MPO activity is an indicator

of neutrophil infiltration in inflamed colon tissues[26]. Administration of DSS

increased the activity of MPO as compared to the normal mice. SK and AK

administration markedly reduced the concentrations of MPO, COX-2 and iNOS as

compared to DSS-induced mice.

## **SK and its derivatives regulate the expression of COX-2, iNOS and the activation**

### **of NF- $\kappa$ B in colonic tissues**

Transcription factors of the NF- $\kappa$ B family play a vital role in the regulation of genes,

which can influence the immune and inflammatory response[27]. Many evidences

have verified that NF- $\kappa$ B plays an important role in the development of inflammatory

bowel disease. In his disease, NF- $\kappa$ B activation is observed in inflamed colonic

mucosa, in which it leads the production of COX-2 and iNOS. To evaluate the

mechanism of SK, we detected the phosphorylation level of STAT-3, NF- $\kappa$ B p65 and

IκB. The outcome shown in [Fig 5](#) suggested that the phosphorylation levels of STAT-3, NF-κB p65 and IκB were markedly up-regulated in DSS-treated group compared with the control group. While SK and its derivatives can effectively inhibit the activation of NF-κB and STAT-3, thus decreasing the generation of COX-2 and iNOS in the colon homogenates.

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

ZO-1, VCAM-1, Occludin and Claudin-1 proteins play an important role in epithelial tight junction, which can protect mucosa epithelial cells from breaching by the harmful substances, maintaining cellular permeability and integrity, and ensuring homeostasis of internal environment[28]. Therefore, we further investigated the effect of SK and its derivatives on epithelial tight junction proteins. The results suggested that the expression of ZO-1, VCAM-1, Occludin and Claudin-1 were remarkably reduced in DSS group compared to the control group. However, SK and its derivatives can restore the expression of these epithelial tight junction proteins ([Fig 6](#)).

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

Inflammasomes, a multiprotein oligomer, have been widely reported that it can

promote the activation of CASP1/caspase 1 and maturation of proinflammatory cytokine IL1B (interleukin 1,  $\beta$ )[29]. Many researches have revealed that NLRP3 affects several inflammatory disorders to a large extent including colitis. As shown in Fig 7, NLRP3, ASC, caspase-1 and IL-1 $\beta$  expressions in DSS group were significantly increased when compared to the control group. However, these changes were receded by SK and its derivatives with different degrees.

### **SK and its derivatives reduced the mRNA level of cytokines and protease**

Real time-PCR analysis and mRNA expressions of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IFN $\gamma$ , COX-2, IL-10 and CD206 were also detected and the results were shown in Fig 8. The DSS treatment apparently enhanced mRNA levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IFN $\gamma$  and COX-2 as compared to the control group, while SK and its derivatives can reduced the mRNA levels of these pro-inflammatory cytokines. On the contrary, the mRNA levels of anti-inflammatory cytokines (IL-10 and CD206) in the DSS treatment mice were lower than the control group. The subsequent SK administration increased IL-10 and CD206 mRNA effectively.

### **SK and its derivatives attenuate the production of COX-2, NF- $\kappa$ B and NLRP3 in colonic tissues**

Immuno-histochemical expressions of COX-2, NF- $\kappa$ B and NLRP3 in all the groups of mice are shown in Fig 9. The results demonstrated that DSS administration resulted in

increased production of COX-2, NF- $\kappa$ B and NLRP3 in colonic tissues compared to the control group. Usage of SK and its derivatives were able to reduce the expression of COX-2, NF- $\kappa$ B and NLRP3 significantly ( $p < 0.05$ ) as compared to mice induced with DSS.

### **Molecular docking**

Docking study has confirmed that SK and its derivatives anchored in the binding pocket of the protein perfectly through a series of binding mode, which may play a vital role in the conformation with them. As shown in. [S1-S4 Figs](#), the binding mode of the compounds with proteins were stabilized mainly through hydrogen bond that are shown in the 2D and 3D diagrams. The binding energy of the compounds with proteins implies their anti-inflammatory activity. The whole results showed that the docking model had certain credibility and demonstrated that shikonin and its derivatives were potential anti-inflammatory agents compared to the positive drug mesalazine.

### **Discussion**

*Lithospermum erythrorhizon* is a kind of TCM, which has long been used for wound healing in ancient times. Typically, its roots are rich in a variety of bioactive substances, including natural naphthoquinones, which represents a continuing source to mine the therapeutic agents[30-32]. Among the naphthoquinones isolated from

*Lithospermum erythrorhizon*, shikonin has attracted extensive interest due to its multiple biological activities, such as anti-inflammatory[18, 33], wound healing[34], anti-microbial[35], anti-ulcer[36], anti-thrombus[37] and anti-cancer activities[38, 39]. Hitherto, nearly 20 kinds of natural shikonin derivatives have been identified.

The anti-inflammatory effects of natural naphthoquinones have been extensively investigated. For instance, Fan *et al.* had investigated the effectiveness of hydroxynaphthoquinone mixture (HM) isolated from *Arnebia euchroma* on rats with experimental colitis induced by 2, 4, 6-tri-nitrobenzene sulfonic acid (TNBS) in 2013.[24] Their results suggested that HM could remarkably attenuate the clinical and histopathological severity of the TNBS-induced colitis in a dose-dependent manner. In addition, it could also alleviate loss of body weight, hematochezia and inflammation, reduce the macroscopic damage score, and improve the histological signs; moreover, it can evidently reduce the inflammatory infiltration, ulcer area and the severity of goblet cell depletion. Notably, HM, which is isolated from *Arnebia euchroma*, contains seven alkannin derivatives, including alkannin, deoxyalkannin, acetylalkannin,  $\beta$ ,  $\beta$ -dimethylacrylalkannin,  $\alpha$ -methylalkannin, isovalerylalkannin and  $\beta$ -acetoxyisovalerylalkannin. In 2012, Isabel *et al.* had reported that the oral administration of shikonin could attenuate the DSS-induced acute ulcerative colitis by blocking the activation of two major targets NF- $\kappa$ B and STAT-3[27]. However, the effectiveness of the commonly used shikonin and its natural derivatives on acute colitis has not been systematically investigated yet.

373 In this study, five representative natural naphthoquinones, namely, 5,  
374 8-dihydroxy-1, 4-naphthoquinone, shikonin, acetyl-shikonin,  $\beta$ ,  
375  $\beta$ -dimethylacryl-shikonin and alkanin, had been selected to treat DSS-induced colitis  
376 in mice. The above results suggested that, the administration of these five natural  
377 naphthoquinones could ameliorate the clinical severity of the wasting disease, prevent  
378 the shortening the length of colorectum, and decrease the loss of body weight,  
379 improve the appearance of feces, and prevent bloody excrements. Such beneficial  
380 effects were further proved by histological evaluation, as evidenced by the markedly  
381 reduced severity and extent of inflamed tissue damage and infiltration of  
382 inflammatory cells. Typically, the relative expression of pro-inflammatory cytokines,  
383 including IL-6, IL-1 $\beta$  and TNF- $\alpha$  in serum, as well as their mRNA levels in colon  
384 tissues, suggested that these five natural naphthoquinones had beneficial effects. As  
385 mentioned earlier, MPO is a marker of neutrophil infiltration[26], which has been  
386 observed to be activated in several experimental colitis models, including the  
387 DSS-induced colitis. Therefore, MPO could be recognized as an indicator to quantify  
388 intestinal inflammation and evaluate the severity of inflammation[26]. In addition, the  
389 pro-inflammatory synthases, including iNOS and COX-2, are the vital inflammatory  
390 mediators with vital functions during the pathogenesis of ulcerative colitis[40]. The  
391 five natural naphthoquinones could suppress the MPO, iNOS and COX-2 activities,  
392 and such results were consistent with those found in histological examination, in  
393 which the inflammatory cell infiltration extents in colonic tissues were less than that

in shikonin- and alkannin-treated animals than in 5, 8-dihydroxy-1, 4-naphthoquinone-, acetyl-shikonin- and  $\beta$ ,  $\beta$ -dimethylacryl-shikonin-treated mice. Besides, results of Western blotting had further verified that shikonin could relieve the colitis in mice through inhibiting the activation of NF- $\kappa$ B and NLRP3 inflammasome, as well as the disruption of epithelial tight junction proteins (ZO-1, VCAM-1, Occludin and Claudin-1). Collectively, our work has provided supporting evidence for the clinical application of natural naphthoquinones, especially for shikonin.

## Conclusions

To sum up, through *in vitro* and *in vivo* experiments, we found that naphthoquinone compounds shikonin and some of its derivatives which were isolated from the root of the Chinese herbal medicine *Lithospermum erythrorhizon* could effectively attenuate the severity of DSS-induced colitis. This effect may due to the reduction of pro-inflammatory cytokines release, including IL-6, IL-1 $\beta$  and TNF- $\alpha$  in serum, as well as their mRNA levels in colon tissues, while the expression of anti-inflammatory cytokines (IL-10 and CD206) were increased. Moreover, they could suppress the MPO, iNOS and COX-2 activities, and such results were consistent with those observed in histological examination. Furthermore, changes in the expression of inflammatory regulatory proteins have been detected. These aforementioned results offer a kind of perspective for the potential use of shikonin and its derivatives to treat the IBD.

## 414 **Abbreviations**

415 DSS, dextran sulfate sodium; IL-1 $\beta$ , interleukin1 $\beta$ ; IL-6, interleukin6; TNF- $\alpha$ , tumor  
 416 necrosis factor- $\alpha$ ; IL-10, interleukin10; COX-2, cyclooxygenase-2; MPO,  
 417 myeloperoxidase; iNOS, inducible nitric oxide synthase; ASC, apoptosis-associated  
 418 speck-like protein containing CARD; NLRP3, nod-like receptor protein3; NF- $\kappa$ B,  
 419 nuclear factor-kappa B; TJ, tight junction; IBD, inflammatory bowel disease; CRC,  
 420 colorectal cancer; TCM, traditional Chinese medicine; SK, shikonin; AK, alkanin;  
 421 acetyl-SK, acetyl-shikonin;  $\beta$ ,  $\beta$ -dimethylacryl-SK,  $\beta$ -dimethylacryl-shikonin; DAI,  
 422 disease activity index; HE, haematoxylin & Eosin; RIPA, radio immunoprecipitation  
 423 assay; PVDF, polyvinylidene fluoride; HRP, horseradish peroxidase; UC, ulcerative  
 424 colitis; ZO-1, zonula occludens; VCAM-1, vascular cell adhesion protein 1; HM,  
 425 hydroxynaphthoquinone mixture; TNBS, 2, 4, 6-tri-nitrobenzene sulfonic acid.

## 427 **Acknowledgements**

428 This research was supported by the National Natural Science Foundation of China  
 429 (NSFC) (21702100, 31771413, 31670298, 21907051), the Program for Changjiang  
 430 Scholars and Innovative Research Team in University (IRT\_14R27), and the  
 431 Fundamental Research Funds for the Central Universities (020814380002,  
 432 020814380057).

434 **Author contributions**

435 **Conceptualization:** Yonghua Yang, Hongyan Lin, Guihua Lu, Jinliang Qi.

436 **Data curation:** Zhongling Wen, Yinsong Wang, Jiangyan Fu, Lu Feng, Xinhong Xu,  
437 Tongming Yin.

438 **Formal analysis:** Zhongling Wen, Yinsong Wang, Jiangyan Fu.

439 **Investigation:** Yonghua Yang, Hongyan Lin, Hongwei Han, Wenxue Sun.

440 **Methodology:** Hongyan Lin, Xiaoming Wang, Guihua Lu, Jinliang Qi.

441 **Project administration:** Wenxue Sun, Hongwei Han, Zhaoyue Wang, Minkai Yang.

442 **Resources:** Xiaoming Wang, Guihua Lu, Jinliang Qi.

443 **Supervision:** Yonghua Yang, Hongyan Lin, Guihua Lu, Jinliang Qi.

444 **Validation:** Yinsong Wang, Jiangyan Fu, Yonghua Yang, Hongyan Lin, Guihua Lu,  
445 Jinliang Qi.

446 **Visualization:** Hongwei Han, Hongyan Lin.

447 **Writing – original draft:** Wenxue Sun, Hongyan Lin.

448 **Writing – review & editing:** Yonghua Yang, Hongyan Lin, Guihua Lu, Jinliang Qi.

449

450 **Additional information**

451 **Conflict of interest:** The authors declare no conflict of interests.

## References

1. Farrell RJ, Peppercorn MA. Ulcerative colitis. *Lancet*. 2002; 359(9303):331-40.  
[https://doi.org/10.1016/S0140-6736\(02\)07499-8](https://doi.org/10.1016/S0140-6736(02)07499-8) PMID: 11830216.
2. Stone MA, Mayberry JF, Baker R. Prevalence and management of inflammatory bowel disease: a cross-sectional study from central England. *European journal of gastroenterology & hepatology*. 2003; 15(12):1275-80.  
<https://doi.org/10.1097/01.meg.0000085500.01212.e2> PMID: 14624149.
3. Terzic J, Grivennikov S, Karin E, Karin M. Inflammation and colon cancer. *Gastroenterology*. 2010; 138(6):2101-14.  
<https://doi.org/10.1053/j.gastro.2010.01.058> PMID: 20420949.
4. Triantafillidis JK, Nasioulas G, Kosmidis PA. Colorectal Cancer and Inflammatory Bowel Disease: Epidemiology, Risk Factors, Mechanisms of Carcinogenesis and Prevention Strategies. *Anticancer research*. 2009; 29(7):2727-37. PMID: 19596953
5. Huang LJ, Zhu Q, Lei M, Cao Q. Current use of immunosuppressive agents in inflammatory bowel disease patients in East China. *World Journal of Gastroenterology*. 2009; 15(24):3055-9. <https://doi.org/10.3748/wjg.15.3055> PMID: 19554661; PubMed Central PMCID: PMC2702116.
6. Munk EM, Pedersen L, Floyd A, Norgard B, Rasmussen HH, Sorensen HT. Inflammatory bowel diseases, 5-aminosalicylic acid and sulfasalazine treatment and risk of acute pancreatitis: A population-based case-control study. *Am J*

474 Gastroenterol. 2004; 99(5):884-8.  
 475 <https://doi.org/10.1111/j.1572-0241.2004.04123.x> PMID: 15128355.

476 7. Katz JA. Treatment of inflammatory bowel disease with corticosteroids.  
 477 Gastroenterol Clin N. 2004; 33(2):171-189.  
 478 <https://doi.org/10.1016/j.gtc.2004.02.002> PMID: 15177533.

479 8. Schroder O, Stein J. Low dose methotrexate in inflammatory bowel disease:  
 480 Current status and future directions. Am J Gastroenterol. 2003; 98(3):530-7.  
 481 [https://doi.org/10.1016/S0002-9270\(02\)06027-6](https://doi.org/10.1016/S0002-9270(02)06027-6) PMID: 12650783.

482 9. Khan S, Rupniewska E, Neighbors M, Singer D, Chiarappa J, Obando C.  
 483 Real-world evidence on adherence, persistence, switching and dose escalation  
 484 with biologics in adult inflammatory bowel disease in the United States: A  
 485 systematic review. Journal of clinical pharmacy and therapeutics. 2019;  
 486 44(4):495-507. <https://doi.org/10.1111/jcpt.12830> PMID: 30873648.

487 10. Hanauer SB, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF,  
 488 et al. Maintenance infliximab for Crohn's disease: the ACCENT I randomised  
 489 trial. Lancet. 2002; 359(9317):1541-9.  
 490 [https://doi.org/10.1016/S0140-6736\(02\)08512-4](https://doi.org/10.1016/S0140-6736(02)08512-4) PMID: 12047962.

491 11. Ma C, Huang V, Fedorak DK, Kroeker KI, Dieleman LA, Halloran BP, et al.  
 492 Crohn's disease outpatients treated with adalimumab have an earlier secondary  
 493 loss of response and requirement for dose escalation compared to infliximab: a  
 494 real life cohort study. Journal of Crohn's & colitis. 2014; 8(11):1454-63.

- 495 <https://doi.org/10.1016/j.crohns.2014.05.007> PMID: 24947334.
- 496 12. Lichtenstein GR, Feagan BG, Cohen RD, Salzberg BA, Diamond RH, Price S, et  
 497 al. Serious infection and mortality in patients with Crohn's disease: more than 5  
 498 years of follow-up in the TREAT registry. The American journal of  
 499 gastroenterology. 2012; 107(9):1409-22. <https://doi.org/10.1038/ajg.2012.218>  
 500 PMID: 22890223; PubMed Central PMCID: PMC3438468.
- 501 13. Singh JA, Wells GA, Christensen R, Tanjong Ghogomu E, Maxwell L,  
 502 Macdonald JK, et al. Adverse effects of biologics: a network meta-analysis and  
 503 Cochrane overview. The Cochrane database of systematic reviews.  
 504 2011(2):CD008794. <https://doi.org/10.1002/14651858.CD008794.pub2> PMID:  
 505 21328309.
- 506 14. Shahsavari Z, Karami-Tehrani F, Salami S, Ghasemzadeh M. RIP1K and RIP3K  
 507 provoked by shikonin induce cell cycle arrest in the triple negative breast cancer  
 508 cell line, MDA-MB-468: necroptosis as a desperate programmed suicide  
 509 pathway. Tumour biology : the journal of the International Society for  
 510 Oncodevelopmental Biology and Medicine. 2016; 37(4):4479-91.  
 511 <https://doi.org/10.1007/s13277-015-4258-5> PMID: 26496737.
- 512 15. Chen Y, Chen ZY, Chen L, Zhang JY, Fu LY, Tao L, et al. Shikonin inhibits  
 513 triple-negative breast cancer-cell metastasis by reversing the  
 514 epithelial-to-mesenchymal transition via glycogen synthase kinase  
 515 3beta-regulated suppression of beta-catenin signaling. Biochemical

- 516 pharmacology. 2019; 166:33-45. <https://doi.org/10.1016/j.bcp.2019.05.001>  
517 PMID: 31071331.
- 518 16. Chen X, Yang L, Zhang N, Turpin JA, Buckheit RW, Osterling C, et al.  
519 Shikonin, a component of chinese herbal medicine, inhibits chemokine receptor  
520 function and suppresses human immunodeficiency virus type 1. Antimicrobial  
521 agents and chemotherapy. 2003; 47(9):2810-6.  
522 <https://doi.org/10.1128/aac.47.9.2810-2816.2003> PMID: 12936978; PubMed  
523 Central PMCID: PMC182643.
- 524 17. Guo H, Sun J, Li D, Hu Y, Yu X, Hua H, et al. Shikonin attenuates  
525 acetaminophen-induced acute liver injury via inhibition of oxidative stress and  
526 inflammation. Biomedicine & pharmacotherapy = Biomedecine &  
527 pharmacotherapie. 2019; 112:108704-12.  
528 <https://doi.org/10.1016/j.biopha.2019.108704> PMID: 30818140.
- 529 18. Yoshida LS, Kakegawa T, Yuda Y, Takano-Ohmuro H. Shikonin changes the  
530 lipopolysaccharide-induced expression of inflammation-related genes in  
531 macrophages. Journal of natural medicines. 2017; 71(4):723-34.  
532 <https://doi.org/10.1007/s11418-017-1106-5> PMID: 28699129.
- 533 19. Papageorgiou VP, Assimopoulou AN, Couladouros EA, Hepworth D, Nicolaou  
534 KC. The Chemistry and Biology of Alkannin, Shikonin, and Related  
535 Naphthazarin Natural Products. Angewandte Chemie. 1999; 38(3):270-301.  
536 [https://doi.org/10.1002/\(SICI\)1521-3773\(19990201\)38:3<270::AID-ANIE270>3.0](https://doi.org/10.1002/(SICI)1521-3773(19990201)38:3<270::AID-ANIE270>3.0)

537 .CO;2-0 PMID: 29711637.

538 20. Papageorgiou VP, Assimopoulou AN, Ballis AC. Alkannins and shikonins: a  
539 new class of wound healing agents. Current medicinal chemistry. 2008;  
540 15(30):3248-67. <https://doi.org/10.2174/092986708786848532> PMID:  
541 19075667.

542 21. Malik S, Bhushan S, Sharma M, Ahuja PS. Biotechnological approaches to the  
543 production of shikonins: a critical review with recent updates. Critical reviews in  
544 biotechnology. 2016; 36(2):327-40.  
545 <https://doi.org/10.3109/07388551.2014.961003> PMID: 25319455.

546 22. Papageorgiou VP. Wound healing properties of naphthaquinone pigments from  
547 Alkanna tinctoria. Experientia. 1978; 34(11):1499-501. [https://doi.org/](https://doi.org/10.1007/bf01932375)  
548 10.1007/bf01932375 PMID: 720485.

549 23. Papageorgiou VP, Assimopoulou AN, Samanidou VF, Papadoyannis IN.  
550 Analytical methods for the determination of alkannins and shikonins. Curr Org  
551 Chem. 2006; 10(5):583-622. <https://doi.org/10.2174/138527206776055277>.

552 24. Fan HY, Zhang ZL, Liu K, Yang MY, Lv WH, Che X, et al. Effectiveness of a  
553 hydroxynaphthoquinone fraction from Arnebia euchroma in rats with  
554 experimental colitis. World journal of gastroenterology. 2013; 19(48):9318-27.  
555 <https://doi.org/10.3748/wjg.v19.i48.9318> PMID: 24409058; PubMed Central  
556 PMCID: PMC3882404.

557 25. Farooq SM, Hou Y, Li H, O'Meara M, Wang Y, Li C, et al. Disruption of GPR35

- 558 Exacerbates Dextran Sulfate Sodium-Induced Colitis in Mice. Digestive diseases  
559 and sciences. 2018; 63(11):2910-22. <https://doi.org/10.1007/s10620-018-5216-z>  
560 PMID: 30043283; PubMed Central PMCID: PMC6373462.
- 561 26. Krawisz JE, Sharon P, Stenson WF. Quantitative assay for acute intestinal  
562 inflammation based on myeloperoxidase activity. Assessment of inflammation in  
563 rat and hamster models. Gastroenterology. 1984; 87(6):1344-50 PMID: 6092199.
- 564 27. Andujar I, Rios JL, Giner RM, Miguel Cerda J, Recio Mdel C. Beneficial effect  
565 of shikonin on experimental colitis induced by dextran sulfate sodium in BALB/c  
566 mice. Evidence-based complementary and alternative medicine : eCAM. 2012;  
567 2012:271606-20. <https://doi.org/10.1155/2012/271606> PMID: 23346196;  
568 PubMed Central PMCID: PMC3549420.
- 569 28. Wang C, Liu J, Zhou Y, Feng G. The effect of 7-hydroxycoumarin on dextran  
570 sulfate sodium-induced ulcerative colitis. Basic & clinical pharmacology &  
571 toxicology. 2019; 124(6):749. <https://doi.org/10.1111/bcpt.12948> PMID:  
572 29278445.
- 573 29. Guo W, Liu W, Chen Z, Gu Y, Peng S, Shen L, et al. Tyrosine phosphatase  
574 SHP2 negatively regulates NLRP3 inflammasome activation via  
575 ANT1-dependent mitochondrial homeostasis. Nature communications. 2017;  
576 8(1):2168-81. <https://doi.org/10.1038/s41467-017-02351-0> PMID: 29255148;  
577 PubMed Central PMCID: PMC5735095.
- 578 30. Chen X, Yang L, Oppenheim JJ, Howard MZ. Cellular pharmacology studies of

- shikonin derivatives. *Phytotherapy research* : PTR. 2002; 16(3):199-209.  
<https://doi.org/10.1002/ptr.1100> PMID: 12164262.
31. Hayashi M. [Pharmacological studies of Shikon and Tooki. (2) Pharmacological effects of the pigment components, Shikonin and acetylshikonin]. *Nihon yakurigaku zasshi Folia pharmacologica Japonica*. 1977; 73(2):193-203 PMID: 560339.
32. Qiu HY, Wang PF, Lin HY, Tang CY, Zhu HL, Yang YH. Naphthoquinones: A continuing source for discovery of therapeutic antineoplastic agents. *Chemical biology & drug design*. 2018; 91(3):681-90. <https://doi.org/10.1111/cbdd.13141> PMID: 29130595.
33. Yang Y, Wang J, Yang Q, Wu S, Yang Z, Zhu H, et al. Shikonin inhibits the lipopolysaccharide-induced release of HMGB1 in RAW264.7 cells via IFN and NF-kappaB signaling pathways. *International immunopharmacology*. 2014; 19(1):81-7. <https://doi.org/10.1016/j.intimp.2014.01.003> PMID: 24447680.
34. Yao CH, Chen KY, Chen YS, Li SJ, Huang CH. Lithospermi radix extract-containing bilayer nanofiber scaffold for promoting wound healing in a rat model. *Materials science & engineering C, Materials for biological applications*. 2019; 96:850-8. <https://doi.org/10.1016/j.msec.2018.11.053> PMID: 30606599.
35. Andujar I, Rios JL, Giner RM, Recio MC. Pharmacological Properties of Shikonin - A Review of Literature since 2002. *Planta Med*. 2013;

600 79(18):1685-97. <https://doi.org/10.1055/s-0033-1350934> PMID: 24155261.

601 36. Lesuis N, Befrits R, Nyberg F, van Vollenhoven RF. Gender and the treatment of  
602 immune-mediated chronic inflammatory diseases: rheumatoid arthritis,  
603 inflammatory bowel disease and psoriasis: an observational study. *Bmc Med*.  
604 2012; 10(1). <https://doi.org/Artn 8210.1186/1741-7015-10-82> PMID: 22853635;  
605 PubMed Central PMCID: PMC3414735.

606 37. Andrikopoulos NK, Kaliora AC, Assimopoulou AN, Papapeorgiou VP.  
607 Biological activity of some naturally occurring resins, gums and pigments  
608 against in vitro LDL oxidation. *Phytotherapy Research*. 2003; 17(5):501-7.  
609 <https://doi.org/10.1002/ptr.1185> PMID: 12748987.

610 38. Liang WQ, Cai AZ, Chen GZ, Xi HQ, Wu XS, Cui JX, et al. Shikonin induces  
611 mitochondria-mediated apoptosis and enhances chemotherapeutic sensitivity of  
612 gastric cancer through reactive oxygen species. *Sci Rep-Uk*. 2016; 6.  
613 <https://doi.org/Artn 3826710.1038/Srep38267> PMID: 27905569; PubMed  
614 Central PMCID: PMC5131274.

615 39. Lin HY, Han HW, Sun WX, Yang YS, Tang CY, Lu GH, et al. Design and  
616 characterization of alpha-lipoic acyl shikonin ester twin drugs as tubulin and  
617 PDK1 dual inhibitors. *European journal of medicinal chemistry*. 2018;  
618 144:137-50. <https://doi.org/10.1016/j.ejmech.2017.12.019> PMID: 29268130.

619 40. Itzkowitz SH. Molecular biology of dysplasia and cancer in inflammatory bowel  
620 disease. *Gastroenterology clinics of North America*. 2006; 35(3):553-71.

621 <https://doi.org/10.1016/j.gtc.2006.07.002> PMID: 16952740.

622

624 **Figure captions:**

625 **Fig 1. SK and its derivatives protect the colon from DSS-induced damage. (A)**

626 Macroscopic observation of colon length. **(B)** Bar graph showing colon length.

627  $**p<0.01$  versus control group. **(C)** Pattern of daily weight changes.  $*p<0.05$ ,

628  $**p<0.01$  versus DSS-treated group. **(D)** Disease activity index of mice in each group.

629  $*p<0.05$ ,  $**p<0.01$  versus DSS-treated group. **(E)** Macroscopic observation of spleen

630 size. **(F)** Bar graph showing spleen weight. Data are shown as mean  $\pm$  S.E.M,  $*p<0.05$

631 versus control group (n=6 per group).

632 **Fig 2. SK and its derivatives exhibited a stronger ability in decreasing the**

633 **amount of pro-inflammatory cytokines and increasing the level of**

634 **anti-inflammatory cytokine in serum. (A-D)** The protein level of cytokines IL-6,

635 IL-1 $\beta$ , TNF- $\alpha$  and IL-10 in serum were determined by ELISA (DSS: 3.5%; SK,

636 Naphthoquinone,  $\beta$ ,  $\beta$ -dimethylacryl-SK, Acetyl-SK, AK: 25 mg/kg; Mesalazine: 200

637 mg/kg). **(E-H)** Effects of different concentrations of SK on the levels of cytokines

638 IL-6, IL-1 $\beta$ , TNF- $\alpha$  and IL-10. Data are shown as mean  $\pm$  S.E.M,  $*p<0.05$ ,  $**p<0.01$

639 versus control group;  $\#p<0.05$ ,  $##p<0.01$  versus DSS-treated group (n=6 per group).

640 **Fig 3. Colon histology and inflammation score of mice in each group (H&E**

641 **staining 200 $\times$  and 400 $\times$ ). (A)** The colon histology sections were stained with

642 hematoxylin and eosin. The colon histology sections of DSS-treated group mice

643 revealed sever pathology with observation of submucosal edema with extensive

644 inflammatory cell infiltration. The frame indicates the region magnified in the bottom

panel. **(B)** Inflammation scores of each group. Data are shown as mean  $\pm$  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).

**Fig 4. 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  $\pm$  S.E.M. from three replicates (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus control; #,  $P < 0.05$ ; ##,  $P < 0.01$  versus DSS group).

**Fig 5. Effect of SK and its derivatives on COX-2, iNOS expression, and NF- $\kappa$ B signal pathway in DSS-induced acute colitis.** **(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- $\kappa$ B signal pathway; **(D)** Relative protein expression ratios of were determined by densitometry. Each point represents the mean  $\pm$  S.E.M. from three replicates (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus control; #,  $P < 0.05$ ; ##,  $P < 0.01$  versus DSS group).

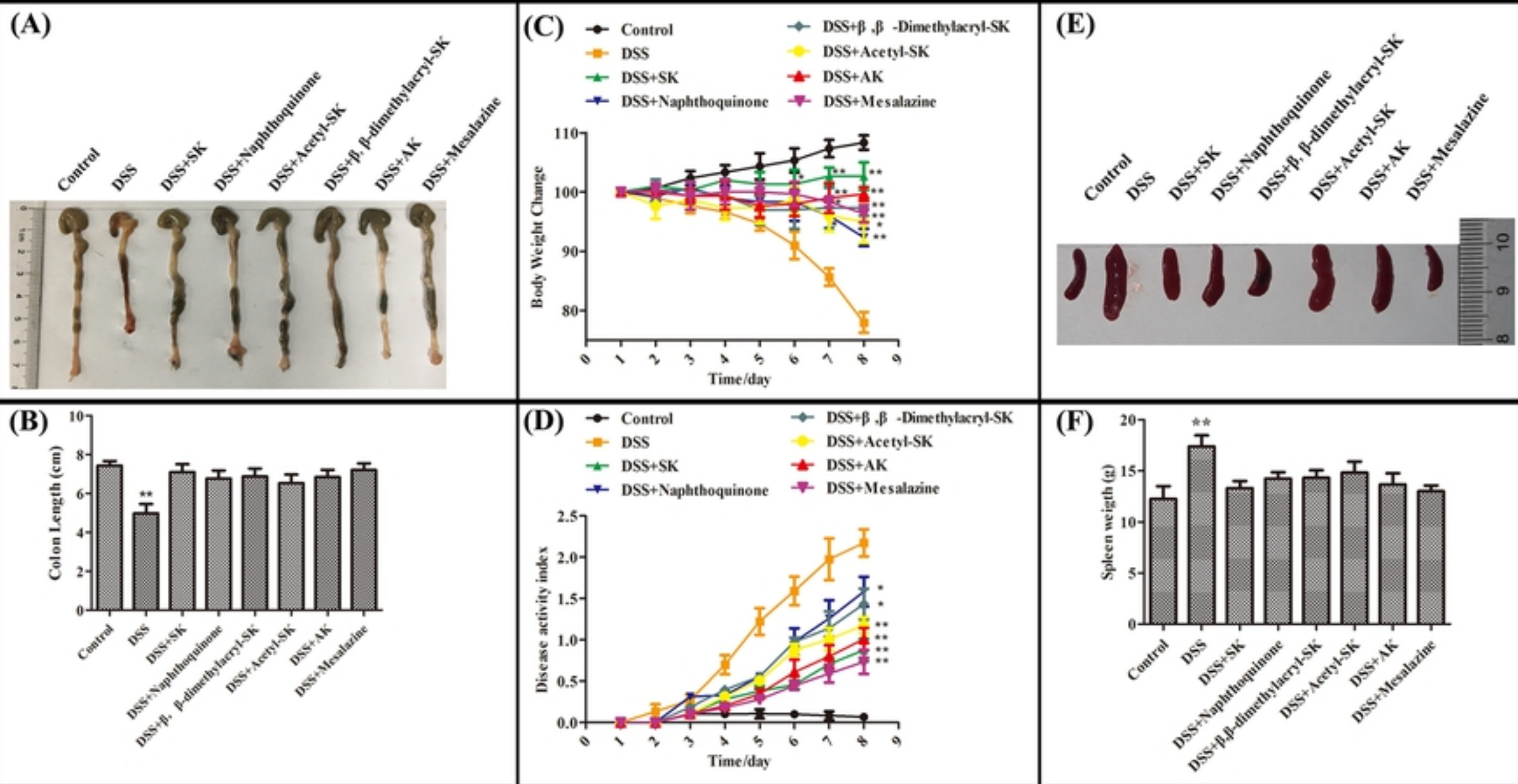
**Fig 6. SK and its derivatives relieved DSS-induced colonic epithelial tight junction disruption in mice.** **(A)** Protein expression of ZO-1, VCAM-1, Occludin and Claudin-1 in the colon tissues were analyzed by Western blot. **(B)** Relative protein expression ratios of were determined by densitometry and normalized to

666 GAPDH. Each point represents the mean  $\pm$  S.E.M. from three replicates (\*,  $P < 0.05$ ;  
667 \*\*,  $P < 0.01$  versus control; #,  $P < 0.05$ ; ##,  $P < 0.01$  versus DSS group).

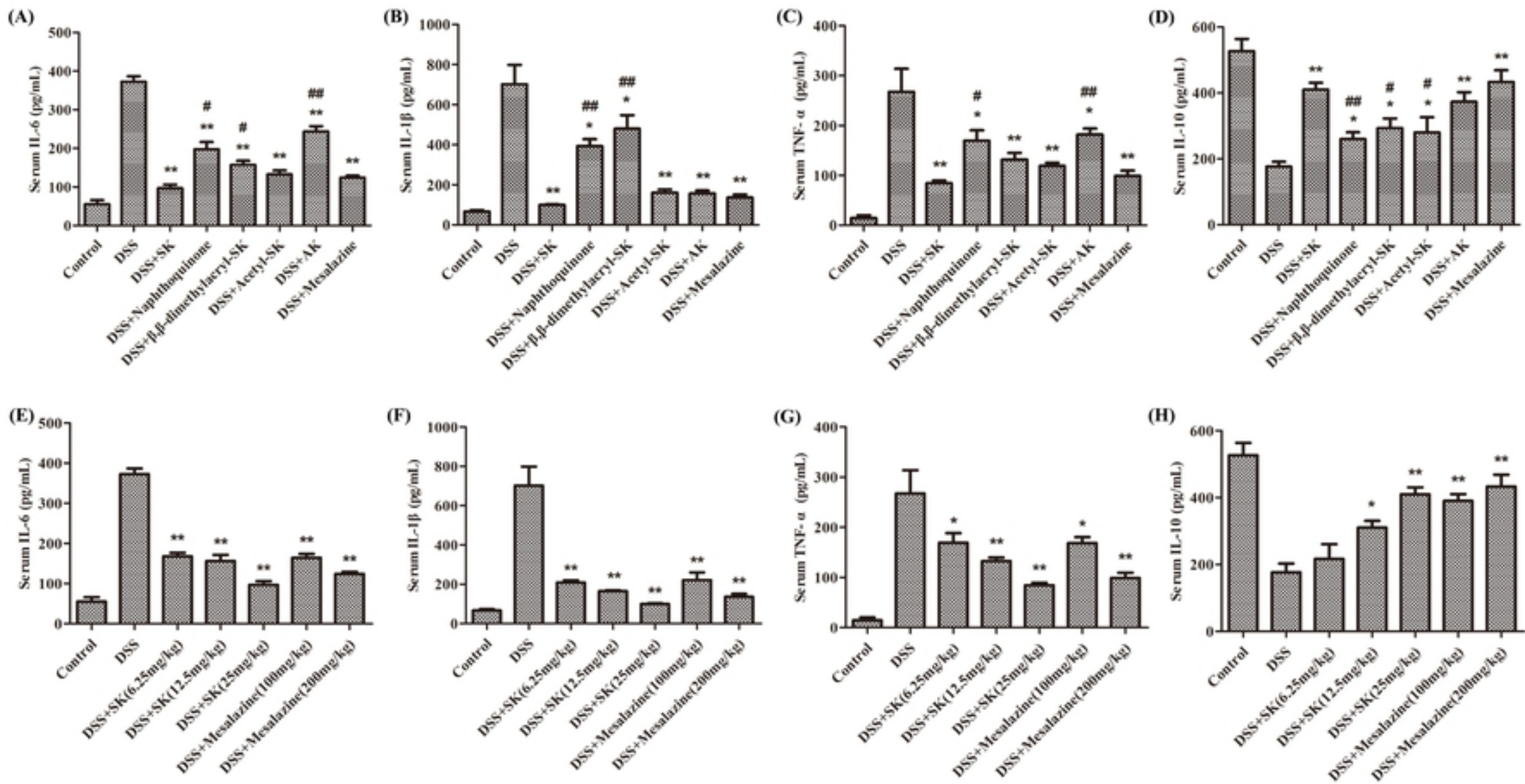
668 **Fig 7. SK and its derivatives reduced the level of NLRP3 inflammasome**  
669 **activation in colonic tissues. (A)** Protein levels of NLRP3, ASC, caspase-1, and  
670 IL-1 $\beta$  were determined by Western blotting.  $\beta$ -Actin was used as a control. **(B)**  
671 Relative protein expression ratios of were determined by densitometry and normalized  
672 to  $\beta$ -Actin. Each point represents the mean  $\pm$  S.E.M. from three replicates (\*,  $P < 0.05$ ;  
673 \*\*,  $P < 0.01$  versus control; #,  $P < 0.05$ ; ##,  $P < 0.01$  versus DSS group).

674 **Fig 8. Real-time PCR quantification of signature genes in inflammation**  
675 **pathways in colon tissues of all groups.** Down-regulation (%) was compared with  
676 vector control. Images are representative of three independent experiments. Data are  
677 mean  $\pm$  S.E.M. of three independent experiments (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus  
678 control; #,  $P < 0.05$ ; ##,  $P < 0.01$  versus DSS group).

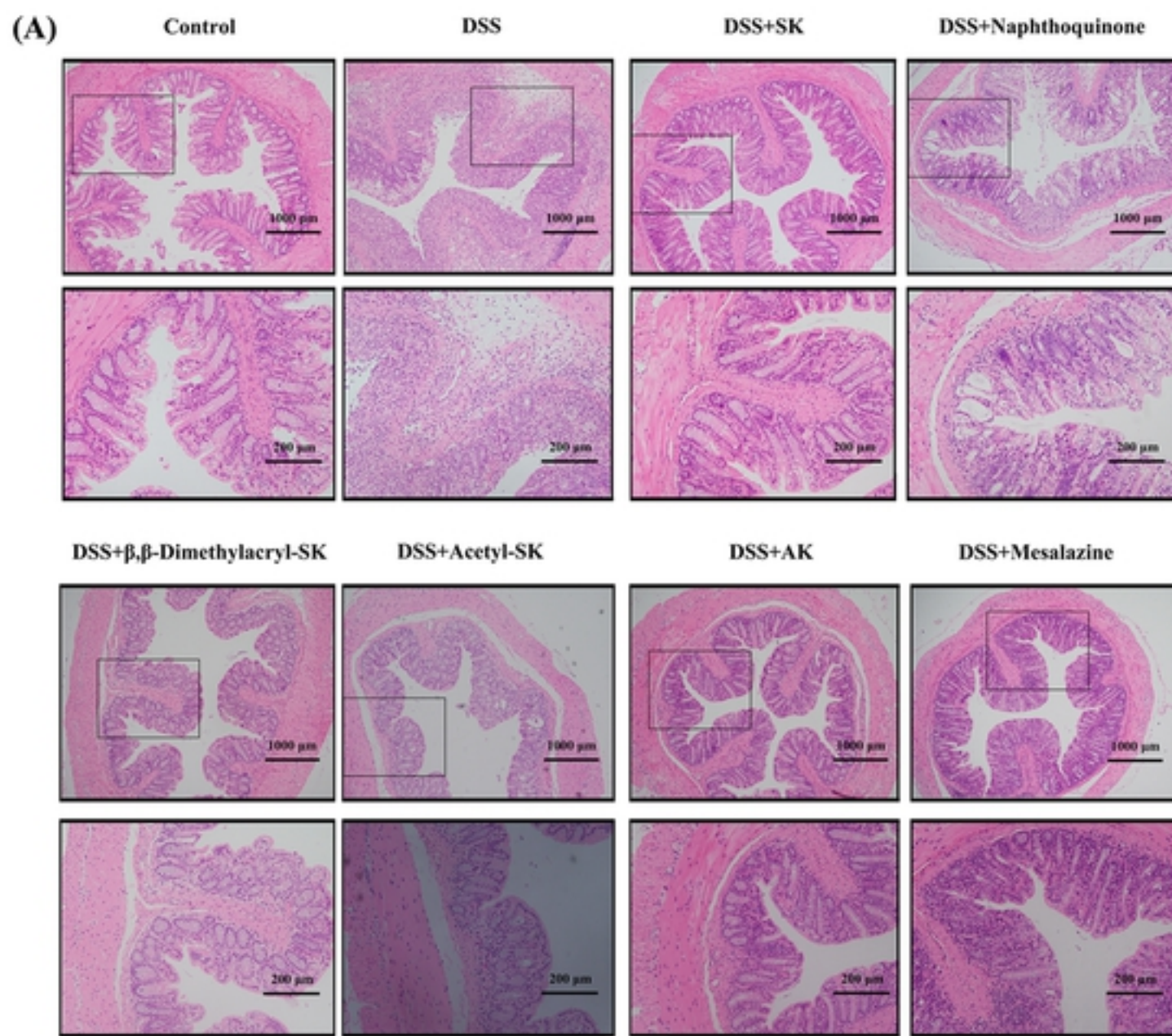
679 **Fig 9. SK and its derivatives attenuate the expression of COX-2, NF- $\kappa$ B and**  
680 **NLRP3. (A)** Immuno-histochemical analysis of COX-2 in each group. **(B)**  
681 Immuno-histochemical analysis of NF- $\kappa$ B in each group. **(C)** Immuno-histochemical  
682 analysis of NLRP3 in each group. The slides were incubated with primary antibody.  
683 After the secondary antibody incubation, the slides were developed with DAB and  
684 counter-stained with hematoxylin. The images were taken at 400 $\times$ magnification. Data  
685 are presented as means  $\pm$  S.E.M. from three replicates (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus  
686 control; #,  $P < 0.05$ ; ##,  $P < 0.01$  versus DSS group).



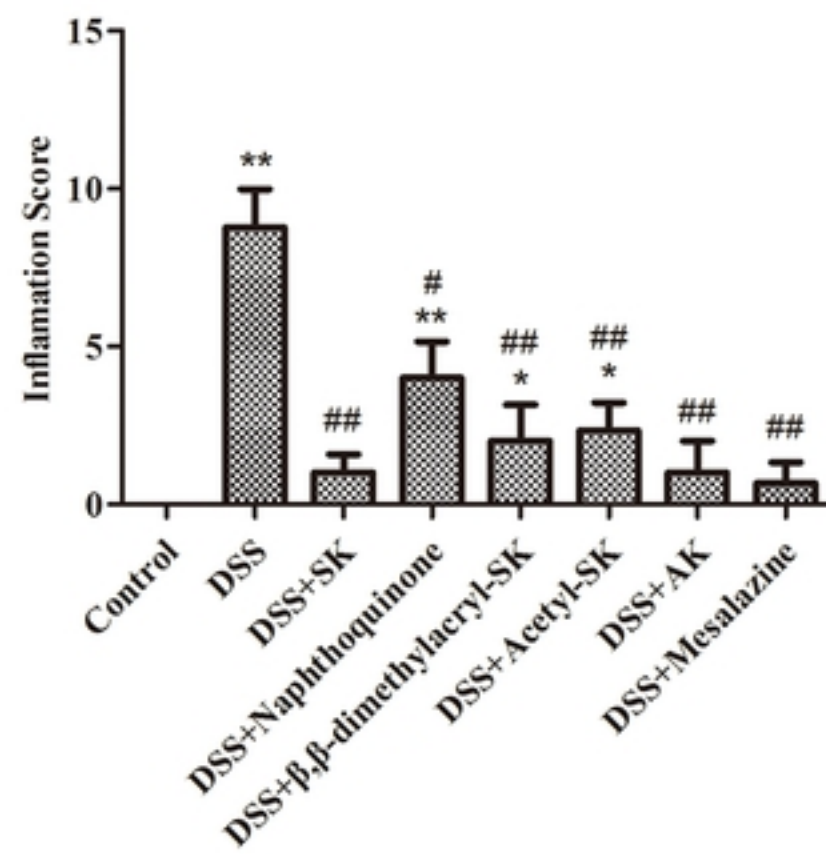
Figure



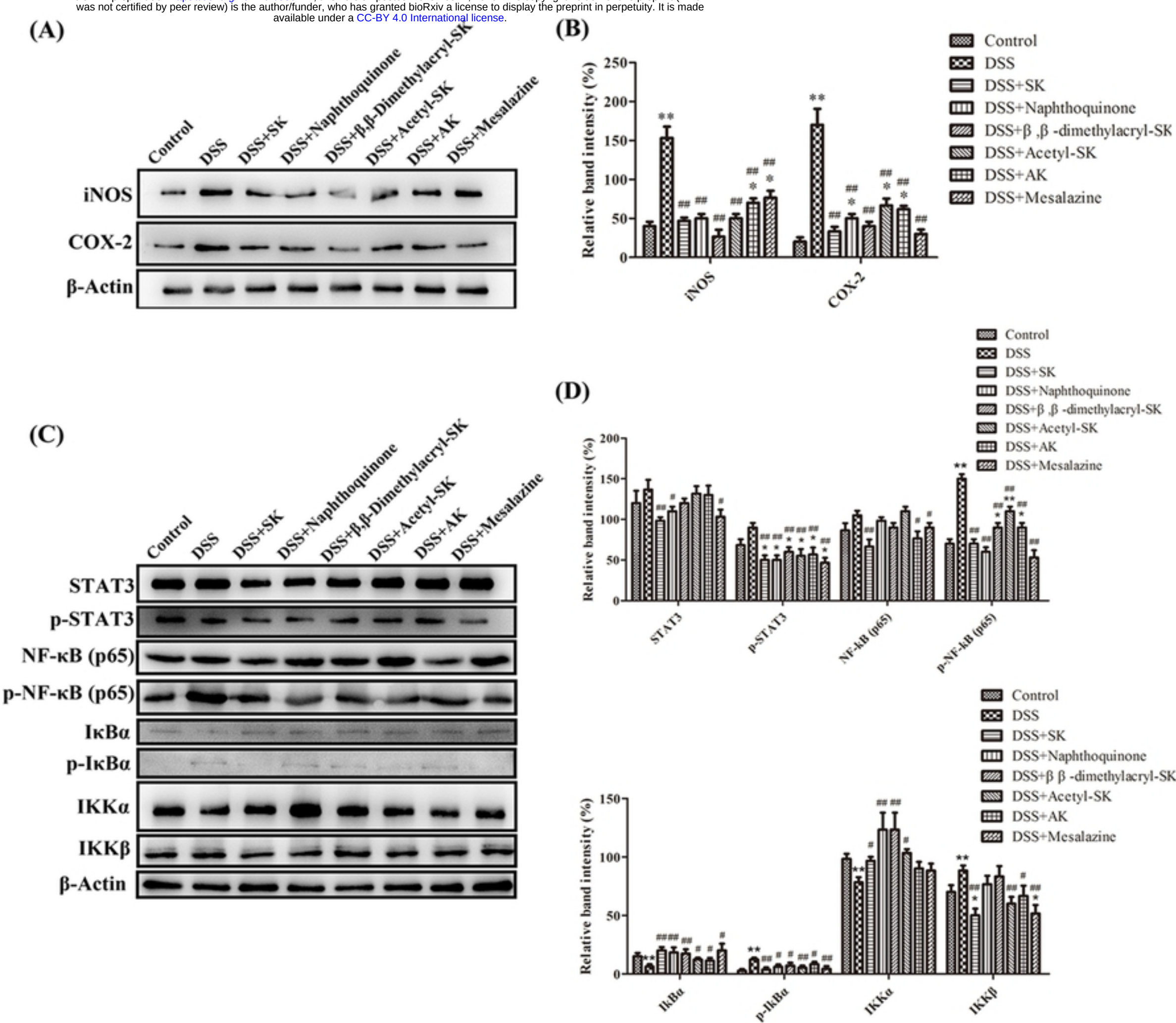
Figure



**(B)**

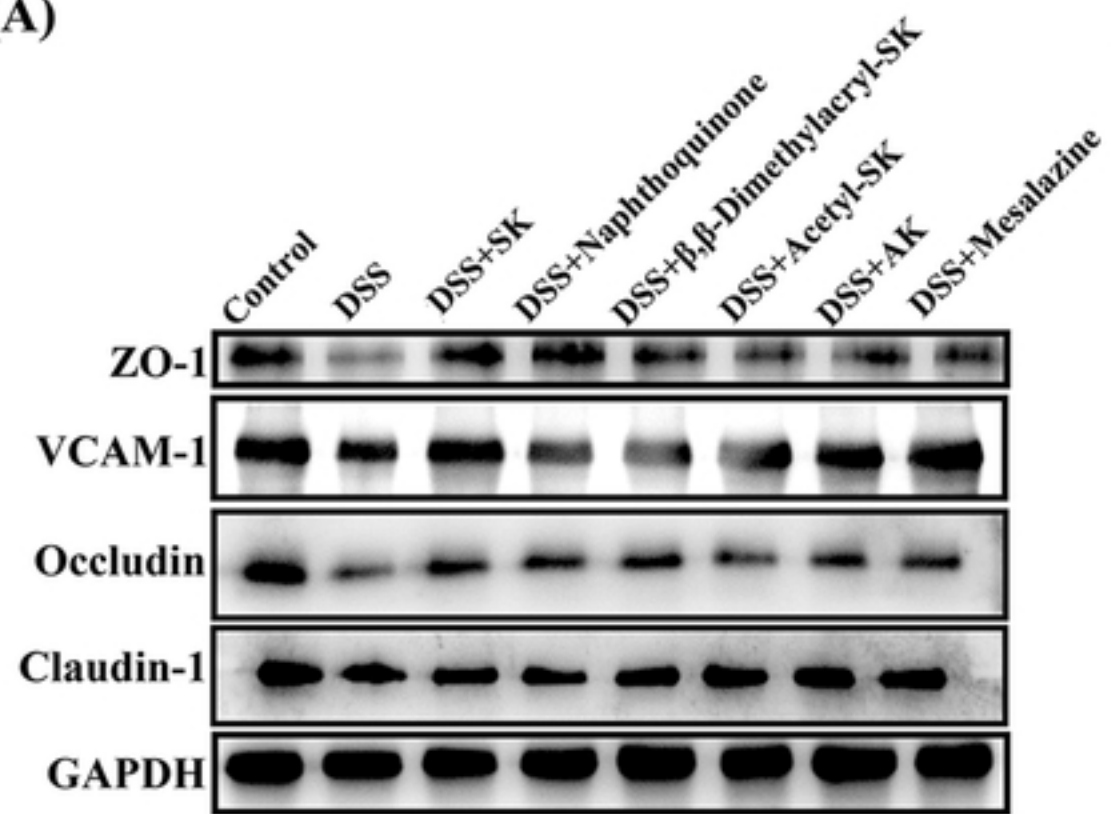


Figure

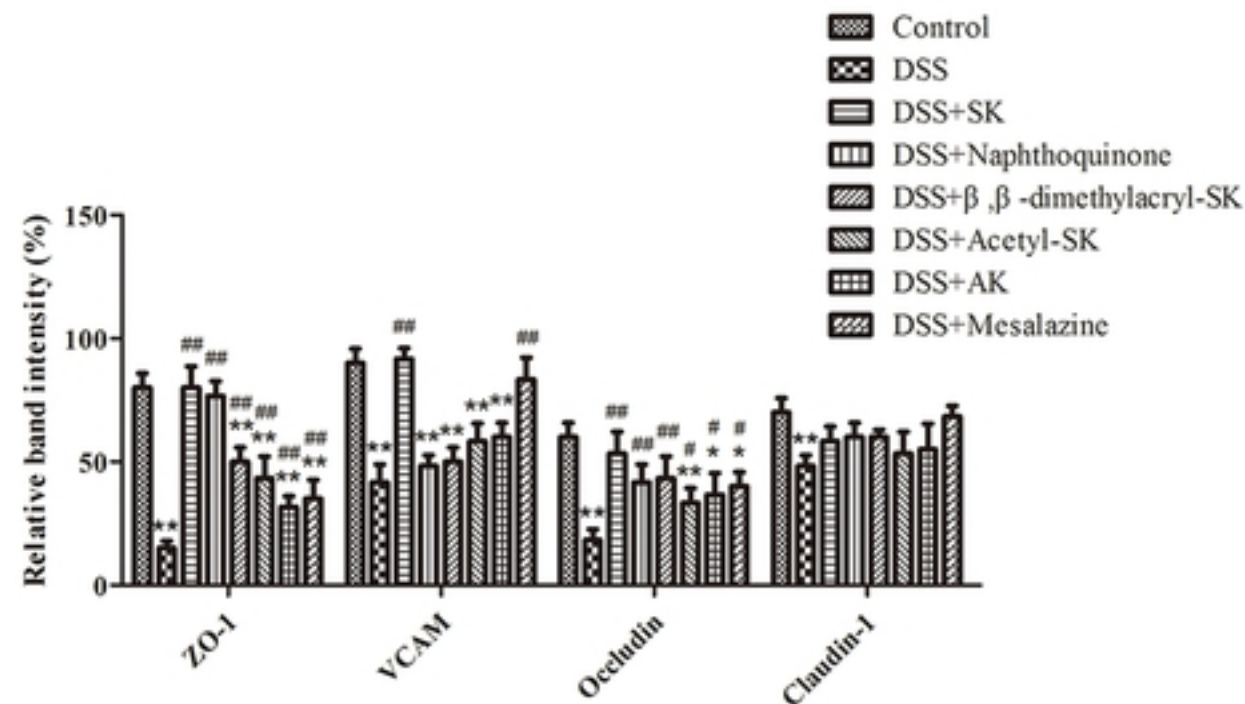


Figure

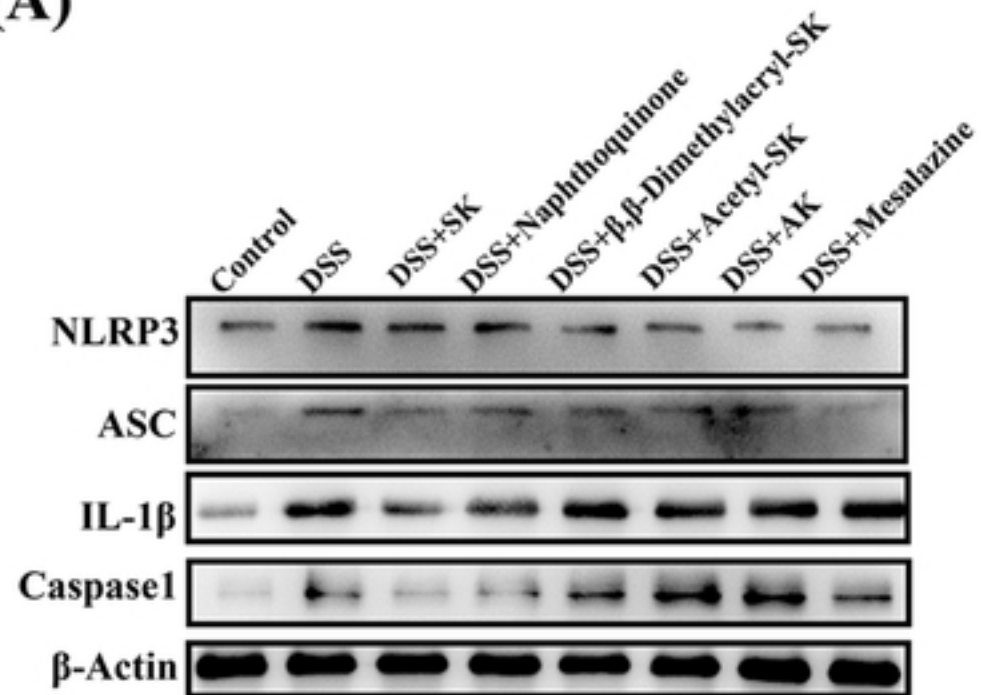
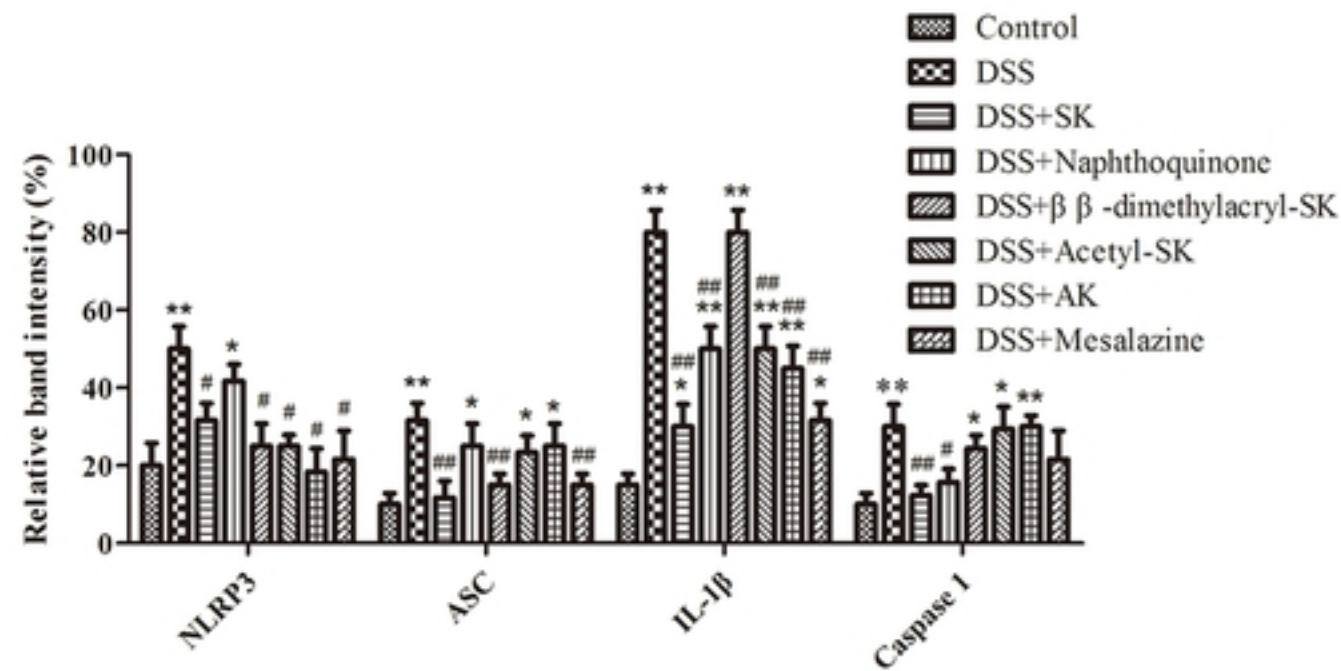
(A)



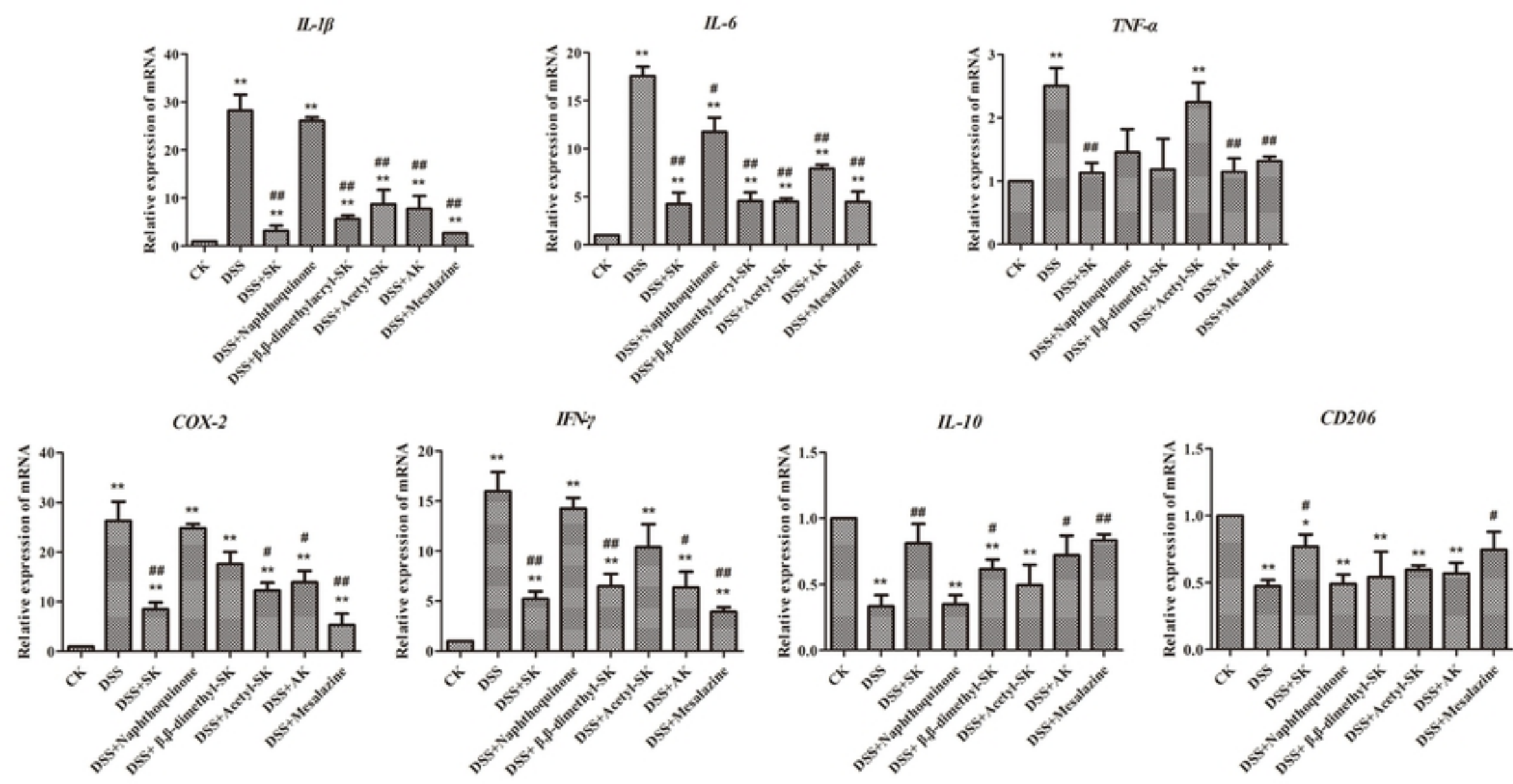
(B)



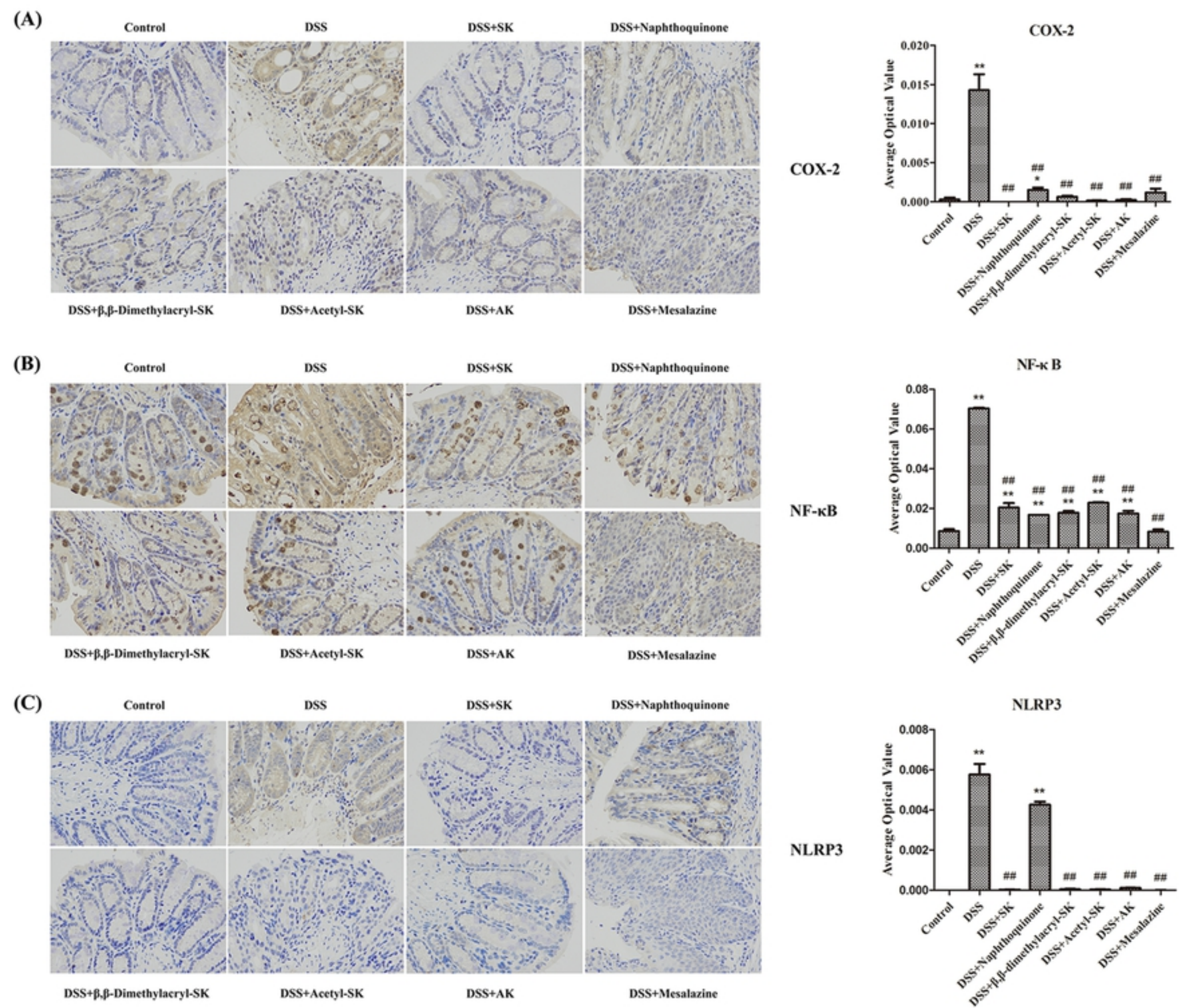
Figure

**(A)****(B)**

Figure



Figure



Figure

**Natural naphthoquinones isolated from *Lithospermum erythrorhizon* suppress dextran sulfate sodium-induced murine experimental colitis**

Wenxue Sun<sup>1,2†</sup>, Hongwei Han<sup>1,2†</sup>, Zhaoyue Wang<sup>1</sup>, Zhongling Wen<sup>1</sup>, Minkai Yang<sup>1</sup>,  
Yinsong Wang<sup>1</sup>, Jiangyan Fu<sup>1</sup>, Lu Feng<sup>1</sup>, Xinhong Xu<sup>1</sup>, Tongming Yin<sup>2</sup>, Xiaoming  
Wang<sup>1</sup>, Guihua Lu<sup>1,3\*</sup>, Jinliang Qi<sup>1,2\*</sup>, Hongyan Lin<sup>1,2\*</sup>, Yonghua Yang<sup>1,2\*</sup>

bioRxiv preprint doi: <https://doi.org/10.1101/830224>; this version posted November 4, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a [CC-BY 4.0 International license](#).

*<sup>1</sup>State Key Laboratory of Pharmaceutical Biotechnology, Institute of Plant Molecular Biology, School of Life Sciences, Nanjing University, Nanjing 210023, PR China*

*<sup>2</sup>Co-Innovation Center for Sustainable Forestry in Southern China, MOE Key Laboratory of Forest Genetics and Biotechnology, Nanjing Forestry University, Nanjing, 210037, PR China*

*<sup>3</sup>School of Life Sciences, Huaiyin Normal University, Huaian 223300, China*

†These authors contributed equally to this work

\*Corresponding authors

\*Corresponding author:

Yong-Hua Yang,

State Key Laboratory of Pharmaceutical Biotechnology,

Nanjing University, Nanjing 210023, PR China.

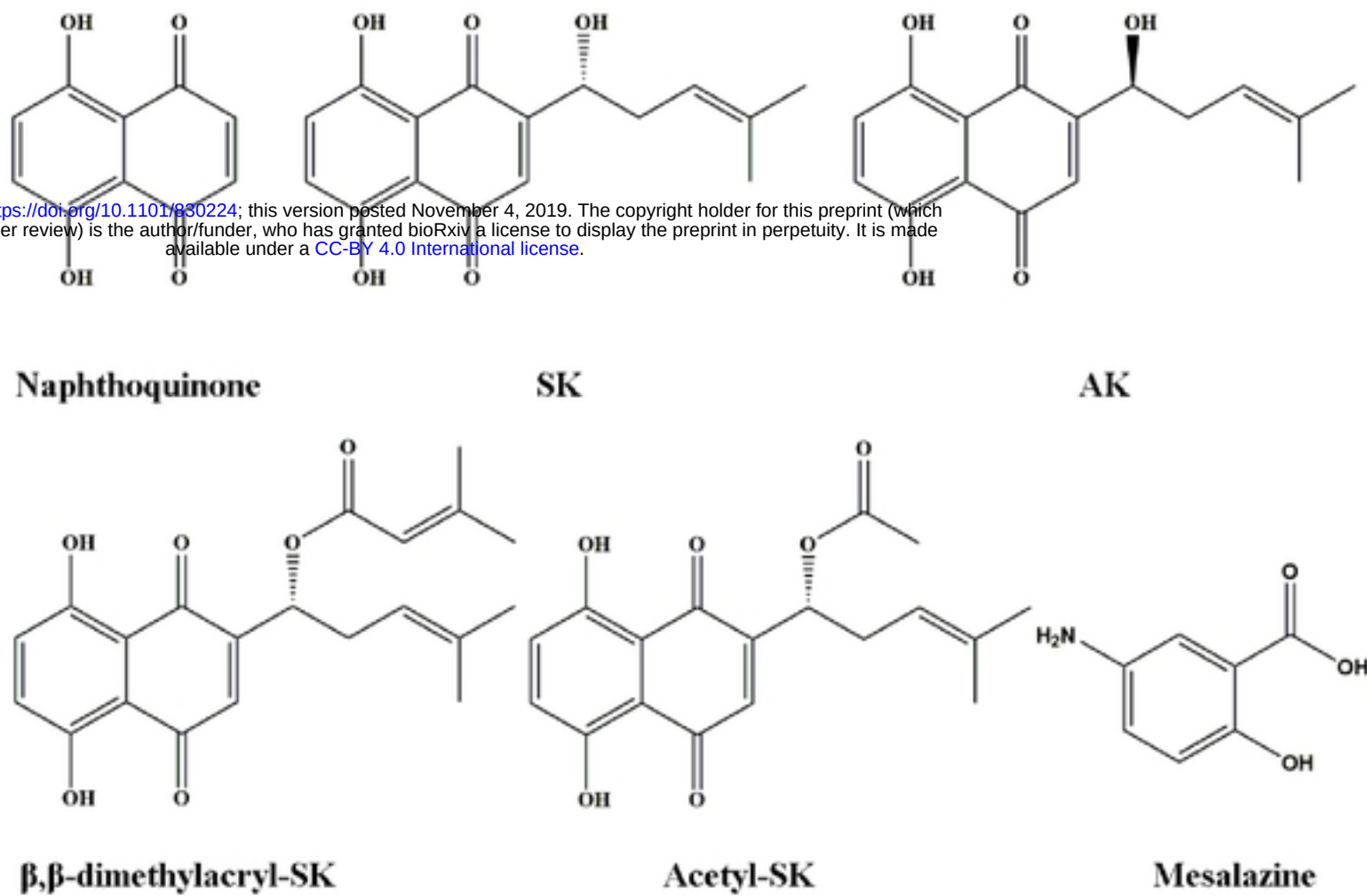
Tel/Fax: 86-25-89686305,

Email: yangyh@nju.edu.cn.

## Supplementary Contents

### S1 Table.

Binding energy of Naphthoquinone, Shikonin or Alkanin,  $\beta$ ,  $\beta$ -dimethylacrylshikonin, Acetylshikonin and Mesalazine with 4C1M, 4DN5, 6NPY, 3O4O, 5U98, 2AZ5, 3OQ3 and 5T5W.



| Compound                                | 4C1M<br>$\Delta G_b(\text{kcal/mol})$ | 4DN5<br>$\Delta G_b(\text{kcal/mol})$ | 6NPY<br>$\Delta G_b(\text{kcal/mol})$ | 3O4O<br>$\Delta G_b(\text{kcal/mol})$ |
|-----------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|
| Naphthoquinone                          | -21.3508                              | -27.0463                              | -30.1348                              | -11.9377                              |
| SK or AK                                | -29.3717                              | -40.5896                              | -44.8991                              | -18.4986                              |
| $\beta$ , $\beta$ -<br>dimethylacryl-SK | -38.1607                              | -52.2836                              | -48.4048                              | -17.6089                              |
| Acetyl-SK                               | -36.7487                              | -47.0860                              | -56.6797                              | -19.9018                              |
| Mesalazine                              | -17.6039                              | -22.6485                              | -26.8171                              | -13.9297                              |
| Compound                                | 5U98<br>$\Delta G_b(\text{kcal/mol})$ | 2AZ5<br>$\Delta G_b(\text{kcal/mol})$ | 3OQ3<br>$\Delta G_b(\text{kcal/mol})$ | 5T5W<br>$\Delta G_b(\text{kcal/mol})$ |
| Naphthoquinone                          | -20.6042                              | -21.5323                              | -21.9813                              | -10.4572                              |
| SK or AK                                | -27.3095                              | -29.4051                              | -25.7536                              | -17.1446                              |

$\beta$ ,  $\beta$ -

|                  |          |          |          |          |
|------------------|----------|----------|----------|----------|
| dimethylacryl-SK | -30.5290 | -34.3772 | -25.5352 | -20.8955 |
| Acetyl-SK        | -28.4046 | -29.2099 | -22.2027 | -17.7823 |
| Mesalazine       | -16.0016 | -18.9920 | -21.0340 | -14.2567 |

## S2 Table.

The standard for the disease activity index (DAI) evaluation.

| Loss of weight (%) | Stool property | Stool occult blood/gross blood stool | score |
|--------------------|----------------|--------------------------------------|-------|
| 0                  | Normal         | Normal                               | 0     |
| 1-5                |                |                                      | 1     |
| 6-10               | Loose stool    | Slight bleeding                      | 2     |
| 11-15              |                |                                      | 3     |
| >15                | Liquid stool   | Blood in stool                       | 4     |

\*: Normal, formed stool; Loose stool, a paste, semi-formed stool that does not adhere to the anus; Liquid stool, a watery stool that can adhere to the anus.

## S3 Table.

Premier sequences of the genes.

| Gene names    | Premier (5'-3')                                                      | Genebank accession no. |
|---------------|----------------------------------------------------------------------|------------------------|
| IL-1 $\beta$  | Forward: CTTCAAGGCAGGCAGTATCACTC<br>Reverse: TGCAGTTGTCTAATGGGAACGT  | NM_008361.4            |
| IL-6          | Forward: TGATGCACTTGCAGAAAACA<br>Reverse: ACCAGAGGAAATTTCAATAGGC     | NM_031168.2            |
| TNF $\alpha$  | Forward: TTGACCTCAGCGCTGAGTTG<br>Reverse: CCTGTAGCCACGTCGTAGC        | NM_013693.3            |
| IL-10         | Forward: AGAGAAGCATGGCCCAGAAA<br>Reverse: ACACCTTGGTCTTGGAGCTT       | NM_008337.4            |
| IFN- $\gamma$ | Forward: GCTGATCCTTTGGACCCTC<br>Reverse: AGAGCTGCAAAGCCAAGATG        | NM_010552.3            |
| CD206         | Forward: CTTCACTCTTCGGGCCTTTGG<br>Reverse: TGACCACTCCTGCTGCTTTA      | NM_008625.2            |
| COX2          | Forward: CCTGCTGCCCCGACACCTTCAACAT<br>Reverse: CAGCAACCCGGCCAGCAATCT | NM_011198.4            |
| GAPDH         | Forward: CACTCACGGCAAATTCAACGGCAC<br>Reverse: GACTCCACGACATACTCAGCAC | NM_001289726.1         |

## Representative <sup>1</sup>H NMR spectra

### *5,8-Dihydroxynaphthalene-1,4-dione (Naphthoquinone, PubChem CID: 10141)*

Red powder, Mp: 227 °C. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ 12.41 (s, 2H, -OH), 7.15 (s, 4H, Ar-H). <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ 172.85 (s, 1C, 4C, 5C, 8C), 134.65 (s, 2C, 3C, 6C, 7C), 111.87 (s, 9C, 10C). ESI-TOF, calcd for C<sub>10</sub>H<sub>6</sub>O<sub>4</sub> ([M+H]<sup>+</sup>), 191.03, found 190.96. Anal. Calcd for C<sub>10</sub>H<sub>6</sub>O<sub>4</sub>: C, 63.16; H, 3.18; O, 33.65; Found: C, 63.08;

bioRxiv preprint doi: <https://doi.org/10.1101/330224>; this version posted November 4, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

### *(R)-5,8-Dihydroxy-2-(1-hydroxy-4-methylpent-3-en-1-yl)naphthalene-1,4-dione*

#### *(Shikonin, PubChem CID: 479503)*

Red powder, Mp: 147-148 °C. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ 12.60 (s, 1H, -OH), 12.49 (s, 1H, -OH), 7.20 (t, J = 7.2 Hz, 2H, Ar-H), 7.18 – 7.17 (m, 1H, -C-CH=C), 5.21 (dd, J = 8.0, 6.9 Hz, 1H, -OH), 4.93 – 4.90 (m, 1H, -C-CH=C), 4.12 (dd, J = 14.3, 7.1 Hz, 1H, -O-CH), 2.67 – 2.62 (m, 1H, -CH<sub>2</sub>), 2.39 – 2.33 (m, 1H, -CH<sub>2</sub>), 1.76 (s, 3H, -CH<sub>3</sub>), 1.66 (s, 3H, -CH<sub>3</sub>). <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ 180.66 (s, 8C), 179.86 (s, 5C), 165.48 (s, 4C), 164.87 (s, 1C), 151.44 (s, 7C), 137.48 (s, 6C), 132.41 (s, 14C), 132.30 (s, 2C), 131.87 (s, 3C), 118.44 (s, 13C), 112.03 (s, 9C), 111.55 (s, 10C), 68.35 (s, 11C), 35.68 (s, 12C), 25.97 (s, 15C), 18.10 (s, 16C). ESI-TOF, calcd for C<sub>16</sub>H<sub>16</sub>O<sub>5</sub> ([M+H]<sup>+</sup>), 289.10, found 288.78. Anal. Calcd for C<sub>16</sub>H<sub>16</sub>O<sub>5</sub>: C, 66.66; H, 5.59; O, 27.75; Found: C, 66.65; H, 5.58; O, 27.72.

### *(S)-5,8-Dihydroxy-2-(1-hydroxy-4-methylpent-3-en-1-yl)naphthalene-1,4-dione*

#### *(Alkanin, PubChem CID: 32465)*

Red powder, Mp: 149 °C. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ 12.60 (d, J = 4.8 Hz, 1H, -OH), 12.50 (d, J = 5.0 Hz, 1H, -OH), 7.21 (t, J = 7.2 Hz, 2H, Ar-H), 7.19 – 7.17

(m, 1H, -C-CH=C), 5.21 (ddd, J = 8.1, 2.7, 1.3 Hz, 2H, -OH, -O-CH), 4.92 (dd, J = 7.8, 4.3 Hz, 1H, -C-CH=C), 2.68 – 2.63 (m, 1H, -CH<sub>2</sub>), 2.36 (dd, J = 14.8, 7.6 Hz, 1H, -CH<sub>2</sub>), 1.76 (s, 3H, -CH<sub>3</sub>), 1.66 (s, 3H, -CH<sub>3</sub>). <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ 180.66 (s, 8C), 179.86 (s, 5C), 165.48 (s, 4C), 164.87 (s, 1C), 151.44 (s, 7C), 137.48 (s, 6C), 132.41 (s, 14C), 132.30 (s, 2C), 131.87 (s, 3C), 118.44 (s, 13C), 112.03 (s, 9C), 111.55 (s, 10C), 68.35 (s, 11C), 35.68 (s, 12C), 25.97 (s, 15C), 18.10 (s, 16C). ESI-TOF, calcd for C<sub>16</sub>H<sub>16</sub>O<sub>5</sub> ([M+H]<sup>+</sup>), 289.10, found 288.78. Anal. Calcd for C<sub>16</sub>H<sub>16</sub>O<sub>5</sub>: C, 66.66; H, 5.59; O, 27.75; Found: C, 66.67; H, 5.62; O, 27.71.

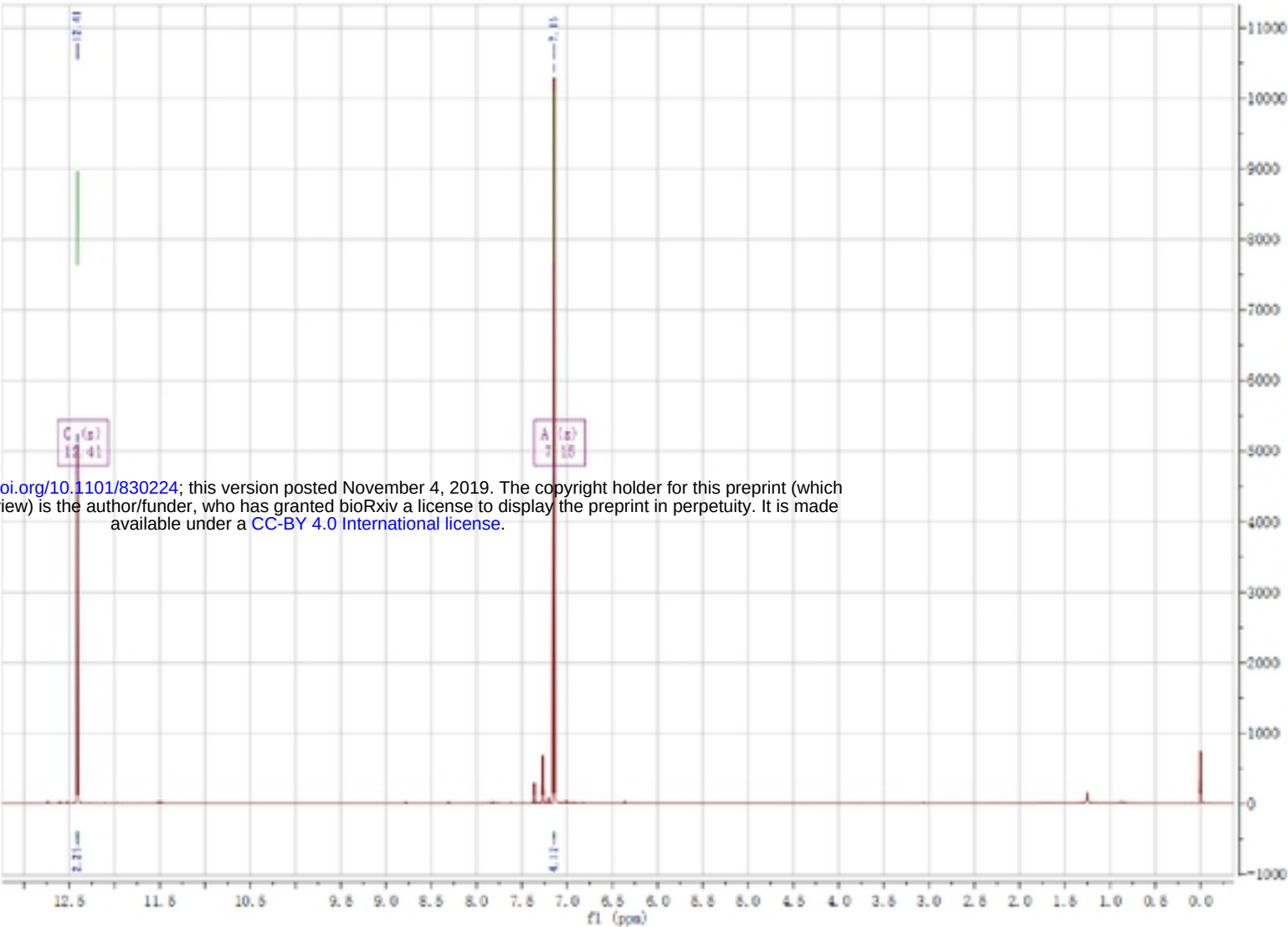
*(R)-1-(5,8-Dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-en-1-yl 3-methylbut-2-enoate* (**β, β-dimethylacrylshikonin**, PubChem CID: 479499)

Red powder, Mp: 103-105 °C. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ 12.60 (s, 1H, -OH), 12.44 (s, 1H, -OH), 7.18 (s, 1H, -C-CH=C), 7.00 – 6.97 (m, 2H, Ar-H), 6.03 (ddd, J = 18.2, 7.3, 4.0 Hz, 1H, -C-CH=C), 5.79 – 5.77 (m, 1H, -O-CH-), 5.15 (t, J = 7.3 Hz, 1H, -C-CH=C), 2.63 (dt, J = 11.7, 6.6 Hz, 1H, -CH<sub>2</sub>-), 2.48 (dt, J = 14.9, 7.3 Hz, 1H, -CH<sub>2</sub>-), 2.15 (d, J = 10.1 Hz, 3H, -CH<sub>3</sub>), 1.94 (d, J = 0.8 Hz, 3H, -CH<sub>3</sub>), 1.69 (s, 3H, -CH<sub>3</sub>), 1.58 (s, 3H, -CH<sub>3</sub>). <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ 179.03 (s, 8C), 177.53 (s, 5C), 166.79 (s, 17C), 166.25 (s, 4C), 165.27 (s, 1C), 159.01 (s, 19C), 149.05 (s, 7C), 135.86 (s, 6C), 132.60 (s, 14C), 132.45 (s, 2C), 131.60 (s, 3C), 118.01 (s, 13C), 115.26 (s, 18C), 111.87 (s, 9C), 111.60 (s, 10C), 68.63 (s, 11C), 32.90 (s, 12C), 27.59 (s, 21C), 25.77 (s, 16C), 20.38 (s, 20C), 17.97 (s, 15C). ESI-TOF, calcd for C<sub>21</sub>H<sub>22</sub>O<sub>6</sub> ([M+H]<sup>+</sup>), 371.14, found 370.10. Anal. Calcd for C<sub>21</sub>H<sub>22</sub>O<sub>6</sub>: C, 68.09; H, 5.99; O, 25.92; Found: C, 68.09; H, 5.98; O, 25.91

*(R)-1-(5,8-Dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-en-1-yl acetate (Acetylshikonin, PubChem CID: 479501)*

Red powder, Mp: 86 °C. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ 12.59 (s, 1H, -OH), 12.43 (s, 1H, -OH), 7.19 (s, 2H, Ar-H), 6.99 (s, 1H, -C-CH=C), 6.02 (dd, J = 7.2, 4.6 Hz, 1H, -O-CH-), 5.12 (t, J = 7.3 Hz, 1H, -C-CH=C), 2.65 – 2.60 (m, 2H, -CH<sub>2</sub>-), 2.14 (s, 3H, -CH<sub>3</sub>), 1.69 (s, 3H, -CH<sub>3</sub>), 1.58 (s, 3H, -CH<sub>3</sub>). <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ 178.23 (s, 8C), 176.71 (s, 5C), 169.79 (s, 17C), 167.48 (s, 4C), 166.96 (s, 1C), 148.22 (s, 7C), 136.14 (s, 6C), 132.90 (s, 14C), 132.74 (s, 2C), 131.47 (s, 3C), 117.67 (s, 13C), 111.83 (s, 9C), 111.57 (s, 10C), 69.53 (s, 11C), 32.83 (s, 12C), 25.78 (s, 16C), 21.00 (s, 18C), 17.95 (s, 15C). ESI-TOF, calcd for C<sub>18</sub>H<sub>18</sub>O<sub>6</sub> ([M+H]<sup>+</sup>), 331.34, found 331.12. Anal. Calcd for C<sub>18</sub>H<sub>18</sub>O<sub>6</sub>: C, 65.45; H, 5.49; O, 29.06; Found: C, 65.40; H, 5.58; O, 29.03.

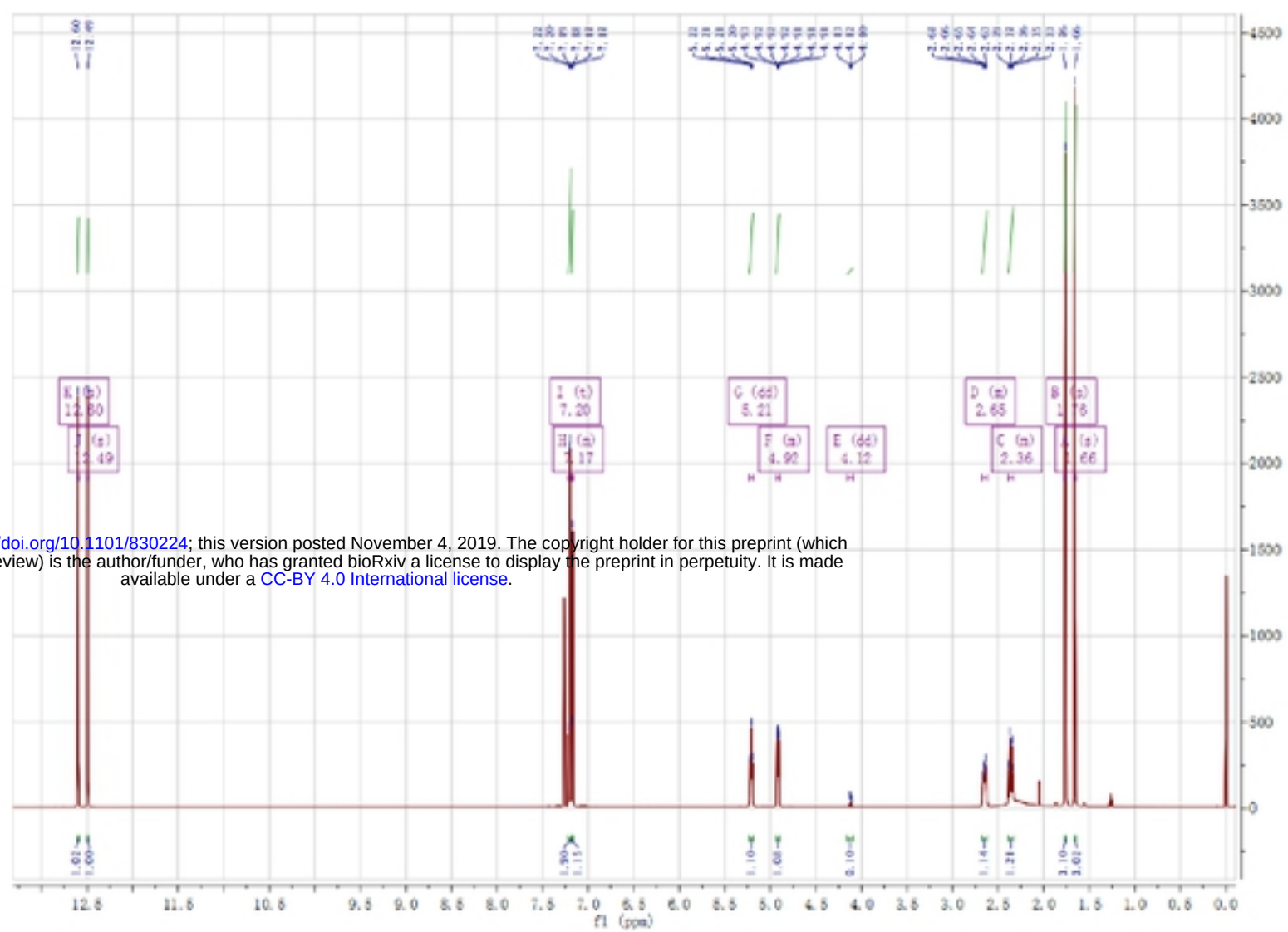
Representative <sup>1</sup>H NMR spectra



bioRxiv preprint doi: <https://doi.org/10.1101/830224>; this version posted November 4, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a [CC-BY 4.0 International license](#).

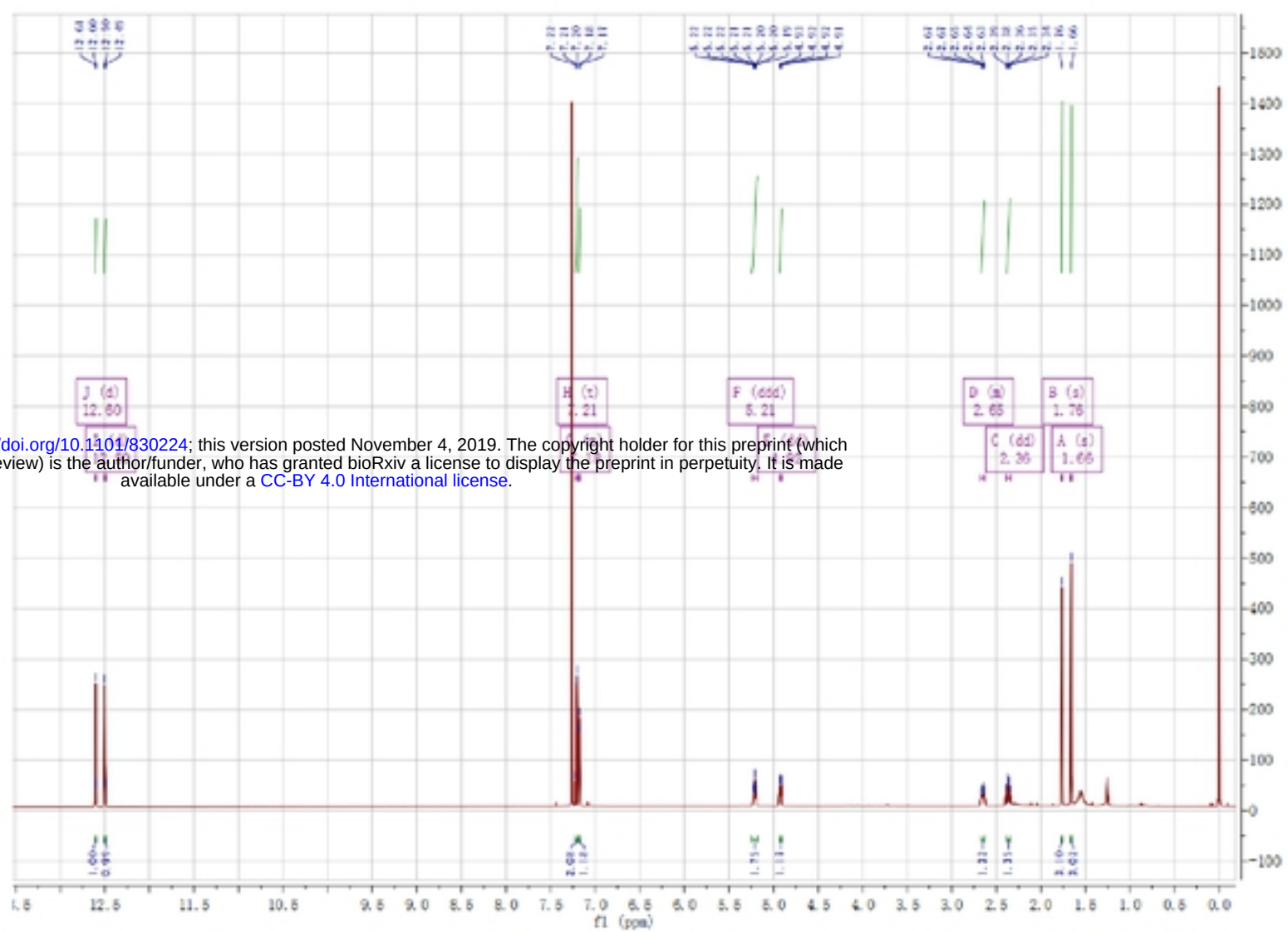
<sup>1</sup>H NMR of Compound **Naphthoquinone**

bioRxiv preprint doi: <https://doi.org/10.1101/830224>; this version posted November 4, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a [CC-BY 4.0 International license](#).



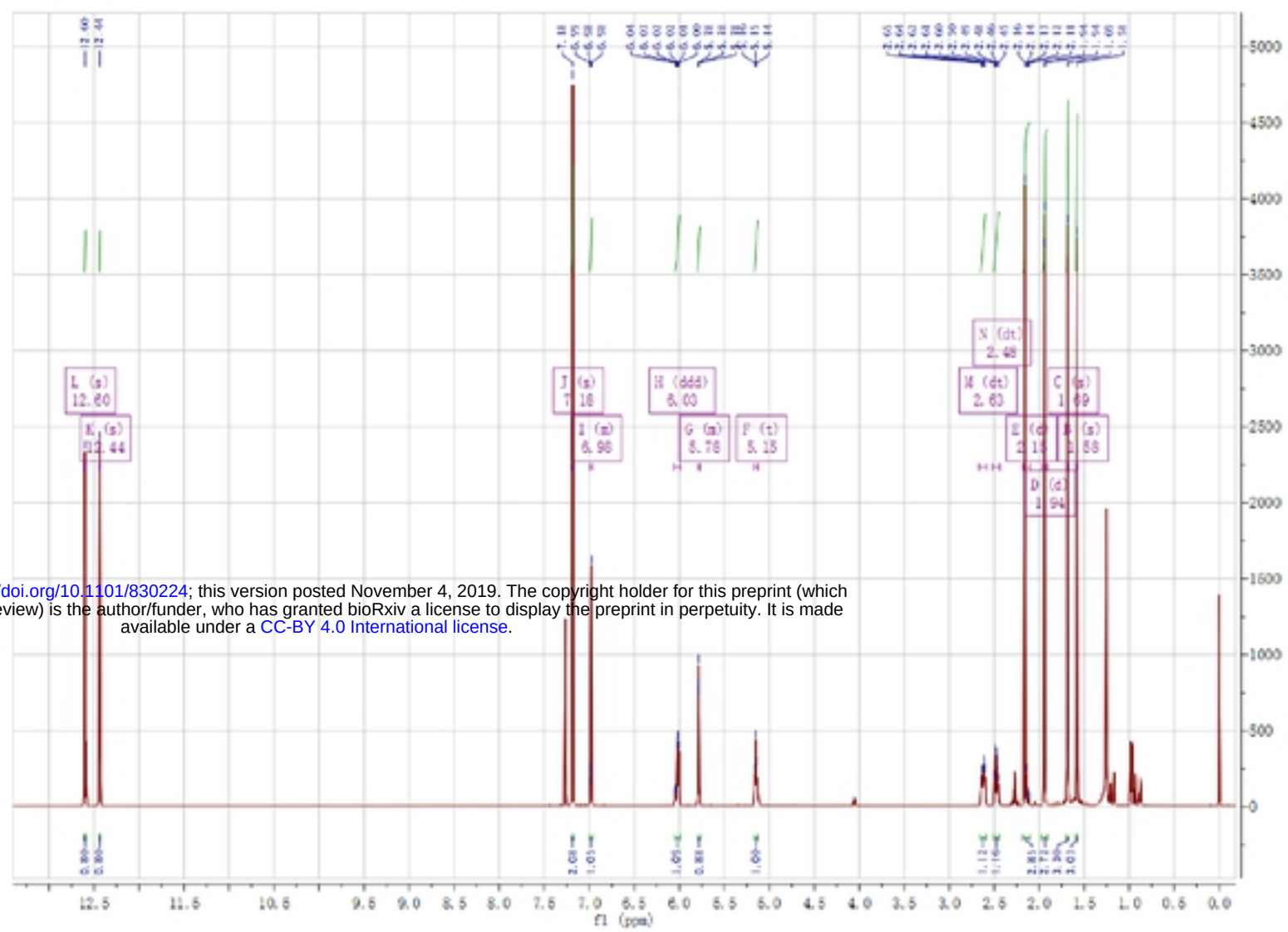
<sup>1</sup>H NMR of Compound Shikonin

bioRxiv preprint doi: <https://doi.org/10.1101/830224>; this version posted November 4, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a [CC-BY 4.0 International license](#).



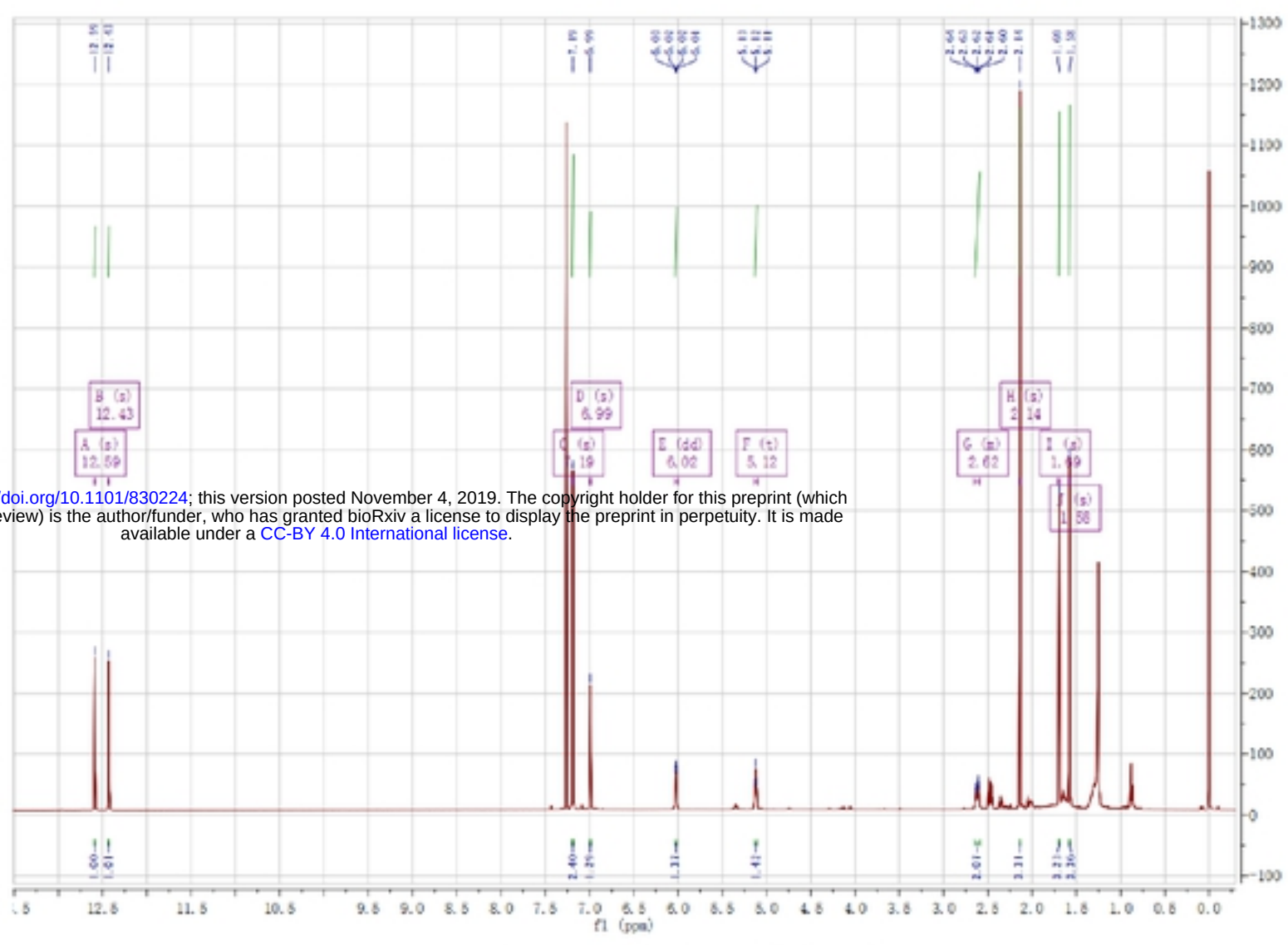
<sup>1</sup>H NMR of Compound **Alkanin**

bioRxiv preprint doi: <https://doi.org/10.1101/830224>; this version posted November 4, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a [CC-BY 4.0 International license](#).



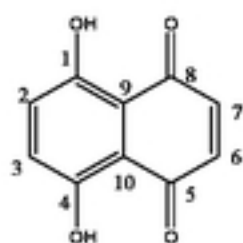
<sup>1</sup>H NMR of Compound  $\beta$ ,  $\beta$ -dimethylacrylshikonin

bioRxiv preprint doi: <https://doi.org/10.1101/830224>; this version posted November 4, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a [CC-BY 4.0 International license](#).

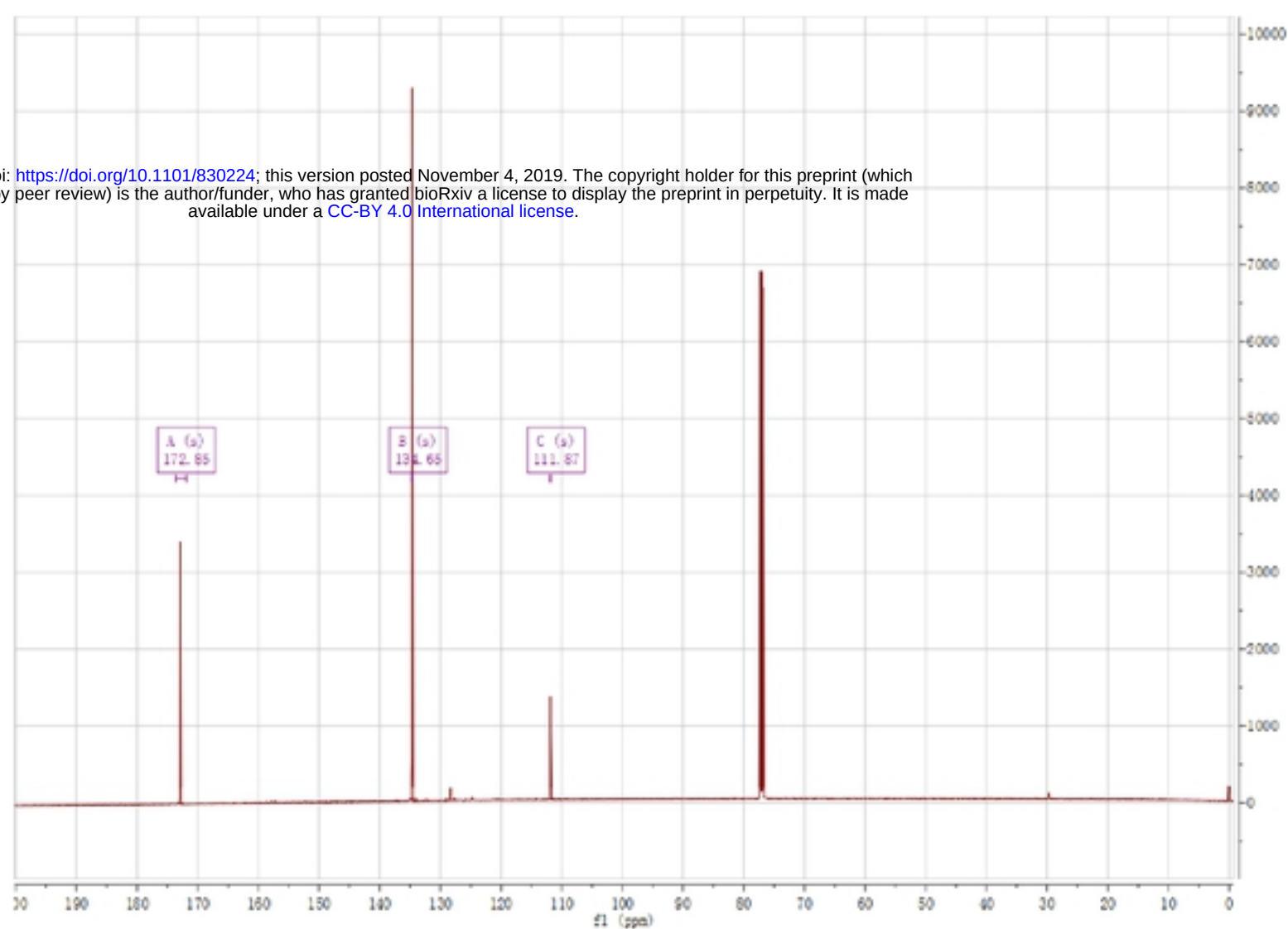


<sup>1</sup>H NMR of Compound Acetylshikonin

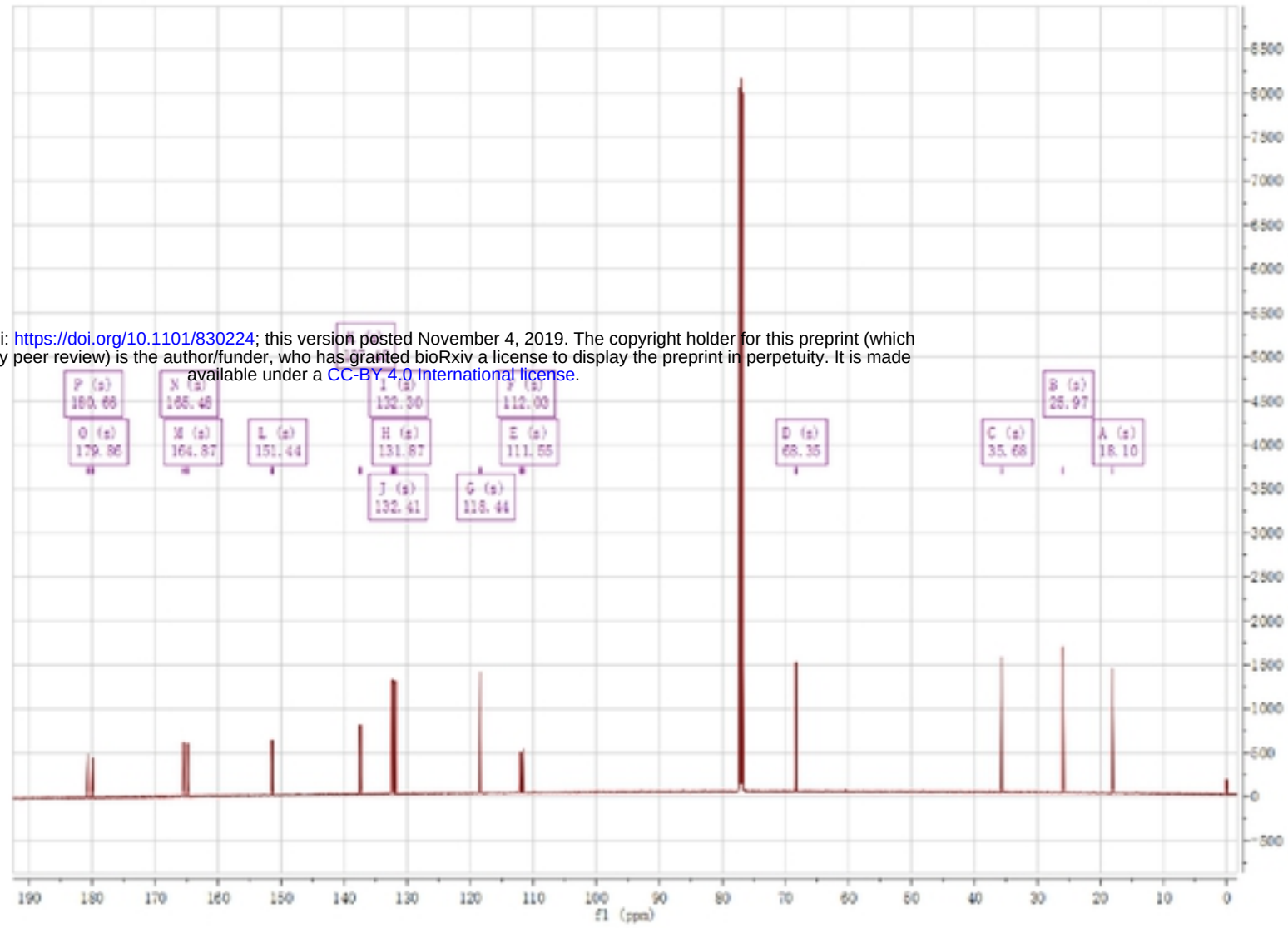
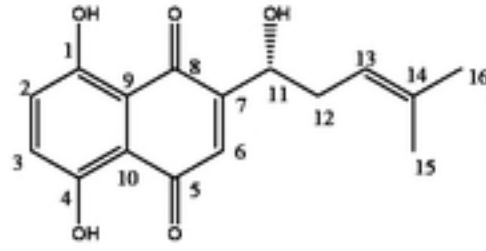
## Representative $^{13}\text{C}$ NMR spectra



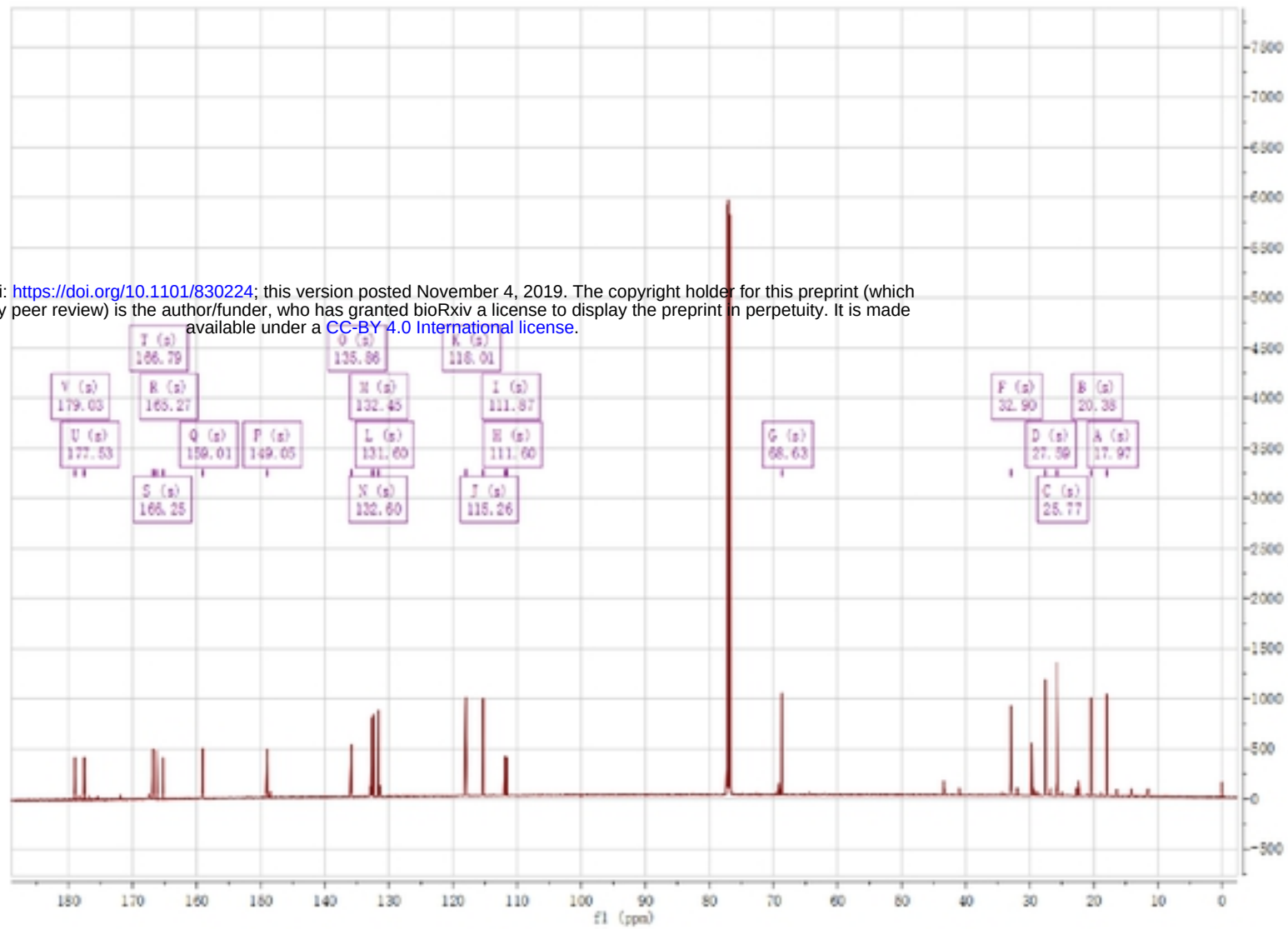
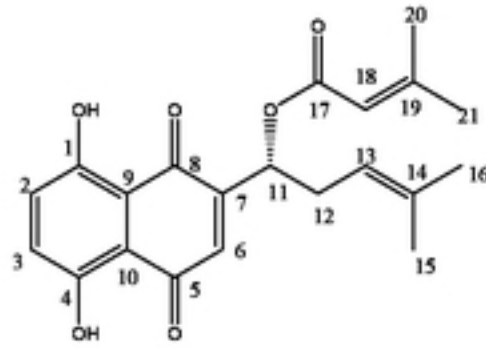
bioRxiv preprint doi: <https://doi.org/10.1101/830224>; this version posted November 4, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a [CC-BY 4.0 International license](#).



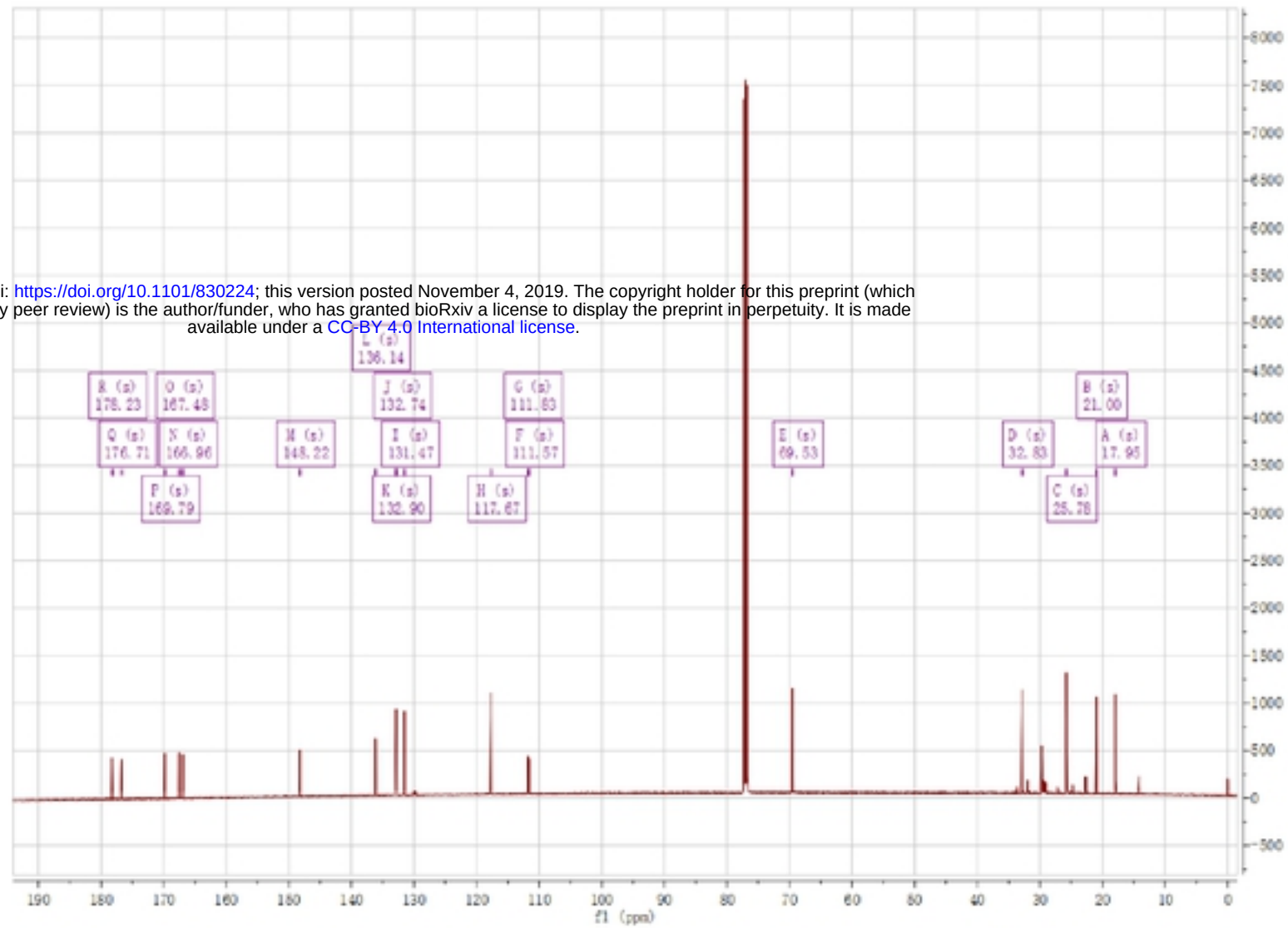
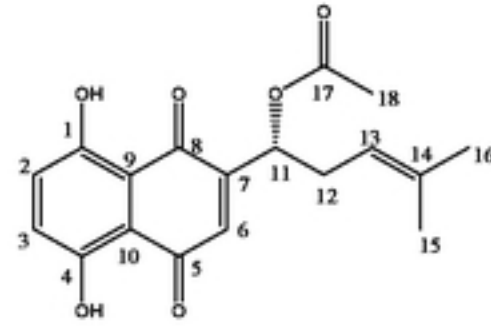
$^{13}\text{C}$  NMR of Compound **Naphthoquinone**



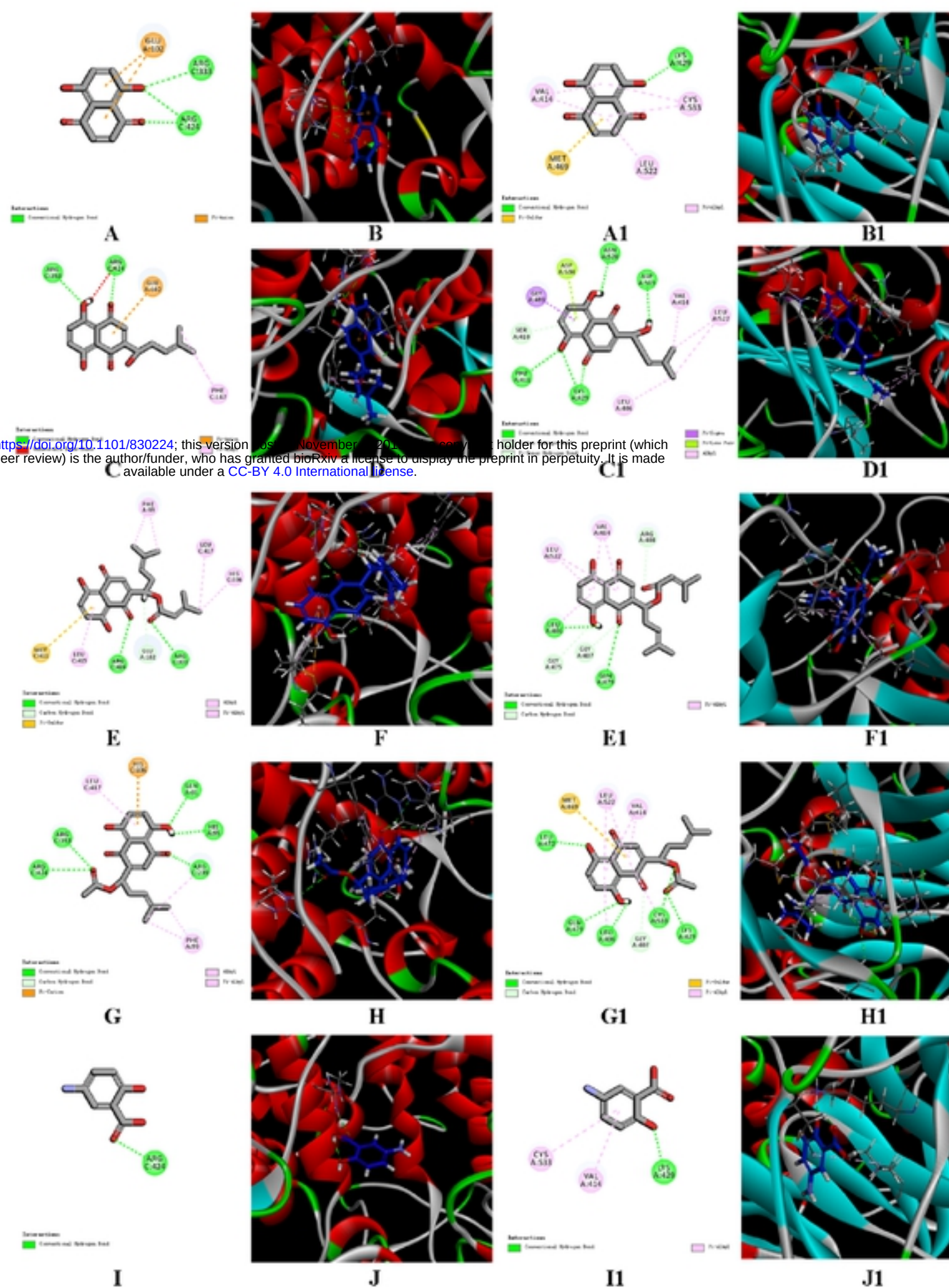
<sup>13</sup>C NMR of Compound **Shikonin**



<sup>13</sup>C NMR of Compound  $\beta$ ,  $\beta$ -dimethylacrylshikonin



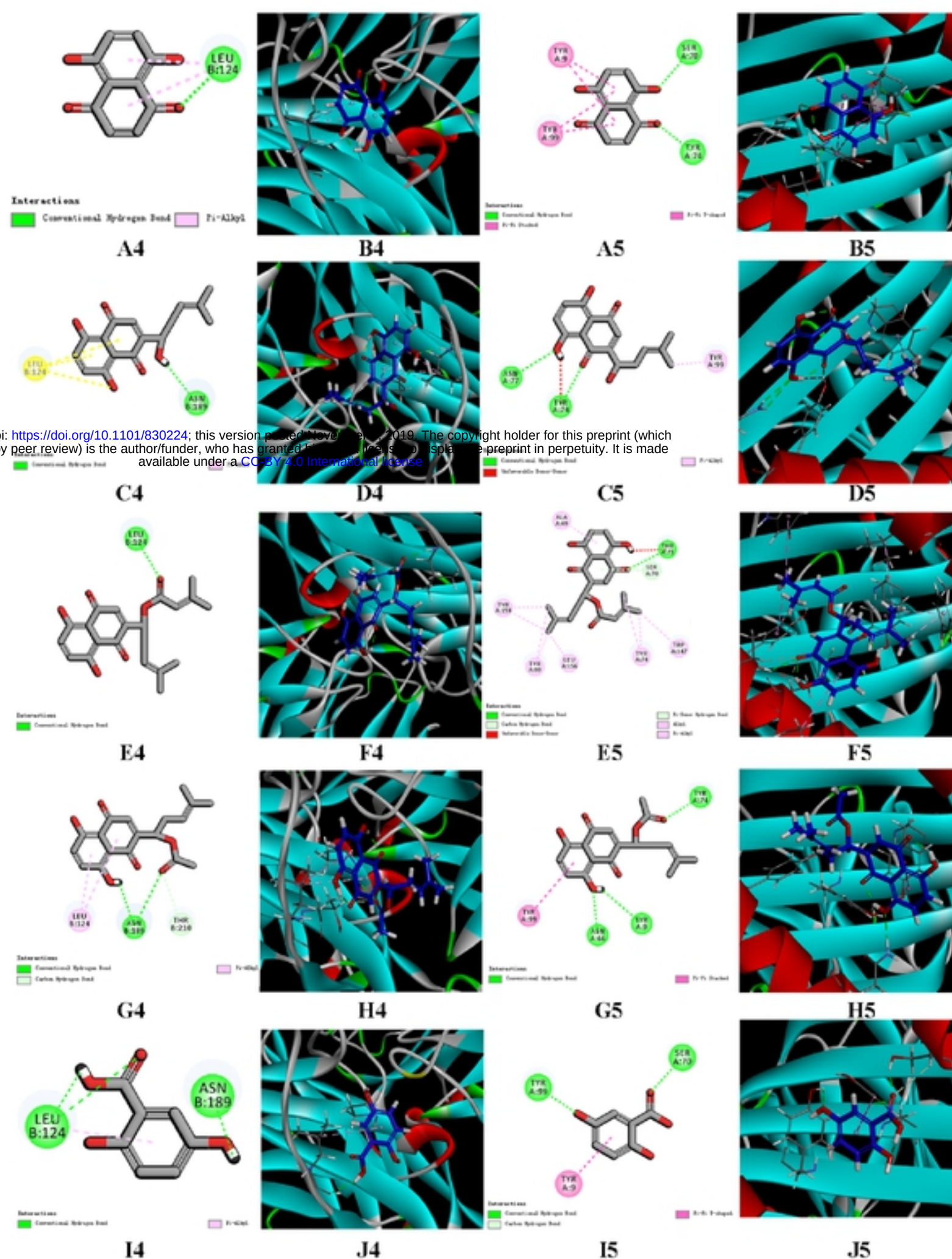
<sup>13</sup>C NMR of Compound **Acetylshikonin**



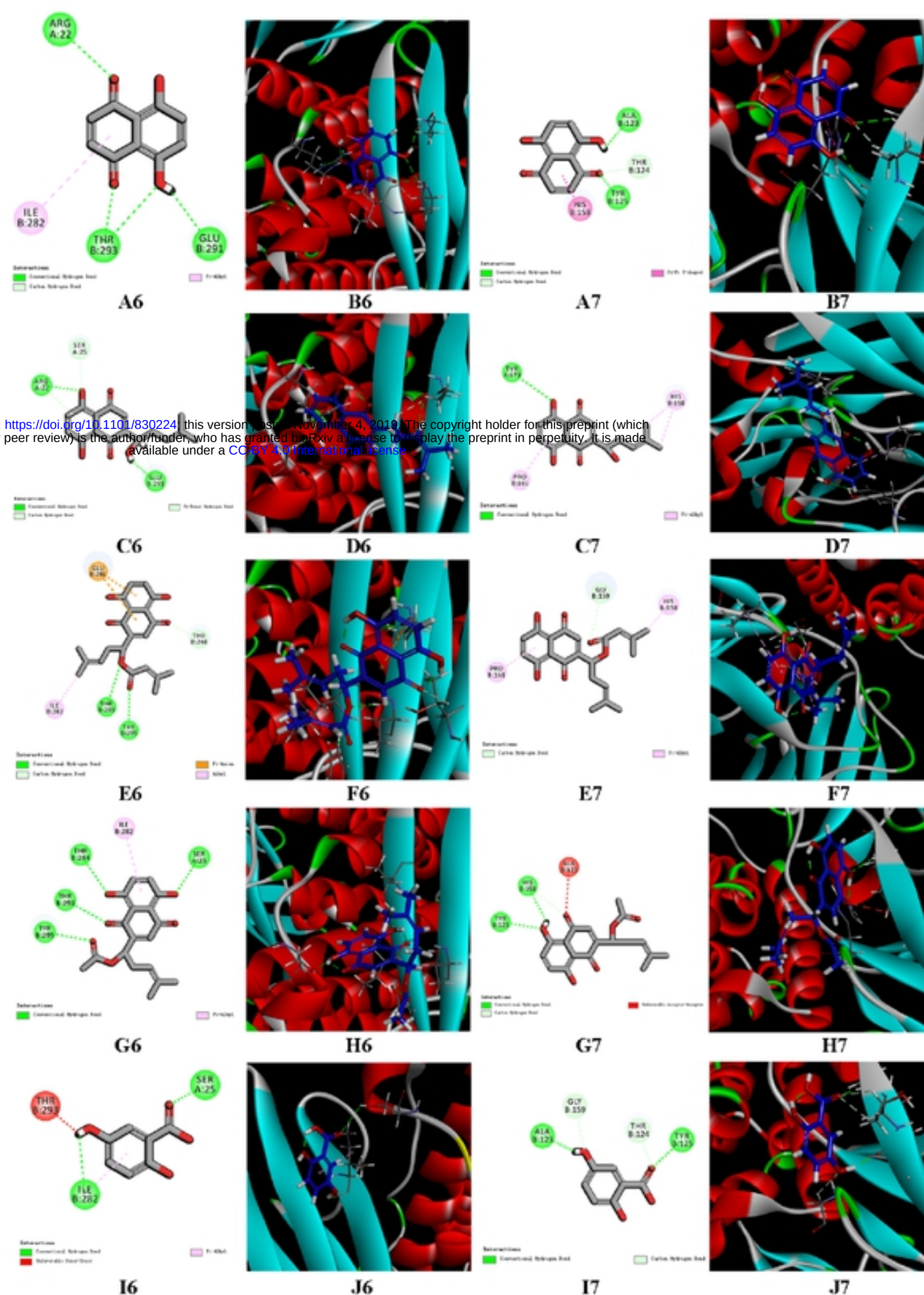
**S1 Fig.** Two and Three Dimensional Molecular Docking Model of Compound Mesalazine, Shikonin and its derivatives with MPO (Entry 4C1M in the Protein Data Bank in the right half part) and with NF-κB (Entry 4DN5 in the Protein Data Bank in the left half part).

<https://doi.org/10.1101/830224>; this version posted November 4, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

**S2 Fig.** Two and Three Dimensional Molecular Docking Model of Compound Mesalazine, Shikonin and its derivatives with NLRP3 (Entry 6NPY in the Protein Data Bank in the right half part) and with TNF- $\alpha$  (Entry 2AZ5 in the Protein Data Bank in the left half part).



**S3 Fig.** Two and Three Dimensional Molecular Docking Model of Compound Mesalazine, Shikonin and its derivatives with IL-1 $\beta$  (Entry 3O4O in the Protein Data Bank in the right half part) and with IL-6 (Entry 5U98 in the Protein Data Bank in the left half part).



**S4 Fig.** Two and Three Dimensional Molecular Docking Model of Compound Mesalazine, Shikonin and its derivatives with IFN $\gamma$  (Entry 3OQ3 in the Protein Data Bank in the right half part) and with IL-10 (Entry 5T5W in the Protein Data Bank in the left half part).