Thieme

# Shikonin Prevents Early Phase Inflammation Associated with Azoxymethane/Dextran Sulfate Sodium-Induced Colon Cancer and Induces Apoptosis in Human Colon Cancer Cells


## Authors

**Isabel Andújar[1,2,3*], Alberto Martí-Rodrigo[1*], Rosa María Giner[1], José Luis Ríos[1], María Carmen Recio[1]**

## Affiliations

1 Departament de Farmacologia, Universitat de València, Valencia, Spain
2 FISABIO-Fundación Hospital Universitario Dr. Peset, Valencia, Spain (Present address)
3 Departamento de Ciencias Biomédicas. Universidad Europea de Valencia, Valencia, Spain



## Key words

*Lithospermum erythrorhizon*, Boraginaceae, inflammatory bowel disease, shikonin, ulcerative colitis, *in vivo*

**received** December 27, 2017
**revised** March 21, 2018
**accepted** March 26, 2018

## Bibliography

**DOI** https://doi.org/10.1055/a-0599-1145
Published online April 11, 2018 | Planta Med 2018; 84: 674–683 © Georg Thieme Verlag KG Stuttgart · New York | ISSN 0032-0943



## Correspondence

Prof. María Carmen Recio
Departament de Farmacologia, Facultat de Farmàcia, Universitat de València
46100 Burjassot, Valencia, Spain
Phone: + 34 963 54 32 83, Fax: + 34 963 54 49 43
maria.c.recio@uv.es

## Correspondence

Dr. Isabel Andújar
Departament de Farmacologia, Facultat de Farmàcia, Universitat de València
46100 Burjassot, Valencia, Spain
Phone: + 34 963 54 32 83, Fax: + 34 963 54 49 43
isabel.andujar@uv.es



## ABSTRACT

Shikonin is the main active principle in the root of *Lithospermum erythrorhizon*, widely used in traditional Chinese medicine for its anti-inflammatory and wound healing properties. Recent research highlights shikonin's antitumor properties and capacity to prevent acute ulcerative colitis. The aim of the present study was to evaluate the ability of shikonin to prevent, *in vivo*, the early phases of colorectal cancer development, with special focus on its cytotoxic mechanism *in vitro*. We employed the azoxymethane/dextran sulfate sodium model of colitis in Balb/C mice. Body weight and drinking were monitored throughout the experiment, and length of colon and lesions of the colon were recorded on termination of the experiment in all of the experimental groups. Colons underwent histological evaluation and biochemical analyses [myeloperoxidase activity assay, measurement of interleukin-6, evaluation of proinflammatory enzymes (cyclooxygenase-2 and inducible nitric oxide synthase), and nuclear factor-κB activation by Western blot]. Caco-2 cells were used to evaluate, *in vitro*, the effect of shikonin on proliferation, cytotoxicity, cell cycle, and apoptosis. Our results reveal that shikonin significantly protected the intestinal tissue of our animals by preventing the shortening of the colorectum and ulcer formation in a dose-dependent manner. Shikonin attenuated the expression of cyclooxygenase-2 and inducible nitric oxide synthase, and myeloperoxidase activity, and inhibited the production of interleukin-6 and activation of nuclear factor-κB. It induced Bcl-2 and inhibited caspase 3. In conclusion, shikonin acts as a chemopreventive agent in the azoxymethane/dextran sulfate sodium model through inhibition of the proinflammatory milieu generated during the disease, an important risk factor in cancer development.


* Isabel Andújar and Alberto Martí-Rodrigo contributed equally to this study.

**ABBREVIATIONS**

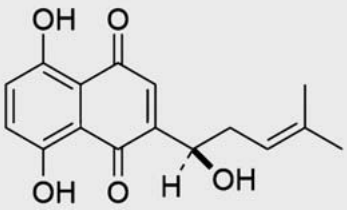
| | |
|---|---|
| ANOVA | one-way analysis of variance |
| AOM | azoxymethane |
| COX-2 | cyclooxygenase-2 |
| CRC | colorectal cancer |
| DSS | dextran sulfate sodium |
| FITC | fluorescein isothiocyanate |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| IBD | inflammatory bowel disease |
| IL-6 | interleukin-6 |
| iNOS | inducible nitric oxide synthase |
| MPO | myeloperoxidase |
| NF-κB | nuclear factor-κB |
| SEM | standard error of the mean |
| STAT3 | signal transducer and activator of transcription-3 |
| TGF-β1 | transforming growth factor-β1 |
| UC | ulcerative colitis |

## Introduction

CRC is a major health problem and a leading cause of death in industrialized countries [1]. It is now well accepted that chronic inflammation is a main factor in the development of cancer, since the continuous presence of proinflammatory cytokines such as IL-6 or TNF-α contribute to the carcinogenic process by modifying the normal survival, growth, proliferation, and differentiation of intestinal stromal cells through the alteration of genes that regulate these processes, such as p53 and Bcl2, among others [2–4]. In addition, the overexpression of proinflammatory enzymes, such as COX-2 or iNOS, favors this tumorigenic microenvironment by increasing oxidative stress and inhibiting endogenous mechanisms of DNA repair [5]. NF-κB activation has also been demonstrated to be essential in tumor growth and progression [6], which further highlights the importance of inflammation in CRC development.

IBD, and especially long-time established UC, represents an example of a disorder that increases the risk of CRC considerably. This risk depends on several factors, such as the duration of the preexisting colitis, its anatomic extension, and the degree of inflammation [7]. It is estimated that the risk of CRC development in a patient with UC can rise as high as 40% after 40 years of disease, while 50–80% of colonic neoplasms may remain undetected during a routine colonoscopy [7], which highlights the urgent need to develop effective chemopreventive drugs.

Traditional medicine based on the use of medicinal plants and their products has provided first-line drugs for centuries, and has been an important source of therapeutic agents developed by the pharmaceutical industry [8]. The dry root of *Lithospermum erythrorhizon* Siebold et Zucc. (Boraginaceae), also known as Zicao, contains the naphtoquinone shikonin (▶ **Fig. 1**) as its principal active molecule. Various preparations containing shikonin are still used today for medicinal and cosmetic purposes in Asia, with Zicao being a commonly used anticancer herbal medicine in China [9]. Shikonin has been studied in a mouse model of acute UC [10] with promising results: oral administration of this naphthoquinone prevented UC development through the downregulation of Th1 responses together with the blockade of two major IBD targets, NF-κB and STAT3. Moreover, as previously published, shikonin stimulates the migration of intestinal epithelial cells *in vitro* through a TGF-β1-dependent process [11], a mechanism that probably complements its anti-inflammatory activity demonstrated *in vivo* to prevent the development of UC by ulcers in the epithelial monolayer. The number of studies highlighting the potential anticancer activity of shikonin and shikonin-containing mixtures is increasing exponentially, and, although the precise underlying mechanism is still undetermined, it seems clear that this naphthoquinone is capable of inhibiting or modulating several molecular targets associated with cancer, as well as activating multiple cell death pathways [12].



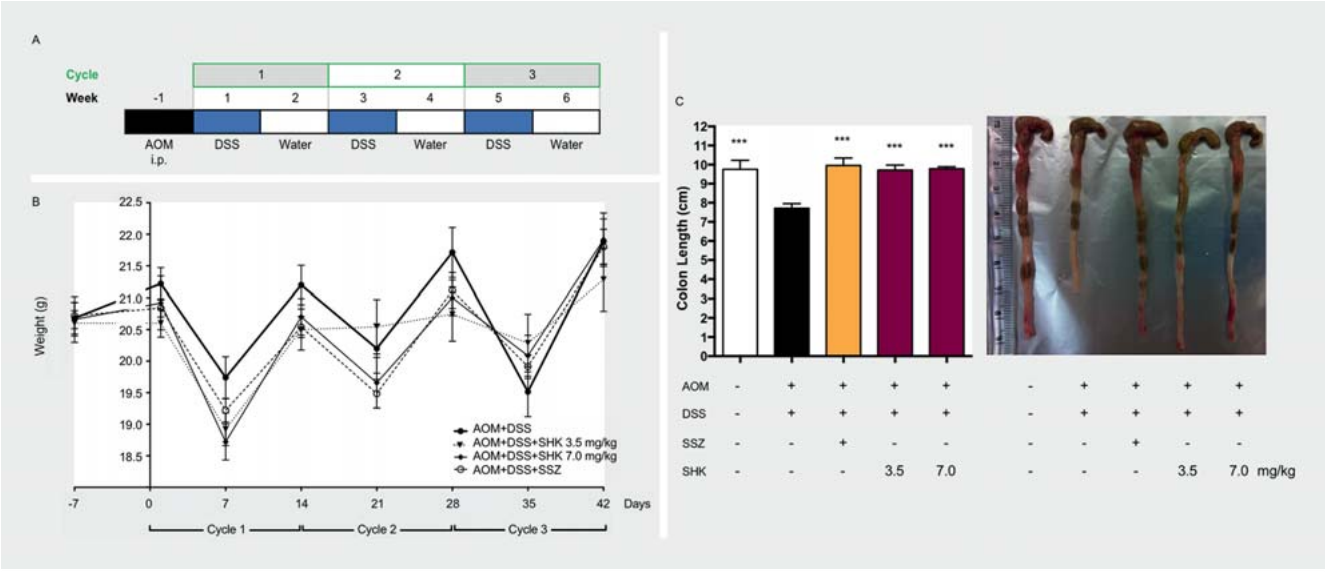

▶ **Fig. 1** Chemical structure of shikonin.

In light of these antecedents, the aim of this study was to evaluate the ability of shikonin to prevent the early phases of colorectal cancer (before tumors are visible) *in vivo*, and to explore its cytotoxic mechanism *in vitro*.

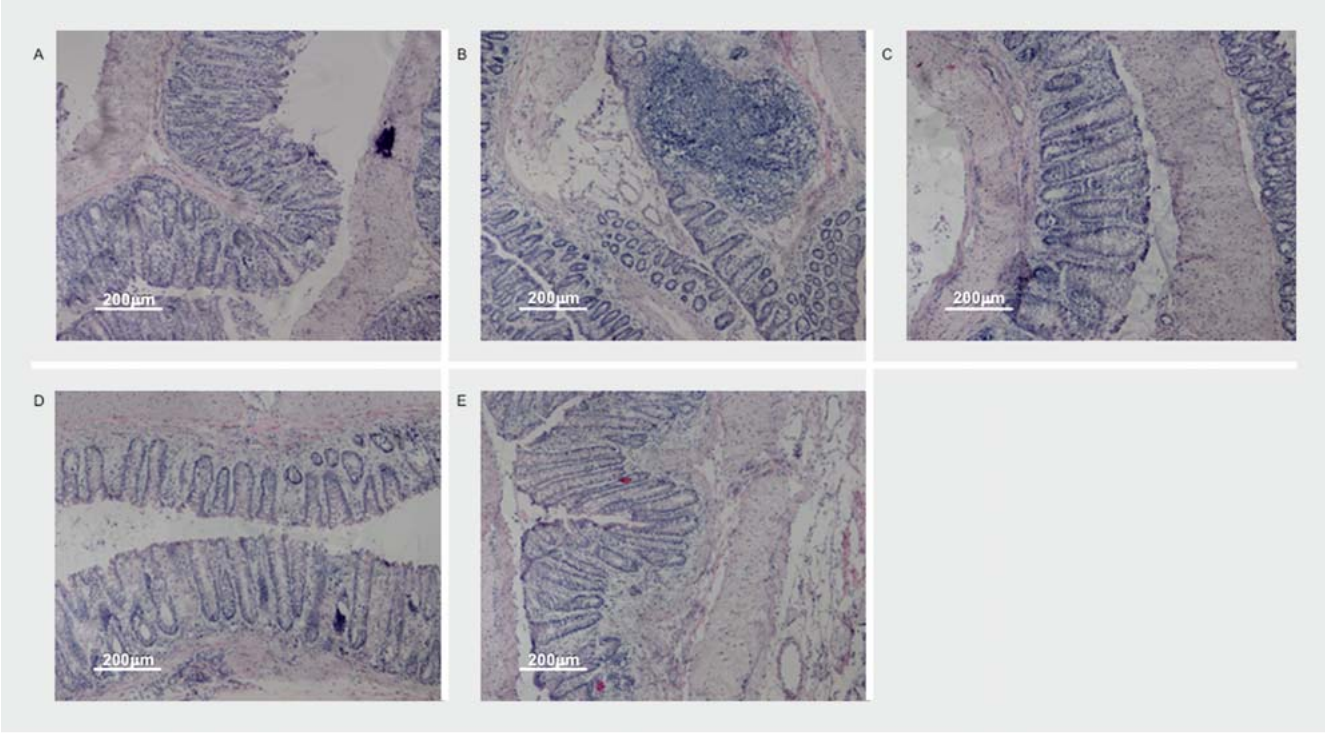
## Results

The weight of the animals during the experimental period was not significantly affected by any of the treatments administered (▶ **Fig. 2A, B**). The weekly fluctuations observed, with weight loss during the odd-numbered weeks and weight gain during the even-numbered weeks, coincided with the cycles of DSS administration. Colitis was exacerbated on the days on which DSS was administered, while there was a temporal remission of inflammation, which favored weight gain, on the days on which animals received water only.

Nevertheless, both sulfasalazine (positive control) and shikonin treatment significantly protected the intestinal tissue, preventing the shortening of the colorectum caused by the development of the disease, and impeding ulcer formation (▶ **Fig. 2C**). Moreover, histologic analysis showed a marked loss of the structure of the microvilli, crypt distortion, *lamina propria* erosion, and erosion of the mucosal membrane, together with massive infiltration of inflammatory cells, in the group of mice treated with AOM/DSS (▶ **Fig. 3B**). In the case of sulfasalazine-treated mice (▶ **Fig. 3C**), the crypt structure was, in general, conserved and there was less inflammatory cell infiltration than in the AOM/DSS group. In addition, in some areas of the colon we detected crypt elongation, which is a consequence of regeneration mechanisms working

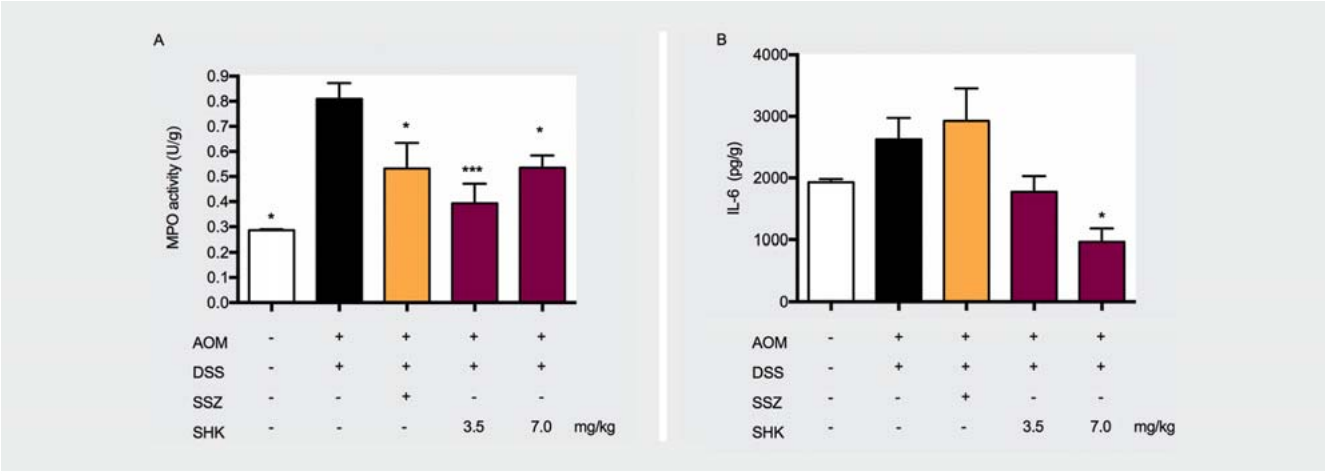



▶ **Fig. 2** Experimental protocol and macroscopic results. **A** Schematic representation of the AOM/DSS experimental protocol. **B** Evolution of weight during the experimental protocol. **C** Effect of shikonin on colon length. Statistical analysis was performed using ANOVA followed by Dunnett's t-test; $***p < 0.001$ vs. AOM/DSS group (n = 10).

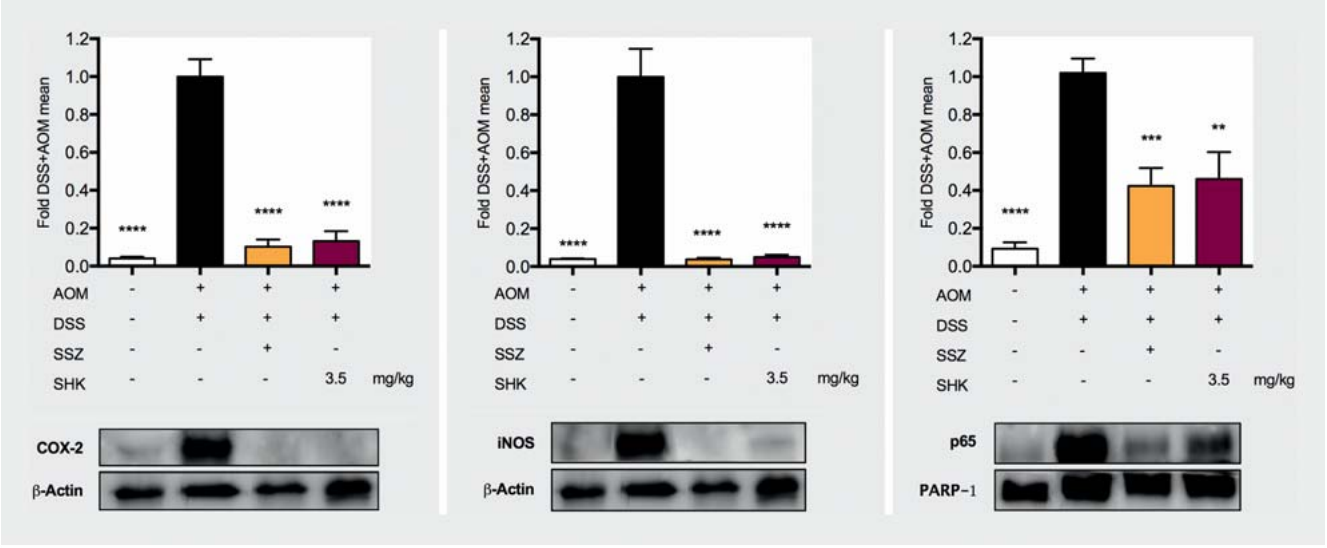



▶ **Fig. 3** Effect of shikonin treatment on histological parameters. Three representative colonic hematoxylin/eosin sections: mice received fresh tap water (**A**), AOM/DSS (**B**), AOM/DSS + sulfasalazine treatment (**C**), AOM/DSS + shikonin 3.5 mg/kg (**D**), or AOM/DSS + shikonin 7 mg/kg (**E**) (n = 10).

faster than the shedding process, which causes cells to accumulate in the villi and to become elongated as a result. Shikonin-treated mice (▶ **Fig. 3 D, E**) exhibited a clear dose-response. Those receiving the lower dose displayed more inflammatory cell infiltration together with partial loss of the mucosa, and partial loss and nonfunctional elongation of the crypts. In contrast, mice treated with the highest dose showed lower cell infiltration and crypts that were better conserved and functional when undergoing elon-




▶ **Fig. 4** Effect of shikonin on neutrophil infiltration and IL-6 production. **A** Mucosal MPO levels were measured to evaluate the effect of shikonin on the number of neutrophils infiltrating the colon. MPO activity is expressed as U/g, that is, the amount of enzyme required to convert 1 µmol of $H_2O_2$ to water in 1 min, expressed per gram of wet tissue. **B** Effect of shikonin on IL-6 production in the colon. *P < 0.05, ***p < 0.001; significantly different from the AOM/DSS group, determined by means of ANOVA followed by Dunnett's t-test (n = 7).

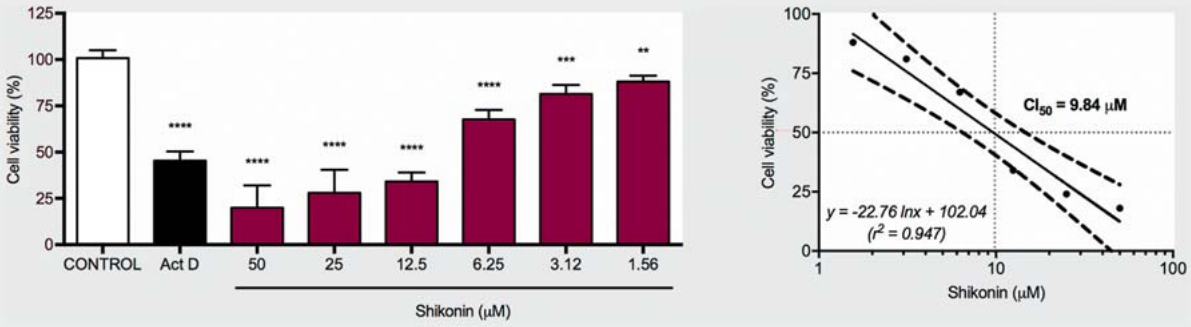



▶ **Fig. 5** Effect of shikonin treatment on proinflammatory mediators in colonic tissue. **A** Effect of shikonin on COX-2 expression. **B** Effect of shikonin on iNOS expression. **C** Effect of shikonin on nuclear translocation of the NF-κB p65 subunit. The histograms at the top represent the data derived from the Western blots following densitometry analysis (mean of the relative OD ± S. E.M); consider the DSS + AOM group as having 100% expression. Levels were normalized against β-actin or the PARP-1 antibody. **P < 0.01, ***p < 0.001, ****p < 0.0001; significantly different from the AOM/DSS group, determined by means of ANOVA followed by Dunnett's t-test (n = 6–8).

gation processes. MPO activity is an indicator of the extent of an inflammatory process, as it is an indirect determination of neutrophil infiltration, and we found that it was significantly decreased by shikonin and sulfasalazine (▶ **Fig. 4A**).

Proinflammatory cytokines play an essential role in the development of CRC. The pleiotropic cytokine IL-6 has been linked to the pathogenesis of sporadic and inflammation-associated CRC, as demonstrated by several experimental and clinical studies [13]. Shikonin (7 mg/kg) blocked AOM/DSS-induced IL-6 production, with similar levels detected in treated and untreated (sham) groups (▶ **Fig. 4B**). However, sulfasalazine did not have an effect on IL-6 production.

According to our results, exposure to AOM/DSS causes a strong expression of COX-2 and iNOS, which is prevented by shikonin and by sulfasalazine (▶ **Fig. 5A, B**). NF-κB plays an essential role in immune and inflammatory responses, as demonstrated by the fact that the promoter and enhancing regions of genes coding for inflammatory mediators have binding sites for this transcription factor [14]. In this sense, patients with inflammatory bowel disease show high NF-κB activation in *lamina propria* biopsies as




▶ **Fig. 6** Evaluation of the cytotoxic effect of shikonin in Caco-2 cells. Differences between each group and the control group were determined by means of ANOVA followed by Dunnett's t-test. *P < 0.05, **p < 0.01, ***p < 0.001; ns: not significantly different (n = 8).

well as elevated levels of macrophages and epithelial cells [15, 16]. Accordingly, we verified that levels of p65 were markedly higher in AOM/DSS-treated animals than in the vehicle group, an increase that was blocked by shikonin treatment (▶ **Fig. 5C**).

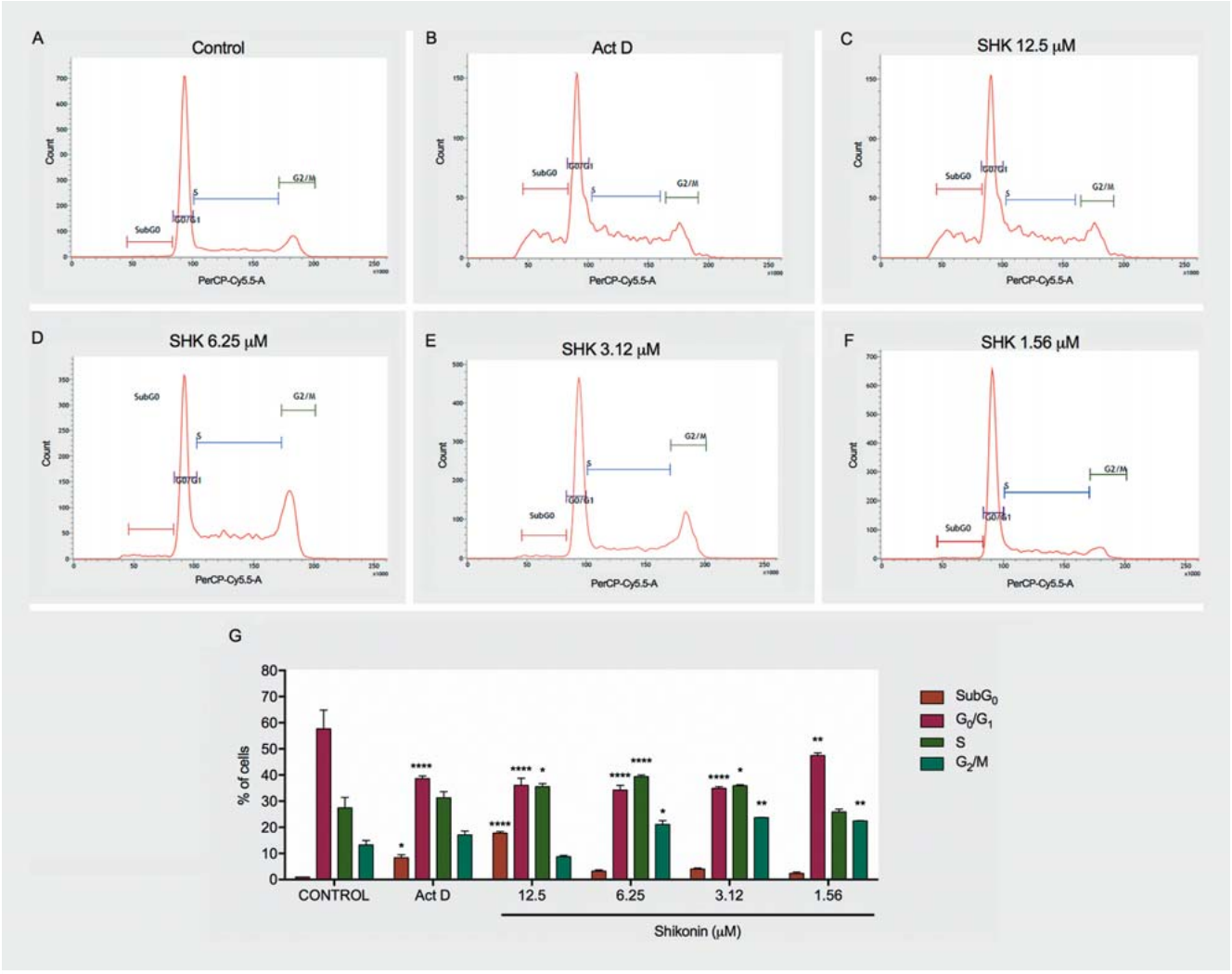
The MTT cytotoxicity assay revealed a dose-dependent *in vitro* cytotoxic effect of shikonin ($IC_{50}$ = 9.84 μM) (▶ **Fig. 6**). To investigate whether shikonin was affecting cell cycle regulation, we determined its effect in Caco-2 cells by flow cytometry and compared it to the effect of the known cytotoxic compound actinomycin D. Twenty-four-hour exposure of Caco-2 cells to shikonin resulted in a statistically significant decrease of cell subpopulations in the preparatory phases of the cycle, $G_0/G_1$ and $G_2/M$, accompanied by an increase in the S phase, in a concentration-dependent manner. These results reveal that shikonin induces the metabolic activation of these cells, which is favorable in the treatment of cancer, as it reduces the percentage of cells that remain quiescent in the $G_0/G_1$ phase, increasing their susceptibility to cytotoxic treatments (▶ **Fig. 7**).

Finally, as seen in the cell cycle analysis, shikonin exerted a pro-apoptotic effect, as demonstrated by the increase of the subpopulation of Caco-2 cells during the $SubG_0$ phase; in other words, it increased the fragmentation of DNA. Since this increase could also have been caused by a direct cytotoxic effect like that which occurs in necrotic processes, we stained cells with propidium iodide and FITC-Annexin V to differentiate apoptotic and necrotic cells. Shikonin significantly and dose-dependently exerted a proapoptotic effect on Caco-2 cells (▶ **Fig. 8**). This is in line with the known effects of shikonin on other cancer cell lines [17, 18]. Moreover, to delve into the mechanism of action by which shikonin induces apoptosis in these cells, we employed Western blot to analyze the expression of the antiapoptotic protein Bcl-2 and the proapoptotic caspase 3. As demonstrated in ▶ **Fig. 9**, treatment with shikonin inhibited the expression of Bcl-2 and induced the activation of caspase 3 is a dose-dependent manner, all of which indicates that shikonin stimulates the apoptotic process by acting on the proapoptotic machinery of the cells.

## Discussion

It is a proven fact that the risk of developing CRC is higher in patients with IBD than in the rest of the population [19]. The discomfort associated with a colonoscopy, used indiscriminately as a population screening method, has favored an increased interest in the use of chemopreventive strategies that reduce the risk of cancer, extending their benefits to IBD patients. Since chronic inflammation is a clear procarcinogenic factor, it seems logical that anti-inflammatory agents have potential as chemopreventive agents. However, to date, no anti-inflammatory drug has demonstrated this property, with the exception of 5-amino-salicylic acid (5-ASA) and related immunosuppressive thiopurines [20]. In this sense, further studies are needed to confirm the chemopreventive effect of other anti-inflammatory drugs in spontaneous colorectal cancer prevention. On this premise, we have studied the chemopreventive effect of shikonin, a natural product with proven anti-inflammatory [10, 21] and wound healing [11] activities, in a model in which the synergic effects of AOM (the tumor-inducing agent) and DSS (which produces severe colitis due to its toxicity to the epithelial lining of the colon) reproduce the initial acute inflammatory phase that precedes the carcinogenesis that occurs in human CRC [1]. We have observed how the said phase was clearly reproduced, especially in the distal part of the colon (as occurs in humans), where an inflammatory process could be observed in the mucosa and submucosa, together with damage to and loss of the majority of the crypts. In line with previous research [22], sulfasalazine administration to mice treated with AOM/DSS partially blocked this inflammatory precancerous process, as did treatment with shikonin.

The activity of the disease is linked to the influx of neutrophils in the mucosa and consequently in the intestinal lumen, which leads to the formation of abscesses in the crypts. As would be expected in a subchronic process, leukocyte infiltration, largely responsible for the pathogenesis of UC, was markedly increased in AOM/DSS-treated mice, evident in the increase of MPO activity in the colonic tissue of these animals. It is known that, in the progression of UC-associated CRC, there is a massive infiltration of neutrophils into the *lamina propria* and submucosa [23], and that an ele-

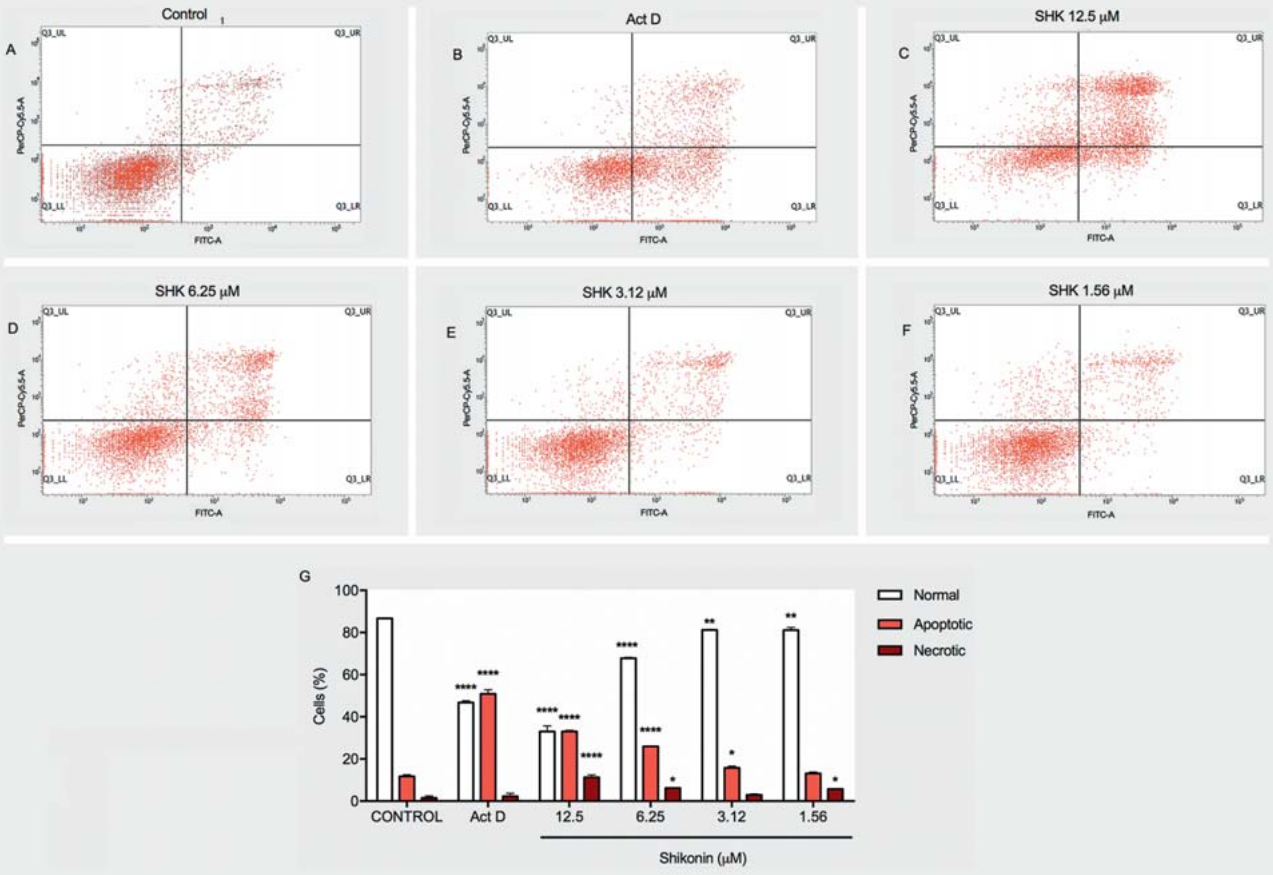



▶ **Fig. 7** The cell cycle of Caco-2 cells (**A**) left untreated, (**B**) treated with actinomycin D-treated, or (**C–F**) treated with increasing doses of shikonin was analyzed by flow cytometry. The histogram (**G**) represents the % of cells in each phase of the cell cycle for each treatment. Significant differences in each phase of each treated group with respect to the control group were determined by means of a two-way analysis of variance followed by Dunnett's t-test, where $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ (n = 8).

vated ratio of neutrophil-to-lymphocyte predicts a significantly higher risk of death in colon cancer [24]. Moreover, it has been demonstrated that neutrophil depletion decreases the different parameters of DSS-induced colitis [25] and that administration of neutrophil-neutralizing antibodies after the last DSS cycle markedly reduces the number and size of tumors and decrease the expression of chemokines, matrix metalloproteinase-9, and neutrophil elastase in an AOM/DSS model [24]. In our experiments, shikonin significantly reduced this infiltration to an even greater extent than sulfasalazine. This depleted number of tumor-associated neutrophils would result in a reduction in neutrophil-produced tumorigenic mediators (such as metalloproteinases), therefore interfering with the progression of chronic colitis to CRC.

As De Robertis M et al. [1] described, key components of cancer-promoting inflammation that have been investigated in the AOM/DSS model include proinflammatory cytokines, such as IL-6 and TNF-$\alpha$, COX-2, and master transcription factors, such as NF-$\kappa$B and STAT-3. Clinical and experimental data show that IL-6 contributes considerably to both sporadic and colitis-associated colorectal cancer [13]. In our experimental procedure, sulfasalazine did not prevent the increase of IL-6 induced by AOM/DSS-treatment; in contrast, shikonin did significantly inhibit this cytokine. Growing evidence supports a critical role for IL-6 signaling during CRC development, and so therapeutics that target this cytokine are viewed as promising options for the treatment of IBD [13, 26].

COX-2 and iNOS were also inhibited by shikonin treatment in our experiments. The expression of COX-2 is increased in 40% of colorectal adenomas and in 80% of CRCs [27], mainly in neoplastic epithelial cells [28], and is associated with tumor development and progression. iNOS, on the other hand, is activated in large bowel neoplasms and dysplasia within 20 weeks of the initiation of AOM/DSS treatment [1]. In our experiment, the expression of these two enzymes was significantly increased in the colitic group when compared to the untreated (sham) group, as expected.

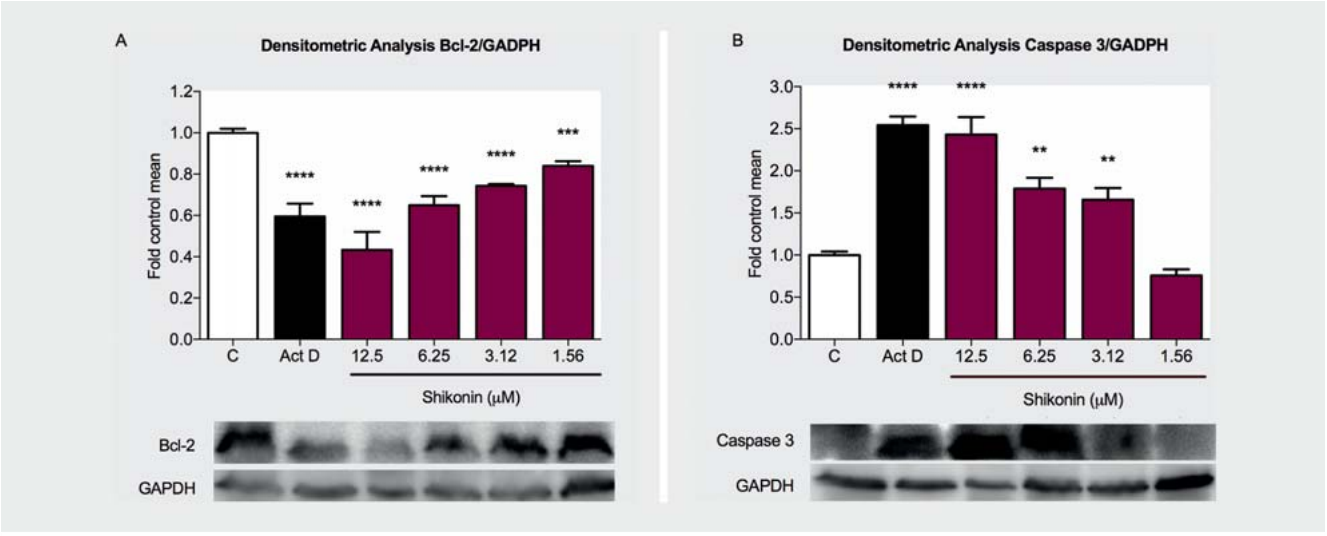



▶ **Fig. 8** Shikonin triggers apoptosis in Caco-2 cells. Normal, apoptotic, and necrotic cells were assessed by flow cytometry using Annexin V and propidium iodide (PI) staining. Cells in the lower left quadrant (Annexin V-FITC$^-$/PI$^-$) are normal, those in the lower right quadrant (Annexin V-FITC$^+$/PI$^-$) are early apoptotic cells, and those in the upper right quadrants (Annexin V-FITC$^+$/PI$^+$) are late apoptotic or necrotic. (**A**) Left untreated, (**B**) treated with actinomycin D-treated, or (**C–F**) treated with increasing doses of shikonin. The histogram (**G**) represents the data obtained from eight independent experiments, expressed as the mean ± S. E. M. Differences between each group and the control group were determined by means of a two-way analysis of variance followed by Dunnett's t-test; $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

However, shikonin significantly inhibited the increase of these proinflammatory enzymes to the same extent as the control treatment sulfasalazine. The activation of the transcription factor NF-κB is probably involved in the overexpression of the aforementioned proinflammatory enzymes and cytokine (COX-2, iNOS, and IL-6), as it is implicated in the regulation of inflammatory and immunomodulatory genes, as well as antiapoptotic genes. Shikonin, in the same way as sulfasalazine, significantly inhibits the activation of this transcription factor, the lynchpin of inflammation-associated cancer [29], and consequently leads to a reduction in the expression of proinflammatory cytokines, adhesion molecules, and antiapoptotic genes [7].

Our *in vitro* results are consistent with this scenario. *In vitro* shikonin induces the proapoptotic Bcl-2 and inhibits the antiapoptotic caspase 3. Although previous studies carried out by other groups [17, 18] have demonstrated shikonin's cytotoxic effect in other cell lines, this is the first time that the cytotoxic effect of shikonin has been demonstrated in the human epithelial colorectal adenocarcinoma cell line Caco-2. Our results confirm those of other authors who claim that shikonin modifies the cell cycle of cancer cells through the accumulation of these cells during the $G_0$ phase, which decreases the subpopulations of $G_0/G_1$, S, and $G_2/M$ in human melanoma cells (A375-S2) and murine melanoma (B16F10) [18, 30]. The reduction of the population of cells in a quiescent phase ($G_0$) increases the susceptibility of these cells to conventional anticancer treatments. In fact, studies in chemoresistant cell lines (MCF-7/Adr) combining shikonin with other reference antitumor drugs (such as paclitaxel or vincristine) have demonstrated the chemosensitizing ability of the former, yielding antitumor activity values similar to those presented by the drug alone [17]. It is worth noting that clinical studies in patients with advanced lung cancer treated with conventional chemotherapy together with shikonin show a decrease in proliferation and tumor growth, longer survival, and a general improvement of their immune functionality and quality of life thanks to a decrease in the general perception of pain, improvement of appetite, and weight gain [31].

In conclusion, shikonin acts as a chemopreventive agent in the AOM/DSS model of UC-associated CRC through inhibition of the




▶ **Fig. 9** Modulation of the apoptotic machinery by shikonin treatment. The histograms at the top represent the data derived from the Western blots following densitometry analysis. The bottom panels show an example of Western blot following probing with the corresponding antibody. Levels were normalized against the GAPDH antibody. **A** Effect of shikonin on Bcl-2 expression. **B** Effect of shikonin on caspase-3 activation. $^{**}P < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$; significantly different from the control group, determined by means of ANOVA followed by Dunnett's t-test (n = 5).

proinflammatory milieu generated during the disease and known to be an important risk factor in cancer development. Moreover, previous studies have demonstrated that shikonin has wound healing properties *in vivo* [32,33] and *in vitro* in intestinal epithelial cell lines [11]. We hypothesize that this wound healing capacity is also at play in the UC model, contributing to the healing of ulcers and therefore reducing the bacterial influx from the intestinal lumen, which in turn diminishes inflammation. Finally, we propose that shikonin inhibits or modulates cellular targets associated with cancer and activates multiple cell death pathways either directly or indirectly [12], and that this cytotoxic effect contributes to the positive results of shikonin in this model. Although these two last hypotheses need to be confirmed by more in-depth studies in the model, we consider that the present evidence points to shikonin as a highly promising therapeutic agent.

## Materials and Methods

### Chemicals

Shikonin (≥ 98 % purity by HPLC) was purchased from TCI Europe. DSS (molecular weight 36–50 kD; MP Biomedicals LLC), sulfasalazine (≥ 98 % purity by HPLC), AOM (≥ 98 % purity by HPLC), and actinomycin D (≥ 95 % purity by HPLC) were purchased from Sigma-Aldrich. All chemical and biochemical reagents used in the buffers and experimental protocols were purchased from Fluka Chemika-Biochemika, Baker, Panreac, and Sigma-Aldrich.

### Animals

Female Balb/C mice weighing 18–20 g (Janvier) were used for the *in vivo* experiments. All animals were fed a standard diet ad libitum and housed under a 12-h light/dark cycle at 22 ± 3 °C and 60 % humidity. Housing conditions and all *in vivo* experiments were approved by the Institutional Ethics Committee of the University of Valencia, Spain (2015/VSC/PEA/00023; approved January 23, 2015).

### Induction of colorectal cancer associated with chronic ulcerative colitis and treatment with shikonin

We employed the AOM/DSS model of colitis-associated colorectal carcinogenesis [7,34], as outlined in ▶ **Fig. 2A**. In brief, after 1-week acclimatization, female Balb/C mice were injected with AOM (7.5 mg/kg, i. p.) and randomly assigned to four treatment groups (10 animals/group): one group was left untreated, a second group received the standard drug sulfasalazine (100 mg/kg), and the two remaining groups were treated with shikonin at two different doses (3.5 and 7.0 mg/kg). Doses were selected based on previous experiments [10]. Both sulfasalazine and shikonin were dissolved in normal drinking water before administration. Doses were calculated based on the estimation that mice drink about 6 mL of water/day. DSS treatment began one week after AOM injection and continued for three cycles. Each cycle consisted of 7 days of 1.5 % DSS (w/v) dissolved in fresh tap water ad libitum followed by 7 days without DSS. During the 14 days that each cycle lasted, both shikonin and sulfasalazine were administered on a continuous basis. A fifth group (six animals; sham group) was left untreated, injected with saline (instead of AOM), and drank normal drinking water throughout the experiment. Since the aim of the present study was to establish the effect of shikonin on the initial stages of CRC development, the endpoint of the procedure was established before visible tumors could be observed, at the stage of subchronic colorectal inflammation.

Body weight and drinking were monitored throughout the experiment. No significant difference in fluid consumption between groups was detected.

Upon termination of the experiment, colonic tissues from mid to distal colon were excised, rinsed with cold PBS, and blotted dry. Following gross examination, a piece (approximately 1 cm) of each colon was separated for histological evaluation and the rest was left for biochemical analyses. Those samples set aside for biochemical tests were ground to powder with a mortar and kept at − 80 °C for further analyses.

## Histopathological analysis

Small (approximately 1 cm) frozen sections (4 µm) of excised colonic tissue were fixed in 10% paraformaldehyde in PBS (pH 7.4) and stained with hematoxylin and eosin. Histologic assessment of the degree of inflammation and dysplasia of the colonic mucosa was carried out in a blinded fashion by a pathologist.

## Myeloperoxidase activity assay

MPO activity was determined as previously described [35]. About 40 mg of powdered tissue was weighed for each sample and homogenized in 80 mM $KH_2PO_4/Na_2HPO_4$ buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide. After centrifugation (11 300 *g* for 20 min at 4 °C), 100 µL of PBS, 85 µL of 22 mM $KH_2PO_4/Na_2HPO_4$ buffer, and 15 µL of $H_2O_2$ 0.017% were added to 30 µL of the supernatant. The enzymatic reaction began when adding 20 µL of tetramethylbenzidine hydrochloride. After 3 min at 37 °C the reaction was stopped. Absorbance was read at 630 nm. MPO activity is expressed as the amount of enzyme required to convert 1 µmol of $H_2O_2$ to water in 1 min, expressed per gram of wet tissue.

## Measurement of interleukin-6 in colon tissue

The concentration of IL-6 in the colon was measured using an enzyme-linked immunosorbent assay kit (eBioscience) following the manufacturer's instructions. About 80 mg of dry powdered tissue was dissolved (20%, w/v) in ice-cold PBS buffer containing 0.1% Igepal CA-630, as described in a previous study [36], and a complete mini-EDTA-free protease inhibitor cocktail was added (Roche). Tissues were sonicated for 10 s and shaken on ice for 45 min. The homogenates were centrifuged (20 000 *g* for 10 min at 4 °C) and supernatants were collected and stored at − 80 °C. Protein concentration in the supernatants was determined with the Bradford method.

## Preparation of cytosolic and nuclear protein fractions from colon and analysis by Western blot

Protein extraction from the intestine was performed as described previously [37]. Powdered tissues were homogenized for 1 min with a Polytron PT-2000 (Kinematica) tissue homogenizer in 1.5 mL of ice-cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM phenylmethyl sulphonyl fluoride, 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 µg/mL pepstatin A]. Igepal CA-630 was added to a final concentration of 0.5%. The homogenates were chilled on ice with gentle shaking for 45 min. The membrane fraction was centrifuged at 106 × *g* for 10 min at 4 °C. The supernatant containing the cytosolic fraction was stored at − 80 °C until use. The pellet was resuspended in 500 µL of buffer B (20 mM HEPES pH 7.8, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulphonyl fluoride, 0.5 mM dithiothreitol, 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 µg/mL pepstatin A) and chilled for 30 min on ice with gentle shaking. After centrifugation at 20 800 × *g* for 15 min at 4 °C, the supernatant containing the nuclear fraction was removed and stored at − 80 °C until use.

The presence of proteins in the supernatants was determined by means of the Bradford method, and equal amounts of protein (30 µg) were used. Membranes were incubated with polyclonal antibodies against COX-2 (1 : 1000; Millipore), iNOS (1 : 2000; Millipore), p65 (1 : 500; SC-7151; Santa Cruz Biotechnology), *β*-actin (1 : 10 000; Sigma-Aldrich), or PARP (1 : 500; Sigma-Aldrich). *β*-Actin was used as a cytosolic or total protein loading control and PARP was used as a nuclear protein loading control. The blots were washed and incubated with peroxidase conjugate anti-rabbit, anti-mouse, or anti-goat immunoglobulin G (1 : 12 000 dilution; Sigma-Aldrich). The immunoreactive bands were visualized with the aid of an enhanced chemiluminiscence system (Millipore).

## Cell culture

Human epithelial colorectal adenocarcinoma Caco-2 cells (ATCC; passages 21–33) were used in all *in vitro* experiments. Cells were maintained in DMEM GlutaMAX (Gibco) (1 g/L glucose) supplemented with 20% fetal bovine serum, 1% antibiotics (penicillin (100 U/mL), and streptomycin sulfate (100 mg/mL) in a humidified 5% $CO_2$ atmosphere.

## Proliferation and cytotoxicity assay

The effect of shikonin on proliferation and cytotoxicity was evaluated with the MTT assay [38]. Caco-2 cells were exposed to shikonin (50–1.56 µM) or to actinomycin D (4 µM) in a 96-well microplate and, 24-h post-treatment, 100 µL per well of a 0.5 mg/mL solution of MTT (Sigma-Aldrich) were added. Absorbance was measured at 490 nm with a Victor X3 (Perkin Elmer) plate reader. Untreated cells were arbitrarily assigned 100% viability.

Actinomycin D was used as a reference compound in cell studies, as it inhibits the growth of tumor cells, thus inducing apoptosis through a mechanism that involves the irreversible alteration of gene replication and protein transcription, together with the inhibition of topoisomerase II and an increase in the production of reactive oxygen species [39].

## Preparation of cell samples and Western blot analysis

Caco-2 cells were plated at a density of $5 \times 10^5$ cells per well in a 6-well cell culture plate along with 2 mL of culture medium and then incubated for 24 h. The cells were pretreated with shikonin or actinomycin D and subsequently stimulated with lipopolysaccharide (1 mg/mL) for the specified periods (16–24 h).

For Western blot, cell lysates were obtained with lysis buffer [1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl, and 25 mM Tris, pH 7.4 (Sigma-Aldrich), and a complete mini-EDTA-free protease inhibitor cocktail]. After centrifugation, proteins were determined in the supernatant by the Bradford method. Membranes were incubated with polyclonal antibodies against Bcl-2 (1 : 500,

SC-7382), caspase-3 (1 : 500, SC-22139), or GAPDH (1 : 1000, SC-20357), all purchased from Santa Cruz Biotechnologies. GAPDH was used as a loading control.

### Cell cycle and apoptosis studies by flow cytometry

To study the effect of shikonin on the cell cycle by flow cytometry, a commercial kit was used (BD Cycletest Plus DNA Reagent Kit, BD Biosciences). Samples were analyzed according to the manufacturer's instructions.

Apoptosis was also studied by flow cytometry with the aid of a commercial kit (FITC-Annexin V Apoptosis Detection Kit I, BD Biosciences).

### Software

Images for all Western blot experiments were acquired with the image analysis system LAS-3000 mini (Fujifilm). Digital images were processed and band density measurements were made with the aid of a Multi Gauge V3.0 software package (Fujifilm).

### Statistical analysis

Statistical analysis was performed by means of ANOVA followed by Dunnett's t-test. The results are presented as the mean ± SEM. GraphPad Prism 4.0 software (GraphPad Software Inc.) was used for all calculations.

## Acknowledgements

This work was supported by the Spanish Government (SAF2009-13059-C03-01) and by the University of Valencia (Especials Accions Grant UV-INV-AE13-139455). I. A. is the recipient of a fellowship from the Generalitat Valenciana (grant number BFPI/2008/040) and ISCIII (Sara Borrell program CD15/00236). The authors thank Brian Normanly for his editing of the manuscript.

## Conflict of Interest

The authors declare no conflict of interest.

## References

[1] De Robertis M, Massi E, Poeta ML, Carotti S, Morini S, Cecchetelli L, Signori E, Fazio VM. The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies. J Carcinog 2011; 10: 9

[2] Popivanova BK, Kitamura K, Wu Y, Kondo T, Kagaya T, Kaneko S, Oshima M, Fujii C, Mukaida N. Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis. J Clin Invest 2008; 118: 560–570

[3] Xie J, Itzkowitz SH. Cancer in inflammatory bowel disease. World J Gastroenterol 2008; 14: 378–389

[4] Rizzo A, Pallone F, Monteleone G, Fantini MC. Intestinal inflammation and colorectal cancer: a double-edged sword? World J Gastroenterol 2011; 17: 3092–3100

[5] Talero E, Sánchez-Fidalgo S, Villegas I, De La Lastra CA, Illanes M, Motilva V. Role of different inflammatory and tumor biomarkers in the development of ulcerative colitis-associated carcinogenesis. Inflamm Bowel Dis 2011; 17: 696–710

[6] Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, Kagnoff MF, Karin M. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 2004; 118: 285–296

[7] Clapper ML, Gary MA, Coudry RA, Litwin S, Chang WCL, Devarajan K, Lubet RA, Cooper HS. 5-aminosalicylic acid inhibits colitis-associated colorectal dysplasias in the mouse model of azoxymethane/dextran sulfate sodium-induced colitis. Inflamm Bowel Dis 2008; 14: 1341–1347

[8] Recio MC, Andújar I, Ríos JL. Anti-inflammatory agents from plants: progress and potential. Curr Med Chem 2012; 19: 2088–2103

[9] Chen X, Yang L, Oppenheim JJ, Howard OMZ. Cellular pharmacology studies of shikonin derivatives. Phyther Res 2002; 16: 199–209

[10] Andújar I, Ríos JL, Giner RM, Cerdá JM, Recio MC. Beneficial effect of shikonin on experimental colitis induced by dextran sulfate sodium in Balb/C mice. Evid Based Complement Alternat Med 2012; 2012: 271606

[11] Andújar I, Ríos JL, Giner RM, Recio MC. Shikonin promotes intestinal wound healing *in vitro* via induction of TGF-$\beta$ release in IEC-18 cells. Eur J Pharm Sci 2013; 49: 637–641

[12] Andújar I, Recio MC, Giner RM, Ríos JL. Traditional chinese medicine remedy to jury: the pharmacological basis for the use of shikonin as an anticancer therapy. Curr Med Chem 2013; 20: 2892–2898

[13] Waldner MJ, Foersch S, Neurath MF. Interleukin-6–a key regulator of colorectal cancer development. Int J Biol Sci 2012; 8: 1248–1253

[14] Ghosh S, Hayden MS. New regulators of NF-*k*B in inflammation. Nat Rev Immunol 2008; 8: 837–848

[15] Rogler G, Brand K, Vogl D, Page S, Hofmeister R, Andus T, Knuechel R, Baeuerle PA, Schölmerich J, Gross V. Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. Gastroenterology 1998; 115: 357–369

[16] Schreiber S, Nikolaus S, Hampe J. Activation of nuclear factor kappa B inflammatory bowel disease. Gut 1998; 42: 477–484

[17] Han W, Li L, Qiu S, Lu Q, Pan Q, Gu Y, Luo J, Hu X. Shikonin circumvents cancer drug resistance by induction of a necroptotic death. Mol Cancer Ther 2007; 6: 1641–1649

[18] Rajasekar S, Park DJ, Park C, Park S, Park YH, Kim ST, Choi YH, Choi YW. *In vitro* and *in vivo* anticancer effects of *Lithospermum erythrorhizon* extract on B16F10 murine melanoma. J Ethnopharmacol 2012; 144: 335–345

[19] Ullman TA, Itzkowitz SH. Intestinal inflammation and cancer. Gastroenterology 2011; 140: 1807–1816

[20] Subramanian V, Logan RF. Chemoprevention of colorectal cancer in inflammatory bowel disease. Best Pract Res Clin Gastroenterol 2011; 25: 593–606

[21] Ríos JL, Recio MC, Escandell JMM, Andújar I. Inhibition of transcription factors by plant-derived compounds and their implications in inflammation and cancer. Curr Pharm Des 2009; 15: 1212–1237

[22] Suzuki S, Sakamoto S, Mitamura T, Sassa S, Kudo H, Yamashita Y. Preventive effects of sulphasalazine on colorectal carcinogenesis in mice with ulcerative colitis. In Vivo 2000; 14: 463–466

[23] Popivanova BK, Kostadinova FI, Furuichi K, Shamekh MM, Kondo T, Wada T, Egashira K, Mukaida N. Blockade of a chemokine, CCL2, reduces chronic colitis-associated carcinogenesis in mice. Cancer Res 2009; 69: 7884–7892

[24] Shang K, Bai YP, Wang C, Wang Z, Gu HY, Du X, Zhou XY, Zheng CL, Chi YY, Mukaida N, Li YY. Crucial involvement of tumor-associated neutrophils in the regulation of chronic colitis-associated carcinogenesis in mice. PLoS One 2012; 7: e51848

[25] Natsui M, Kawasaki K, Takizawa H. Selective depletion of neutrophils by a monoclonal antibody, RP-3, suppresses dextran sulphate sodium-induced colitis in rats. J Gastroenterol Hepatol 1997; 12: 801–808

[26] Rogler G. Where are we heading to in pharmacological IBD therapy? Pharmacol Res 2015; 100: 220–227

[27] Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 1994; 107: 1183–1188

[28] Sinicrope FA, Gill S. Role of cyclooxygenase-2 in colorectal cancer. Cancer Metastasis Rev 2004; 23: 63–75

[29] Li Q, Withoff S, Verma IM. Inflammation-associated cancer: NF-kappaB is the lynchpin. Trends Immunol 2005; 26: 318–325

[30] Wu Z, Wu L, Li L, Tashiro S, Onodera S, Ikejima T. p53-mediated cell cycle arrest and apoptosis induced by shikonin via a caspase-9-dependent mechanism in human malignant melanoma A375-S2 cells. J Pharmacol Sci 2004; 94: 166–176

[31] Guo XP, Zhang XY, Zhang SD. Clinical trial on the effects of shikonin mixture on later stage lung cancer. Chinese J Mod Dev Tradit Med 1991; 11: 598–599, 580

[32] Karayannopoulou M, Tsioli V, Loukopoulos P, Anagnostou TL, Giannakas N, Savvas I, Papazoglou LG, Kaldrymidou E. Evaluation of the effectiveness of an ointment based on Alkannins/Shikonins on second intention wound healing in the dog. Can J Vet Res 2011; 75: 42–48

[33] Papageorgiou VP, Assimopoulou AN, Ballis AC. Alkannins and shikonins: a new class of wound healing agents. Curr Med Chem 2008; 15: 3248–3267

[34] Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. Cancer Sci 2003; 94: 965–973

[35] Suzuki K, Ota H, Sasagawa S, Sakatani T, Fujikura T. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. Anal Biochem 1983; 132: 345–352

[36] Rosengren S, Firestein GS, Boyle DL. Measurement of inflammatory biomarkers in synovial tissue extracts by enzyme-linked immunosorbent assay. Clin Diagn Lab Immunol 2003; 10: 1002–1010

[37] Sugimoto K, Hanai H, Tozawa K, Aoshi T, Uchijima M, Nagata T, Koide Y. Curcumin prevents and ameliorates trinitrobenzene sulfonic acid-induced colitis in mice. Gastroenterology 2002; 123: 1912–1922

[38] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65: 55–63

[39] Sobell HM. Actinomycin and DNA transcription. Proc Natl Acad Sci U S A 1985; 82: 5328–5331