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ORIGINAL ARTICLE

Long-term systemic toxicity of shikonin derivatives in Wistar rats

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Abstract

Context: Although the antitumor, immunomodulatory activities, and other effects of shikonin have been studied for decades, its systemic toxicity *in vivo* remains unclear.

Objective: To estimate the long-term systemic toxicity of shikonin derivatives (ShD) in a rat model.

Materials and methods: The roots of *Arnebia euchroma* (Royle) Johnst. (Boraginaceae) were extracted in ethanol, passed through a molecular sieve, and dried. A microemulsion solution in water was subsequently prepared. Adult Wistar rats were treated with ShD by gavage at concentrations of 200, 400, and 800 mg/kg per day for 90 days or 180 days. Hematological and biochemical examinations were performed, and the vital organs were subjected to pathological analyses.

Results: We did not observe hematological or non-hematological toxicity of ShD at a dose as high as 800 mg/kg per day for 6 months.

Discussion and conclusion: Our findings may offer some beneficial information for the practical application and research of *Arnebia euchroma*. We demonstrated in an animal model that ShD may be safe for usage.

Keywords

Arnebia euchroma, liver, long-term toxicity, heart, kidney

History

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Introduction

Chinese medical herbs designated as “Zicao” are actually dried roots of the borage plants belonging to the Boraginaceae family, *Lithospermum erythrorhizon* Sieb. et Zucc. and *Arnebia euchroma* (Royle) Johnst., which have been used for the treatment of macular eruption, measles, and sore throat for thousands of years in China (Chen et al., 2002). Naphthoquinone pigments, shikonin, and alkannin have been found to be the major components in both the plants. An early study that used an extract from Boraginaceae reported that it could inhibit the propagation of pulmonary carcinomas and improve immune functions (Guo et al., 1991). We also found that shikonin derivatives could protect the immune organs from damage and promote immune responses in tumor-bearing mice (Su et al., 2012). Furthermore, the shikonin extract of *Lithospermum erythrorhizon* induced apoptosis when added to cultures of human colorectal carcinoma cells (Hsu et al., 2004), HL60 cells (Gao et al., 2002; Hsu et al., 2004), and HeLa cells (Wu et al., 2004). The underlying mechanisms of tumor suppression have been identified to induce of apoptosis via signaling pathways that possibly involve reactive oxygen species (Chang et al., 2010), p53, p27, Bcl-2, caspases (Fujii et al., 1992), and inhibition of telomerase and DNA topoisomerase I/II (Hsu et al., 2004).

In addition to antitumor effects, Sasaki et al. (2000) reported that extracts from either *Lithospermum erythrorhizon* or *Arnebia euchroma* had antifungal activity against *Candida albicans in vitro*. Bactericidal research by Shen et al. (2002) demonstrated the activities of this agent against Gram-positive cocci, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci. Ho et al. (2003) showed that an extract of *Arnebia euchroma* diminished Vero cell infection by hepatitis C virus (HCV) in an *in vitro* inoculation model. Additionally, Wang et al. (2009) reported that shikonin had therapeutic effects on lupus nephritis in NZB/W F1 mice. However, no study has evaluated the long-term systemic toxicity of shikonin. Thus, this study was designed to estimate the long-term systemic toxicity of shikonin derivatives in a rat model.

Materials and methods**Animals**

Male and female in-bred Wistar rats were purchased from Changchun Experimental Animal Center and were maintained in a conventional animal housing facility throughout the experiment. The rats were 4-weeks-old, with a qualified permission No. SCXK-2008-0004. All experiments were performed according to Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China and were approved by the Ethics Committee of Jilin University (No. JLU-20080128002).

Shikonin derivatives

Shikonin derivatives (ShD) were prepared as described previously in our laboratory (Su et al., 2012). In brief, the crude *Arnebia euchroma* drug (“Xinjiang Zicao” in Chinese) was purchased from the Changchun Pharmaceutical Group, Co., which is authorized by the State Administration of Traditional Chinese Medicine of the People’s Republic of China to collect (October, verified by Chinese Academy of Medical Sciences), store, and sell Chinese medicinal herbs. The crude *Arnebia euchroma* drug was lixiviated with absolute ethanol, and the majority of the solvent was removed using a rotary evaporator. Subsequently, the solution was filtered, passed through a molecular sieve (Macroprep 1 K; Omega Centrifugal Devices, Port Washington, NY), and dried at 60 °C to obtain the ShD powder. Finally, ShD was collected as a dry crystallized powder. A microemulsion was used to prepare a water-soluble reagent (Shikonin derivative concentration: 1 mg/mL) using 1.08% of polyvinylpyrrolidone (PVP), and the drug was autoclaved and preserved at 4 °C. Acetylshikonin was found to be the main component using the extract characterization procedure described in detail in our previous study (Su et al., 2012).

Acute toxicity study

Forty Kunming mice were used to evaluate acute toxicity of ShD with maximal tolerated dose (MTD) test. After overnight fasting (approximately 10 h), 20 mice comprising an equal number of males and females were treated with ShD at dose of 10 g/kg (3 times per day for 3 days). The remaining 20 mice (equal number of males and females) treated with normal saline were set as control group. After a 2 week observation period, the animals were sacrificed for analyses.

Chronic toxicity study

We used 100 Wistar rats for the chronic toxicological investigation. The rats were randomly divided into 5 groups (20 animals per group) for each of the following treatments: normal control (without treatment), solvent control (PVP only without ShD), and ShD at 200, 400, and 800 mg/kg per day. The solvent or ShD was gavaged once a day for up to 90 days or 180 days (from March 2008 to August 2008), and the body weight of each of the rats was measured every 10 days during this period. Blood was collected for hematological and biochemistry examinations on days 90 and 180. Blood samples were collected from the rats by heart puncture and were added to 2 bottles; one with anticoagulant and one

without (2 mL per bottle). The blood cells were examined with a hemocyte counter (Coulter STKS, Hialeah, FL). Serum samples were separated after overnight coagulation of the blood and were analyzed automatically with the Coulter LX2D (Beckman, Brea, CA). The animals were sacrificed after the blood samples were collected by heart puncture. The organs were removed and fixed in ethanol, and the paraffin sections were stained with hematoxylin–eosin. Pathological analyses were conducted on these organs by two independent investigators blinded to group assignment.

Statistics

Data are expressed as mean \pm standard error of the mean (SEM). The data were analyzed by analysis of variance (ANOVA) using the Statistics Package for Social Science (SPSS) software (Version 16.0) (SPSS Inc., Chicago, IL). The Student–Newman–Keuls (SNK) test was used to assess significant differences between the control and treated groups. *p* Values of <0.05 were considered significant.

Results

Acute toxicity study in mice

No deaths occurred during the 2 week observation period. There was no significant differences in body weights, food consumption, clinical signs, and tissue morphology between ShD treated and control group (data not shown).

General conditions of the rats treated with ShD

Toxicological investigations were performed in an officially authorized national laboratory. The rats were given ShD for up to 90 days or 180 days, and food and water consumption in each group was observed daily during this period. In the first 10 days, the rats ingested an average of 18 g of food and 20 ml of water per animal in each of the 5 groups, with no significant differences between the groups. The food and water consumption increased an average of 1 g and 6 ml, respectively, per 10 days and increased to approximately 25 g and 60 ml, respectively, per day at 3 months after ShD administration. Thereafter, dietary consumption did not change and no significant differences were found among the five groups. The body weight of each rat was measured every 10 days: this value was approximately 100 g before ShD administration, increased to nearly 200 g in 50–60 days and further increased to approximately 300 g on day 100. When treatment ended on day 180, the average body weight of the rats in the 5 groups had increased to approximately 370 g, without an obvious difference among the groups (Table 1).

Table 1. Body weight changes of the rats in different groups.

	0 day	30 day	60 day	90 day	120 day	150 day	180 day
Normal control (g)	96 \pm 2.11	158 \pm 3.42	199 \pm 3.12	266 \pm 5.19	319 \pm 5.78	352 \pm 6.21	370 \pm 7.73
Solvent control (g)	99 \pm 3.19	160 \pm 5.22	199 \pm 4.01	268 \pm 6.17	318 \pm 5.66	351 \pm 5.99	370 \pm 6.93
ShD							
800 mg/kg (g)	98 \pm 3.18	162 \pm 4.32	198 \pm 5.01	269 \pm 5.03	318 \pm 5.99	350 \pm 5.93	371 \pm 6.99
400 mg/kg (g)	101 \pm 3.71	162 \pm 4.99	204 \pm 5.17	271 \pm 4.23	318 \pm 5.07	352 \pm 4.99	369 \pm 7.38
200 mg/kg (g)	100 \pm 3.22	161 \pm 5.29	201 \pm 4.31	273 \pm 4.54	321 \pm 6.16	350 \pm 6.08	373 \pm 7.27

All *p* > 0.05.

Additionally, the glossiness of the coats and the activities of the rats were not obviously different among the 5 groups.

Evaluation of non-hematological and hematological toxicities of ShD in Wistar rats

No rats died during the first 3 months, and 50 rats (10 from each group) comprising an equal number of females and males were analyzed on day 90. During the subsequent 90 days, no rats died as well, and the remaining 50 rats were sacrificed for analysis at the end of the study.

Blood biochemistry was assessed to monitor the functions of the vital organs of the rats, and no obvious abnormality was observed in the rats treated with ShD compared with the control groups (Table 2).

The influence of ShD on the hemogram of the rats was evaluated at 90 day and 180 day after ShD administration. Long-term ShD administration did not affect the peripheral blood cell counts or the functional parameters of these cells (Table 3).

The hematopoietic status of the bone marrow was observed in a histological sample of the sternum. The bone marrow exhibited a normal structure in the ShD-treated groups comparable to those observed in the normal and solvent controls (Figure 1A and E). The morphology and percentages of different cell lines were almost identical in the different groups.

Histological examination of the heart, liver, and kidney of the rats was also conducted in this study (Figure 1). Compared with the normal and solvent groups, no significant aberrations were observed in these organs in the ShD-treated rats at different concentrations. Interestingly, the absence of the so-called “cat-eye” structure of neurons was the only abnormality observed in the brain slices approximately 90 days after ShD administration. However, this feature appeared to be reversible because the typical morphology of neuronal cells was observed thereafter (at 180th day).

Discussion

In this study, shikonin derivatives were extracted by ethanol, and waxy contaminants were deleted using a molecular ultrafiltration sieve rather than ether treatment. Industrially, it may be replaced by binding to a macroporous resin. Hence, the traditional dewaxing procedure that uses a dangerous chemical reagent such as ether should no longer be required. Several protocols are available for facilitating ShD solubilization into water solution. In the present study, the dried shikonin derivative powder was dispersed into a solvent heated at a constant temperature of 60 °C, leading to formation of a stable microencapsulation reagent. The shikonin solution was maintained at 4 °C for over 1 year without water–oil dissociation, and it was stored as a form of dried, bright violet crystal for a long period. PVP is a microencapsulating solvent with a high molecular weight, and its water solution can adequately dissolve the shikonin crystals. The profile of this preparation did not differ from those of the preparations from the original ShD of *Arnebia euchroma* in thin-layer silicon G chromatography, indicating that the solubility of shikonin

Table 2. Blood biochemistry examinations of the rats treated with ShD.

Groups	TP g/L	ALB g/L	ALT U/L	ALP U/L	AST U/L	BUN mmol/L	CR umol/L	CHO mmol/L	Glu mmol/L	TBIL umol/L
90 days										
Normal control	67.08 ± 4.82	30.61 ± 2.09	46.42 ± 7.35	198.60 ± 63.47	205.30 ± 32.61	7.82 ± 0.75	60.37 ± 8.81	2.21 ± 0.25	7.10 ± 0.98	6.53 ± 0.77
Solvent control	67.19 ± 4.67	29.77 ± 2.70	46.33 ± 8.14	197.50 ± 61.43	201.70 ± 34.91	7.69 ± 0.86	59.27 ± 9.20	2.16 ± 0.35	7.02 ± 1.26	6.78 ± 0.76
ShD										
800 mg/kg	67.39 ± 4.30	30.24 ± 2.23	45.89 ± 7.19	200.50 ± 52.85	207.40 ± 53.24	7.83 ± 1.05	61.17 ± 11.07	2.08 ± 0.17	7.06 ± 1.01	6.67 ± 0.79
400 mg/kg	67.05 ± 5.78	31.02 ± 1.97	46.63 ± 7.26	198.50 ± 51.12	201.80 ± 46.93	7.68 ± 0.98	60.33 ± 11.55	2.05 ± 0.18	6.89 ± 0.99	6.71 ± 0.71
200 mg/kg	66.21 ± 4.23	29.99 ± 2.81	45.81 ± 8.94	203.70 ± 73.08	196.10 ± 37.03	7.79 ± 1.16	59.56 ± 13.20	1.99 ± 0.18	6.95 ± 1.14	6.60 ± 0.74
180 days										
Normal control	67.01 ± 4.23	30.92 ± 2.47	46.71 ± 10.99	203.66 ± 59.83	190.30 ± 65.35	7.74 ± 0.91	59.38 ± 12.11	2.11 ± 0.19	7.01 ± 0.89	7.15 ± 0.88
Solvent control	66.86 ± 3.18	31.83 ± 2.36	46.38 ± 11.71	202.70 ± 60.25	190.20 ± 62.05	7.61 ± 0.84	60.35 ± 12.02	2.11 ± 0.21	6.82 ± 0.92	7.02 ± 0.90
ShD										
800 mg/kg	66.13 ± 5.21	30.68 ± 2.37	46.09 ± 11.74	203.70 ± 60.79	190.40 ± 69.60	7.61 ± 0.69	60.17 ± 10.09	1.95 ± 0.18	7.06 ± 0.94	7.04 ± 0.93
400 mg/kg	65.86 ± 3.74	30.45 ± 2.90	46.10 ± 9.05	205.70 ± 70.17	191.70 ± 60.98	7.73 ± 0.77	58.28 ± 11.50	1.96 ± 0.21	6.92 ± 0.99	6.93 ± 0.59
200 mg/kg	67.36 ± 2.84	30.89 ± 2.04	45.56 ± 7.65	209.50 ± 65.64	192.10 ± 56.03	7.63 ± 0.88	57.87 ± 11.36	0.91 ± 0.20	6.90 ± 1.23	7.06 ± 0.65

TP, total protein; ALB, albumin; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CR, creatinine; CHO, cholesterol; Glu, glucose; TBIL, total bilirubin. All $p > 0.05$.

Table 3. Values of blood cell counts of rats treated with ShD.

Groups	WBC $\times 10^9/L$	Lymphocyte (%)	Monocyte (%)	PMNs (%)	RBC $\times 10^{12}/L$	HGB g/L	MCV	PLT $\times 10^9/L$
90 days								
Normal control	11.22 \pm 3.12	75.99 \pm 5.18	7.02 \pm 0.81	16.88 \pm 4.78	6.99 \pm 0.43	129.64 \pm 9.47	39.30 \pm 2.01	688.23 \pm 147.20
Solvent control	11.10 \pm 3.42	76.09 \pm 5.29	7.07 \pm 0.75	16.84 \pm 5.18	7.03 \pm 0.52	128.60 \pm 9.68	38.33 \pm 2.68	680.60 \pm 149.90
ShD								
800 mg/kg	11.10 \pm 3.72	75.87 \pm 4.50	7.15 \pm 0.79	16.98 \pm 4.65	7.01 \pm 0.53	131.30 \pm 9.82	38.64 \pm 3.04	700.10 \pm 138.10
400 mg/kg	11.70 \pm 3.47	75.69 \pm 5.03	7.12 \pm 0.69	17.19 \pm 4.88	7.04 \pm 0.45	134.50 \pm 10.30	38.26 \pm 2.80	689.00 \pm 157.00
200 mg/kg	11.30 \pm 2.93	75.18 \pm 4.86	7.25 \pm 1.07	17.57 \pm 4.17	7.02 \pm 0.44	131.20 \pm 8.35	38.21 \pm 1.73	683.10 \pm 148.90
180 days								
Normal control	11.40 \pm 3.15	75.47 \pm 5.27	7.22 \pm 0.84	16.52 \pm 4.88	6.81 \pm 0.61	132.75 \pm 0.86	38.43 \pm 3.77	693.78 \pm 128.40
Solvent control	11.60 \pm 4.15	76.69 \pm 4.92	7.19 \pm 0.80	16.12 \pm 4.62	6.88 \pm 0.66	133.90 \pm 0.09	38.26 \pm 3.27	688.70 \pm 125.90
ShD								
800 mg/kg	11.20 \pm 2.93	75.40 \pm 8.13	7.24 \pm 0.64	17.36 \pm 7.79	6.85 \pm 0.60	132.90 \pm 1.05	38.50 \pm 2.37	692.80 \pm 115.50
400 mg/kg	11.10 \pm 5.34	75.74 \pm 5.12	7.30 \pm 0.76	16.96 \pm 4.64	6.83 \pm 0.76	132.40 \pm 0.95	38.47 \pm 2.29	696.10 \pm 133.60
200 mg/kg	11.70 \pm 4.57	76.46 \pm 5.35	7.14 \pm 0.88	16.40 \pm 5.43	6.89 \pm 0.73	132.00 \pm 1.02	38.50 \pm 2.00	695.50 \pm 128.00

WBC, white blood cells; PMNs, polymorphonuclear leucocytes; RBC, red blood cell; HGB, hemoglobin; MCV, mean corpuscular volume; PLT, platelet. All $p > 0.05$.

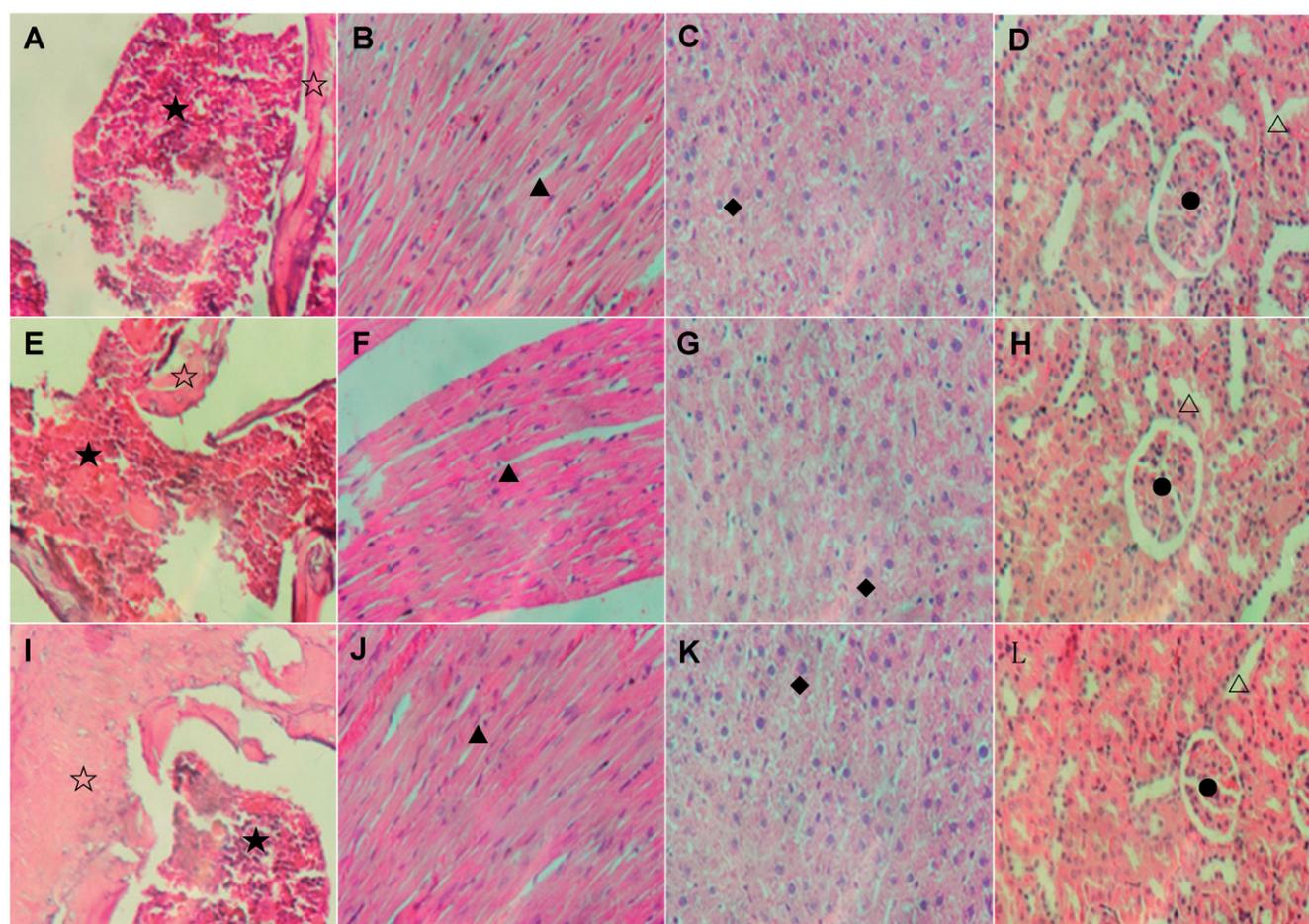


Figure 1. Pathological analyses of the vital organs of Wistar rats in different groups. Wistar rats of both ShD treated groups and control groups were kept for 90 or 180 days, then they were sacrificed and the organs were removed for pathological analyses. A to D: sternum, heart, liver and kidney of rats without treatment. E to H: sternum, heart, liver and kidney of rats treated with solvent. I to L: sternum, heart, liver and kidney of rats treated with ShD at the concentration of 800 mg/kg. No significant abnormality and difference were found among the different groups. *: bone marrow; ☆: bone; ▲: myocardial cell; ◆: hepatocyte; ●: glomerulus; △: renal tubule.

was improved without changes in the other physical properties of the shikonin fraction.

The systemic toxicity of shikonin *in vivo* remains unclear although *Arnebia euchroma* has been studied for decades. Thus, we performed this investigation to evaluate the long-term toxicity of ShD. In chronic toxicity studies, the top dose

should not exceed 1000 mg/kg body weight/day, and 2- to 4-fold intervals are frequently optimal for setting the descending dose levels. Therefore, we set 3 dose levels: 800, 400, and 200 mg/kg. The smallest daily dosage of the agent was 200 mg/kg, which was estimated to be 20-fold of that in the pharmacokinetic observation. For chronic toxicity studies of

biotechnology-derived pharmaceuticals, duration of 6 months has generally been accepted for regulatory approval (Clarke et al., 2008). As a result, we chose 6 months in the present study. Quality of life, hematological examinations, clinical biochemistry assessment, and organ pathology were investigated in this study.

Both hematological examinations and clinical biochemistry determinations are routinely performed in clinical practice because they are closely related to the selection, dose, and duration of the drug. Hence, it is very important to monitor hematological and biochemical changes during the development of a new drug, and measurement of these parameters is required for chronic toxicity studies, as instructed in the OECD guideline for testing of chemicals.

We observed no acute toxicity of ShD to mice in this study. In the chronic toxicity study, ShD did not affect either peripheral blood cells or bone marrow at a concentration as high as 800 mg/kg during the 6-month administration period. Additionally, ShD administration did not influence the functions of vital organs (e.g., the liver and kidney), as demonstrated by blood biochemistry and organ pathological analyses. These results suggest that ShD did not induce either hematological or non-hematological toxicity in Wistar rats.

Chemotherapy is one of the main therapeutic strategies for cancer, but it usually causes severe toxic effects such as leukopenia and other systemic discomforts. In previous studies, we demonstrated *in vitro* that ShD significantly inhibits proliferation of tumor cell lines such as SMMC-7721, HeLa, and HepA₂₂ at concentrations of 2.5–10 µg/mL, whereas it does not interfere with the growth of normal cell lines such as HEK93 and NIH3T3 at the same concentrations (Wang et al., 2007; Yan et al., 2007). Further, we found that ShD inhibits the growth of transplantable tumors *in vivo* as effectively as chemotherapy with improved life quality in Swiss mice (Su et al., 2012), which suggests that the mechanisms of shikonin differ from those of chemotherapy. Shikonin may inhibit the production of monokines such as tumor necrosis factor α (Staniforth et al., 2004) as well as down-regulate the expression of chemokine receptors (Chen et al., 2003). In conclusion, our results in an animal model indicate that ShD may be safe for usage *in vivo*.

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Declaration of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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