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Hematopoietic effect of small molecular fraction of *Polygoni multiflori* Radix Praeparata in cyclophosphamide-induced anemia mice

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[ABSTRACT] The aim of this study is to investigate the protective effects of a small molecular fraction (SMF) of *Polygoni multiflori* Radix Praeparata (PMRP) in a cyclophosphamide (CTX) induced anemia mouse model. Small molecular fraction of PMRP was prepared and identified by high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF-MS). In pharmacology, we examined the peripheral hemogram and thymus and spleen index. The content of granulocyte-macrophage colony-stimulating factor (GM-CSF) in serum was mensurated by enzyme-linked immunosorbent assay (ELISA); The level of superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (T-AOC), and malondialdehyde (MDA) in serum and spleen tissue homogenate were detected, and glutathione peroxidase (GSH-PX) was assayed in spleen. The results show that SMF can significantly accelerate the recovery of peripheral hemogram, increase the activity of antioxidant enzymes and GM-CSF in serum and spleen. SMF also increases the number of spleen cells, improves bone marrow pathology. In conclusion, the SMF of PMRP promoted the recovery of hematopoietic function in a CTX-induced anemia mouse, which can support SMF to be used as an adjunct to chemotherapy to counteract its side effects.

[KEY WORDS] *Polygoni multiflori* Radix Praeparata; Small molecular fraction; Cyclophosphamide; Hemopoiesis

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Introduction

Anemia is a common blood disorder involving a lack of healthy red blood cells or hemoglobin resulting from erythrocyte damage, decreased or faulty erythrocyte production, or blood loss [1]. Hematopoietic damage is a common side effect in the course of chemotherapy. Its specific manifestations are anemia such as leukopenia and thrombocytopenia, which often leads to the termination of radiotherapy and chemotherapy. Cyclophosphamide (CTX), a frequently-used chemotherapeutic agent for treating cancer, is sufficiently hemotoxic to deplete hematopoietic stem cells in the bone marrow and cause a reduction in circulating peripheral blood cells which results in aplastic anemia [2-5]. For the anemia caused by chemotherapeutic drugs, there is no specific medicine to improve the hemogram. Therefore, it is especially important to find drugs that can restore multiple hematopoietic cells.

Polygoni multiflori Radix Praeparata (PMRP) is the root of *Polygonum multiflorum* Thunb., a member of the family Polygonaceae [6-7]. The traditional effect of *Polygonum multiflorum* is believed to replenish liver and kidney, and benefit blood. The major ingredients of PMRP are stilbenes, quinones, flavonoids, and polysaccharides [7-9]. Pharmacological studies have shown that PMRP and its active ingredients can improve immune function [10], exhibit antioxidative and antiaging activity [11-13], and so on. The small molecular components such as flavonoid and phenolic acid constituents of PMRP exhibit significant antioxidative activity [14-16]. At present, it has been reported that polysaccharides isolated from PMRP can be used to alleviate chemotherapy-induced myelosuppression [17-21]. However, it is not clear whether the small molecular components in PMRP can inhibit myelosuppression. And since oxi-

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dative stress injury is an important pathological course of chemotherapy leading to bone marrow suppression, we speculate that the SMF of PMRP may alleviate anemia by regulating oxidative activity and restoring the function of hematopoietic organs.

So, this study investigated the therapeutic effects of SMF on hematopoiesis, oxidative stress, antioxidant enzyme activity, hematopoiesis-related cytokines, the thymus and spleen indexes, and other characteristics in CTX induced anemia model in mice. The components contained in the SMF were identified by HPLC-Q-TOF-MS. It is hoped that the results will support the use of *P. Multiflori* small molecular fraction as an adjunct to chemotherapy to counteract its side effects and to provide a reference for further studies of efficacy, mechanism, and possible clinical applications.

Material and Methods

Reagents

Colla corii Asini (CCA) was purchased from Shandong-huaxin Pharmaceutical Co., Ltd. (No. Z37021276, Shandong, China), cyclophosphamide was obtained from Jiangsu ShengDi Pharmaceutical Co., Ltd. (batch No. H32020857, Jiangsu, China). Granulocyte-macrophage colony-stimulating factor (GM-CSF, No. 20170915), total antioxidant capacity (T-AOC, No. 20170910), superoxide dismutase (SOD, No. 20170917), catalase (CAT, No. 20170915), thiobarbituric acid method malondialdehyde (MDA, No. 20170914), and glutathione peroxidase (GSH-PX, No. 20170915) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Small molecular fraction of Polygoni multiflori Radix Praeparata

Polygoni Multiflori (No. 201506001) was provided by Infinitus Co., Ltd. (Guangdong, China). The material was ground, passed through a 40-mesh sieve, and 100 g of the powder was extracted three times with hot distilled water (10 : 1, *V/W*) for 3 h. The polysaccharides were removed by alcohol precipitation. After concentration, the crude extract solution was adsorbed by D101 macroporous resin, eluted with water and then with ethanol. The ethanol eluent was concentrated under vacuum to recover the organic solvent and freeze dried to obtain SMF.

Identification of compound structures by HPLC-Q-TOF-MS/MS

Prepare the test sample solution by taking an appropriate amount of SMF. The composition of the lyophilized SMF powder was determined by high-performance liquid chromatography (HPLC), and the compounds are identified by quadrupole time-of-flight mass spectrometry (Q-TOF-MS/MS).

Chromatographic conditions: SHIMADZU LC-2010C liquid chromatograph (Kyoto, Japan). Including SHIMADZU quaternary pump, autosampler, column oven, SPD-M20A diode array detector and LC solution data workstation); analytical column was Agela Venusil MP C_{18} (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of (A) 0.1% phosphoric acid aqueous solution and (B) acetonitrile. The HPLC elution condition were optimized as follows: isocratic 6% B (0−6 min), linear gradient from 6% to 10% B (6−10 min), isocratic 10% B (10−15 min), 10%−15% B (15−25 min), 15%−20% B (25−45 min), 20%−30% B (45−70 min), 30%−35% B (70−75 min), 35%−60% B (75−85 min), 60%−65% B (85−90 min), and isocratic 65% B (90−100 min), 65%−100% B (100−105 min), and isocratic 100% B (105−110 min); Flow rate was 1.0 $mL \cdot min^{-1}$; Column temperature was 20 °C; detection wavelength was 254 nm; analysis time was 110 min; injection volume was 10 μL.

ESI-MS/MS analysis conditions: Agilent 1260-6530 HPLC-Q-TOF (Agilent, Singapore), negative ion mode scan. 0.1% phosphoric acid was replaced with 0.1% formic acid in the mobile phase. Ion source parameters: dry gas ion source parameters: dry gas (N2) temperature: 350 °C, flow rate, flow rate 9.0 $L \cdot min^{-1}$; Capillary voltage, 3300 V; atomization pressure; atomization pressure, 40 psi; scanning range *m/z* 100−1000.

Data acquisition was performed using Agilent HPLC-Q-TOF-MS Mass Hunter acquisition software B.04.00 software (Agilent, Santa Clara, CA, USA), and data processing was performed using Mass Hunter Workstation Qualitative Analysis Software B.06.00 software (Agilent, Santa Clara, CA, USA). The elemental composition determination parameters are set as follows: C, 0−50; H, 0−100; O, 0−50; N, 0−3; preliminary assumption that all compounds do not contain elements such as phosphorus, sulfur, bromine and chlorine; The allowable error range is $\pm 5 \times 10^{-6}$.

Animals and experimental design

Male ICR mouse weighing 18−20 g purchased from the YangZhou University Comparative Medical Center, license No. SCXK (su) 2012-0004. The mice were housed at 20 ± 2 ºC and a 12 h alternating light-dark cycle. Food and water were available ad libitum. After a 3-day acclimatization period, the mice were randomly allocated to six groups of nine mice each. One group included normal control (NC) mice. The remaining mice were myelosuppressed by daily intraperitoneal administration of 40 $mg \cdot kg^{-1} \cdot d^{-1}$ CTX for 5 days and then assigned to model control (MC), *Colla corii Asini* (CCA, positive controls), and SMF groups. Mice in SMF groups were given daily oral 2, 4 and 8 $g \cdot kg^{-1}$ doses for 9 days after the first injection of CTX. Mice in the CCA group were administered oral doses of 5 $g \cdot kg^{-1}$. Mice in the NC and MC groups were given saline at the same intervals. On 9th day, the animals were weighed, sacrificed, and the required samples were collected.

Samples collection

On completion of the experimental procedures, body weights were recorded, blood was collected from the orbital sinus into tubes with or without ethylenediaminetetraacetic acid (EDTA). Blood collected into tubes with EDTA was used for peripheral hemogram analysis with an automatic counter system at Sir Run Run Hospital of Nanjing Medical University. The hemogram included red blood cell (RBC), platelet (PLT), hematocrit (HCT), and hemoglobin (Hb) data. Blood collected in tubes without EDTA was centrifuged at 3000 r·min^{-1} for 15 min to obtain serum and stored at −20 ºC for further analysis of physiological and biochemical indexes. Mice were sacrificed by cervical dislocation; thymuses and spleens were removed, cleaned, and weighed. The weight relative to body weight was calculated as organ weight (mg)/body weight (g) and recorded as the organ index. The spleens were stored at −70 ºC for further analysis.

Determination of biochemical indicators

Serum GM-CSF was determined by ELISA following the manufacturer's instructions. Spleen tissue was homogenized in 0.1 g·mL^{-1} wet weight ice-cold physiological saline. The homogenates were centrifuged at 4 ºC for 10 min to obtain supernatants for performing the assays. The levels of T-AOC, SOD, CAT, and MDA in serum and spleen tissue supernatant were determined with commercially available colorimetric assay kits following the manufacturer's instructions.

Bone marrow and spleen histology

Spleen tissue (3 samples per group) was fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), embedded in paraffin, and sectioned at 5 μm for histological analysis. The tissue was stained with hematoxylin and eosin (H&E) and evaluated by light microscopy. Femurs (3 samples per group) were fixed in 4% paraformaldehyde in PBS, decalcified in formic acid-sodium citrate for 5 days, embedded in paraffin, and sectioned at 5 μm. The tissue was stained with H&E and the bone marrow was evaluated by light microscopy.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (San Diego, CA, USA). Data were expressed as means ± SD. One-way analysis of variance and Dunnett's post-hoc test was used for multiple group comparisons. P-values < 0.05 were considered statistically significant; P-values < 0.01 were considered statistically highly significant.

Results

HPLC chromatography and Q-TOF-MS/MS spectrometry identification

The composition of the lyophilized SMF powder was determined by HPLC, which confirmed the presence of stilbene glycosides and other ingredients, as shown in Fig. 1. The total ion current map obtained by qualitative analysis of SMF with HPLC-Q-TOF-MS/MS is shown in Fig. 2. By application of the rules of cleavage described in the literature, integrating the retention time data, and using UV spectrum characteristics, and MS ion fragmentation, 25 chromatographic peaks were identified and their structures were deduced (Fig. 3). The MS spectrometry results and cleavage pathways of the components of SMF are shown in Table 1.

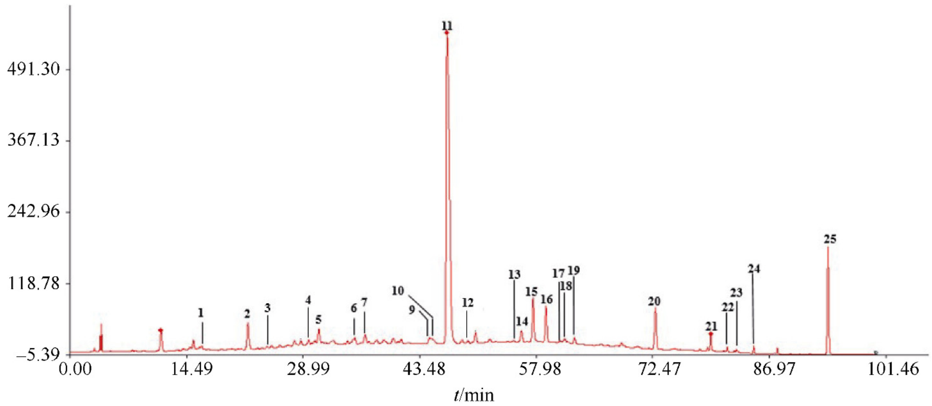

Fig. 1 HPLC chromatogram of SMF showing 25 peaks detected by an evaporative light scattering detector

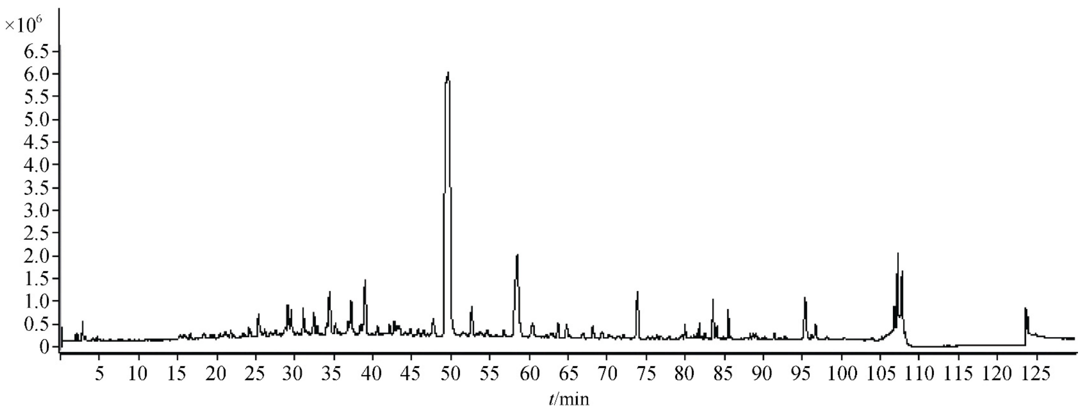

Fig. 2 Total ion flow diagram of SMF by HPLC-Q-TOF-MS/MS qualitative analysis

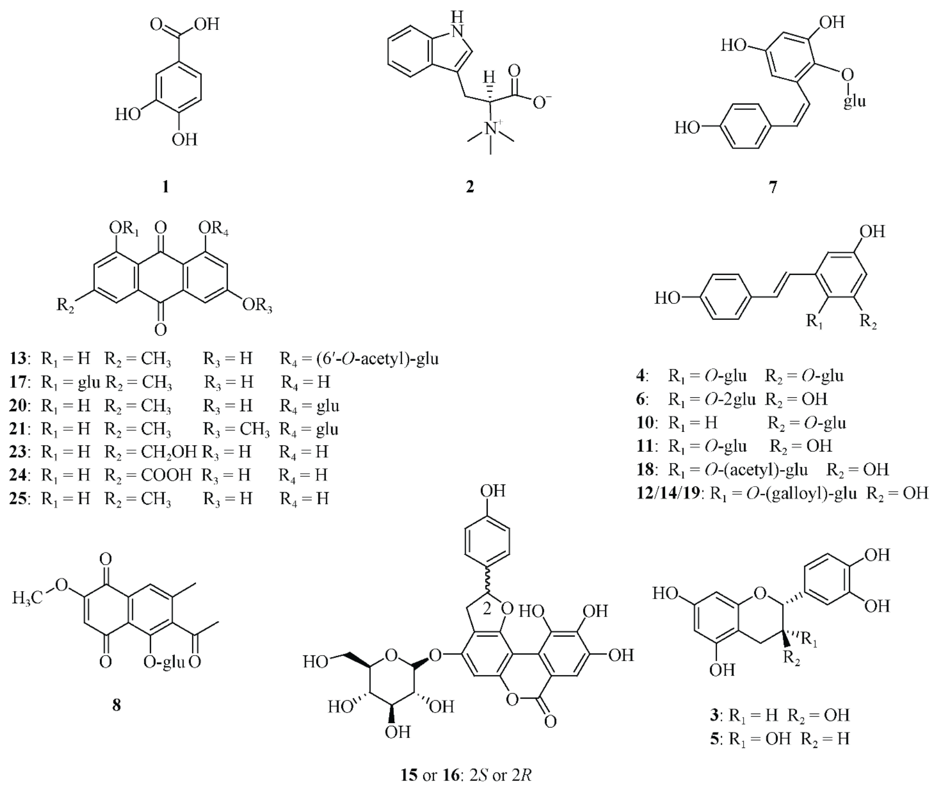

Fig. 3 Structural formulae of the chemical constituents of SMF

Table 1 Mass spectrometry data and cleavage pathways of the components of SMF

Peaks	t_R/min	Formula	MS (*m/z*)	MS/MS (*m/z*)	Error (× 10^{-6})	Identification
1	20.283	$C_7H_6O_4$	153.0189 $[M-H]^-$	109.0 $[M-H-CO_2]^-$	2.81	Protocatechuic acid
2	24.323	$C_{14}H_{18}N_2O_2$	245.1292 $[M-H]^-$	186.0, 142.0, 119.0	1.43	Hypaphorine
3	28.229	$C_{15}H_{14}O_6$	289.0712 $[M-H]^-$ 325.0479 $[M+Cl]^-$	137.0 $[M-H-C_8H_8O_3]^-$ 125.0 $[M-H-C_9H_8O_3]^-$	1.94	Catechin
4	31.764	$C_{26}H_{32}O_{14}$	613.1747 $[M+HCOO]^-$ 603.1476 $[M+Cl]^-$	405.1 $[M-H-Glu]^-$ 243.0 $[M-H-2Glu]^-$	1.77	Polygonimitin C
5	33.583	$C_{15}H_{14}O_6$	289.0710 $[M-H]^-$ 325.0471 $[M+Cl]^-$	137.0 $[M-H-C_8H_8O_3]^-$ 125.0 $[M-H-C_9H_8O_3]^-$	2.63	Epicatechin
6	36.343	$C_{26}H_{32}O_{14}$	567.1705 $[M-H]^-$ 613.1749 $[M+HCOO]^-$ 603.1458 $[M+Cl]^-$	405.1 $[M-H-Glu]^-$ 243.0 $[M-H-2Glu]^-$	2.52	2, 3, 5, 4'-Tetrahydroxystilbene-2-*O*-di-glucoside
7	38.228	$C_{20}H_{22}O_9$	405.1178 $[M-H]^-$	243.0 $[M-H-Glu]^-$ 215.0 $[M-H-Glu]^-$ 173.0 $[M-H-Glu-CH_2CO]^-$ 225, 197, 137, 149, 109	3.22	*cis*-2, 3, 5, 4'-Tetrahydroxy-stilbene-2-*O*-*β*-D-glucoside
8	41.730	$C_{20}H_{22}O_{10}$	421.1121 $[M-H]^-$ 467.1187 $[M+HCOO]^-$ 457.0891 $[M+HCOO]^-$	259.0 $[M-H-Glu]^-$ 241.0, 213.0549, 173.0602	1.89	2-Methoxy-6-acetyl-7-methyljuglone-glucoside
9	45.433	$C_{26}H_{28}O_{12}$	531.1482 $[M-H]^-$	405.1, 243.0, 161.0	4.89	Unknow
10	46.645	$C_{20}H_{22}O_8$	389.1224 $[M-H]^-$ 435.1278 $[M+HCOO]^-$ 425.0983 $[M+Cl]^-$	227.0 $[M-H-Glu]^-$ 185.0 $[M-H-Glu-C_2H_2O]^-$ 143.0 $[M-H-Glu-2C_2H_2O]^-$	4.59	Polydatin (picied)
11	48.531	$C_{20}H_{22}O_9$	405.1172 $[M-H]^-$	243.0 $[M-H-Glu]^-$ 215.0 $[M-H-Glu]^-$ 173.0 $[M-H-Glu-CH_2CO]^-$ 225, 197, 137, 149, 109	4.69	*trans*-2, 3, 5, 4'-Tetrahydroxy-stilbene-2-*O*-*β*-D-glucoside

Continued

Peaks	t_R/min	Formula	MS/(*m/z*)	MS/(*m/z*)	Error (× 10^{-6})	Identification
12	51.325	$C_{27}H_{26}O_{13}$	557.1276 $[M-H]^-$	405.1 $[M-H-C_7H_4O_4]^-$ 243.0 $[M-H-C_7H_4O_4-Glu]^-$ 313.0, 169.0	4.42	2, 3, 5, 4'-Tetrahydroxy-stilbene-2-*O*-(galloyl)-*β*-D-glucoside
13	55.870	$C_{23}H_{22}O_{11}$	473.1070 $[M-H]^-$	311.0 $[M-H-Glu]^-$	4.08	Emodin-8-*O*-(6'-*O*-acetyl)-*β*-D-glucoside
14	56.984	$C_{27}H_{26}O_{13}$	557.1273 $[M-H]^-$	405.1 $[M-H-C_7H_4O_4]^-$ 243.0 $[M-H-C_7H_4O_4-Glu]^-$ 313.0, 169.0	4.95	2, 3, 5, 4'-Tetrahydroxy-stilbene-2-*O*-(galloyl)-*β*-D-glucoside
15	57.991	$C_{27}H_{24}O_{13}$	555.1118 $[M-H]^-$	393.0 $[M-H-Glu]^-$ 349.0 $[M-H-Glu-CO_2]^-$	4.7	Polygonumosides A or B
16	59.675	$C_{27}H_{24}O_{13}$	555.1117 $[M-H]^-$	393.0 $[M-H-Glu]^-$ 349.0 $[M-H-Glu-CO_2]^-$	4.88	Polygonumosides A or B
17	61.863	$C_{21}H_{20}O_{10}$	431.0964 $[M-H]^-$	269.0 $[M-H-Glu]^-$ 241.0 $[M-H-Glu-CO]^-$ 225.0 $[M-H-Glu-CO_2]^-$ 197.0 $[M-H-Glu-CO_2-CO]^-$	4.56	Emodin-1-*O*-*β*-D-glucoside
18	62.806	$C_{22}H_{24}O_{10}$	447.1277 $[M-H]^-$	243 $[M-H-Glu-CO-CH_3]^-$ 225, 197, 173, 137, 109	4.4	2, 3, 5, 4'-Tetrahydroxy-stilbene-2-*O*-(acetyl)-*β*-D-glucoside
19	63.983	$C_{27}H_{26}O_{13}$	557.1285 $[M-H]^-$	405.1 $[M-H-C_7H_4O_4]^-$ 243.0 $[M-H-C_7H_4O_4-Glu]^-$ 313.0, 169.0	2.8	2, 3, 5, 4'-Tetrahydroxy-stilbene-2-*O*-(galloyl)-*β*-D-glucoside
20	73.209	$C_{21}H_{20}O_{10}$	431.0965 $[M-H]^-$	269.0 $[M-H-Glu]^-$ 241.0 $[M-H-Glu-CO]^-$ 225.0 $[M-H-Glu-CO_2]^-$ 197.0 $[M-H-Glu-CO_2-CO]^-$	4.33	Emodin-8-*O*-*β*-D-glucoside
21	79.707	$C_{22}H_{22}O_{10}$	481.0885 $[M+Cl]^-$ 445.1118 $[M-H]^-$	283.0 $[M-H-Glu]^-$ 240.0 $[M-H-Glu-CH_3-CO]^-$ 212.0 $[M-H-Glu-CH_3-2CO]^-$	4.93	Physcion-8-*O*-*β*-D-glucoside
22	82.266	$C_{16}H_{10}O_7$	313.0343 $[M-H]^-$	269.0 $[M-H-CO_2]^-$ 241.0 $[M-H-CO_2-CO]^-$ 225.0 $[M-H-2CO_2]^-$	3.43	Carboxyl emodin
23	83.411	$C_{15}H_{10}O_6$	285.0394 $[M-H]^-$	257.0 $[M-H-CO]^-$ 241.0 $[M-H-CO_2]^-$ 226.0 $[M-H-CO_2-CH_2O]^-$	3.71	Citreorosein
24	85.397	$C_{15}H_8O_7$	299.0188 $[M-H]^-$	255.0 $[M-H-CO_2]^-$ 227.0 $[M-H-CO_2-CO]^-$ 199.0 $[M-H-CO_2-2CO]^-$	3.09	Hydroxyl rhein
25	95.935	$C_{15}H_{10}O_5$	269.0445 $[M-H]^-$	241.0 $[M-H-CO]^-$ 225.0 $[M-H-CO_2]^-$ 181.0 $[M-H-2CO_2]^-$ 210.0 $[M-H-CO_2-CH_3]^-$	–0.19	Emodin

Table 2 Effects of SMF on hematological parameters (mean ± SD, *n* = 9)

Group	RBC (× 10^{12}/L)	Hb (g·L^{-1})	HCT (%)	PLT (× 10^9/L)
NC	8.8 ± 0.4	141.3 ± 6.4	56.1 ± 2.5	1355.0 ± 187.1
MC	7.1 ± 0.2$^{###}$	117.1 ± 5.4$^{###}$	47.2 ± 3.3$^{###}$	825.9 ± 186.3$^{###}$
SMF (2 g·kg^{-1})	7.5 ± 0.4*	121.6 ± 5.0	48.8 ± 2.1	870.0 ± 158.7
SMF (4 g·kg^{-1})	7.7 ± 0.4**	124.2 ± 4.0*	50.6 ± 2.0*	994.8 ± 142.6*
SMF (8 g·kg^{-1})	7.9 ± 0.3***	126.7 ± 6.0**	50.1 ± 2.6	991.6 ± 93.9*
CCA	7.7 ± 0.5**	124.8 ± 6.8*	51.0 ± 2.8*	1027.0 ± 141.2*

$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* normal control mice (NC); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* model control mice (MC), CCA (positive control mice)

Effects of SMF on peripheral blood

As shown in Table 2, the RBC and PLT counts, Hb concentration, and HCT were less in the MC group than in the NC group ($P < 0.001$). The RBC counts were higher in mice treated with 2 ($P < 0.05$), 4 ($P < 0.01$), and 8 ($P < 0.001$) g·kg^{-1} SMF compared with the MC mice. Hb concentrations and PLT counts were higher in mice treated with 4 ($P < 0.05$) and 8 ($P < 0.01$) g·kg^{-1} SMF than in the MC mice. Improvements in hematological parameters was better with 4 and 8 g·kg^{-1} than with 2 g·kg^{-1} SMF.

Effects of SMF on thymus and spleen indexes

As shown in Fig. 4, the thymus and spleen indexes were both much lower in MC mice than in NC mice ($P < 0.001$). Compared with MC mice, SMF did not reverse thymus atrophy, but at each of the tested concentrations it did reverse the CTX-induced decrease in the spleen index ($P < 0.001$).

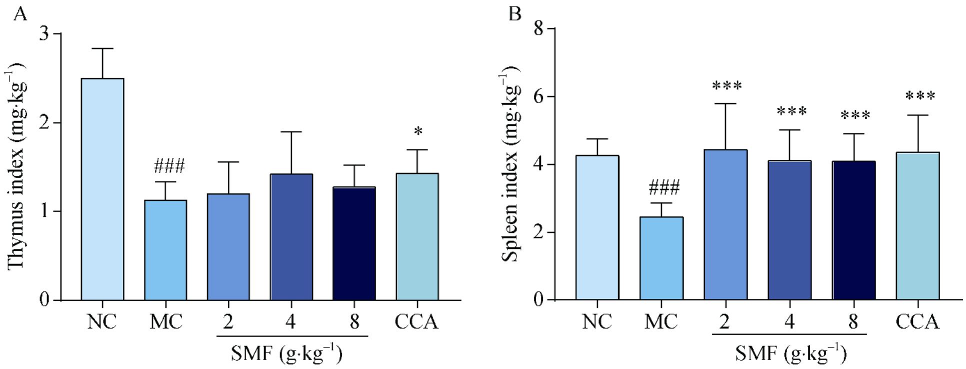

Fig. 4 Effects of SMF on the thymus and spleen indexes in mice with CTX-induced anemia. (n = 9, mean ± SD). A: thymus index; B: spleen index. $^{###}P$ < 0.001 *vs* NC mice; $^{*}P$ < 0.05, $^{***}P$ < 0.001 *vs* MC mice, CCA positive control group

Effects of SMF on GM-CSF hematopoiesis-related cytokines

As shown in Fig. 5, the expression of GM-CSF in serum was inhibited by CTX in MC than in NC mice (P < 0.01), and treatment with 4 or 8 g·kg^{-1} SMF increased serum GM-CSF concentration (P < 0.05), compared with MC mice. Serum GM-CSF was increased by treatment with 2 g·kg^{-1} SMF compared with the MC group but the difference was not significant (P > 0.05).

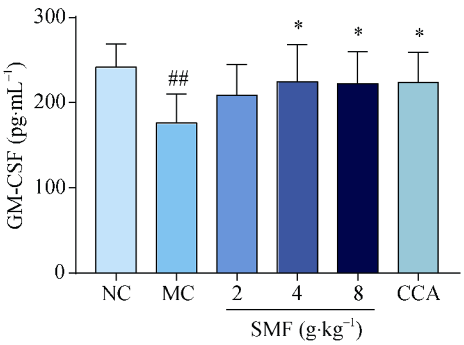

Fig. 5 Effects of SMF on serum GM-CSF in mice with CTX-induced anemia (n = 9, mean ± SD). $^{##}P$ < 0.01 *vs* NC mice; $^{*}P$ < 0.05 *vs* MC mice, CCA positive control group

Effects of SMF on serum CAT, SOD, T-AOC, and MDA

As shown in Fig. 6, CTX significantly changed the serum concentrations of the antioxidants CAT, SOD, T-AOC, and MDA (P < 0.01 or P < 0.001) compared with NC mice. SMF at 2, 4, and 8 g·kg^{-1} increased total antioxidative activity (T-AOC) and the antioxidative activities of CAT and SOD. SMF decreased the accumulation of serum MDA in a dose-dependent manner.

Effects of SMF on CAT, SOD, T-AOC, and MDA in spleen tissue

As shown in Fig. 7, five oxidation-related indicators in spleen tissue were significantly modified by CTX (P < 0.01 or < 0.001) in the NC mice. SMF at 2 and 4 g·kg^{-1} increased SOD and CAT concentration (P < 0.05) and increased SOD, CAT, and GSH-PX concentration at 8 g·kg^{-1} SMF (P < 0.01 or < 0.001) compared with the MC mice. T-AOC activity decreased significantly with CTX-treatment, and SMF at 4 and 8 g·kg^{-1} elevated T-AOC activity. SMF (2 g·kg^{-1}) had a trend of increasing the level of T-AOC in the spleen. SMF decreased the accumulation of spleen MDA in a dose-dependent manner. These results show that SMF ameliorated the oxidative response in MC mice by increasing T-AOC and enhancing antioxidant activity in the spleen tissue of model mice.

Effects of SMF on spleen histopathology

H&E staining (Fig. 8) revealed a dose-dependent effect of SMF on spleen histopathology. A small number of megakaryocytes were observed in the spleen of the blank group, which is a common morphological state in mice. There were fewer spleen bodies in the model group than that in the blank group[0], and the megakaryocytes in the spleen sinus were small. With the increase of concentration in the drug-administered group, the number of spleen bodies gradually returned to normal, and the number of megakaryocytes in the spleen sinus also increased. The number of spleen bodies in the positive drug group was not significantly different from that in the blank group, and moderate or multi-quantity megakaryocytes were observed in the spleen sinus.

Effects of SMF on bone marrow histopathology

As shown in Fig. 9, the trabecular meshwork of the femoral spongy network of the blank group of mice is the hematopoietic cells (erythrocytes, white blood cells and platelets), macrophages, adipocytes or mesenchymal cells, as well as abundant sinusoids. The number of platelets in white blood cells, red blood cells and bone marrow in the model group was significantly reduced compared with the blank group. The number of red blood cells and platelets in the treatment with 2 and 4 g·kg^{-1} SMF group was only slightly reduced, and there was no abnormality in the hematopoietic cells of the system in the 8 g·kg^{-1} group. There were no abnormalities in hematopoietic cells in the positive drug group, and moderate amounts of fat cells were observed.

Discussion

P. Multiflori is generally considered effective in improving hematospermia, supporting liver and kidney function, and strengthening bones and muscles. However, the hematopoietic function and antioxidants of small molecules such as anthraquinone is not clear, so we did this research to explore the

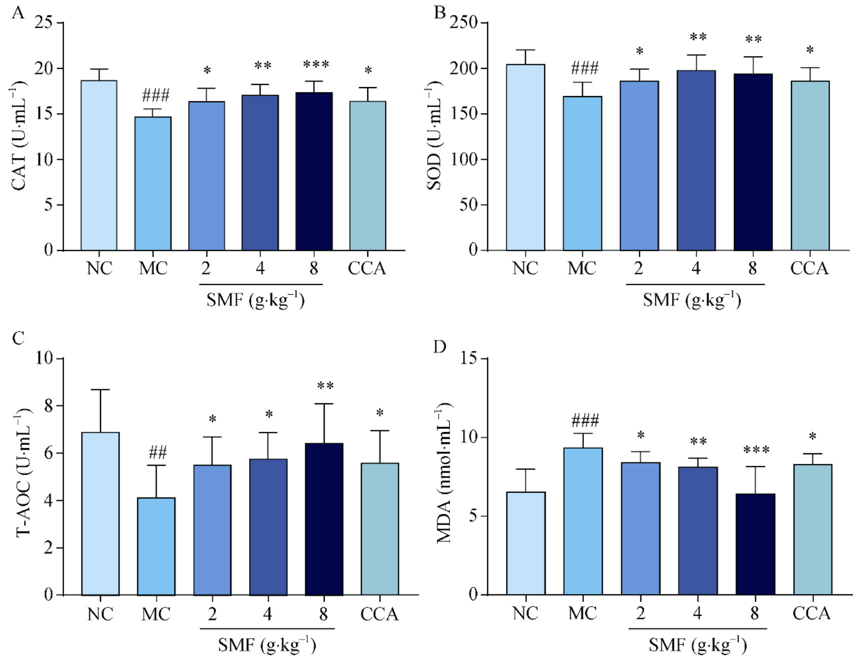

Fig. 6 Effects of SMF on serum antioxidants in mice with CTX-induced anemia (n = 9, mean ± SD). A: CAT; B: SOD; C: T-AOC; D: MDA. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* NC mice; $^{*}P < 0.05$, $^{***}P < 0.001$ *vs* MC mice, CCA positive control group

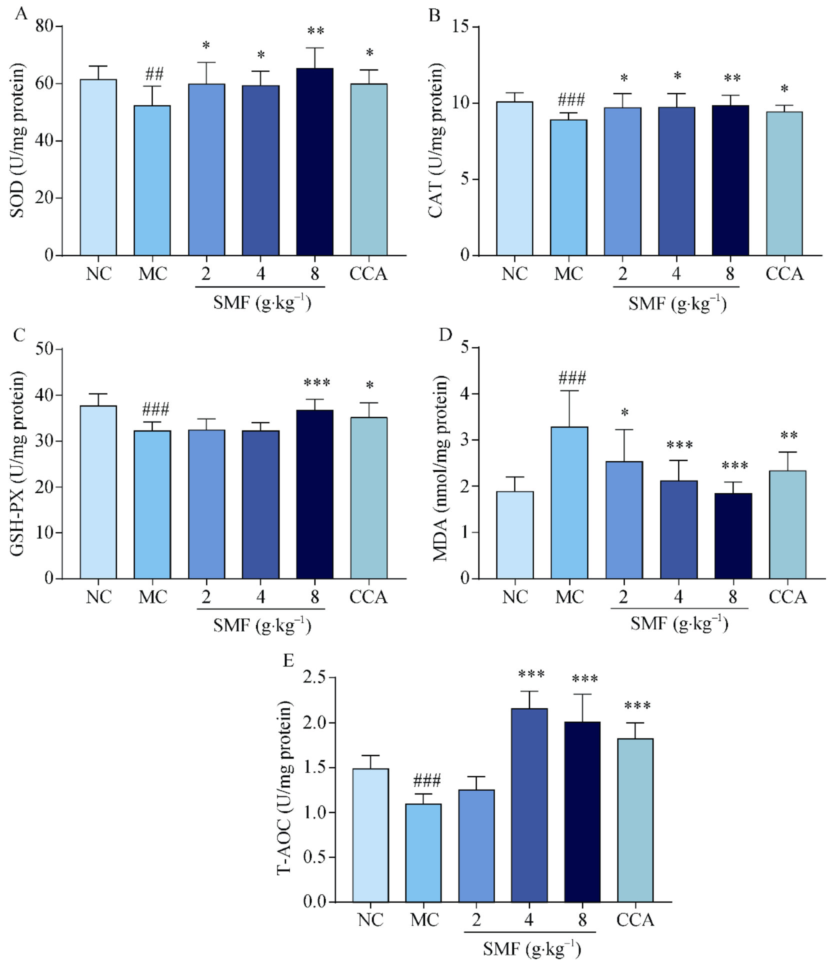

Fig. 7 Effects of SMF on spleen antioxidant profiles in mice with CTX-induced anemia (n = 9, mean ± SD). A: SOD; B: CAT; C: GSH-PX; D: MDA; E: T-AOC. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *vs* NC mice; $^{*}P < 0.05$, $^{***}P < 0.001$ *vs* MC mice, CCA positive control group

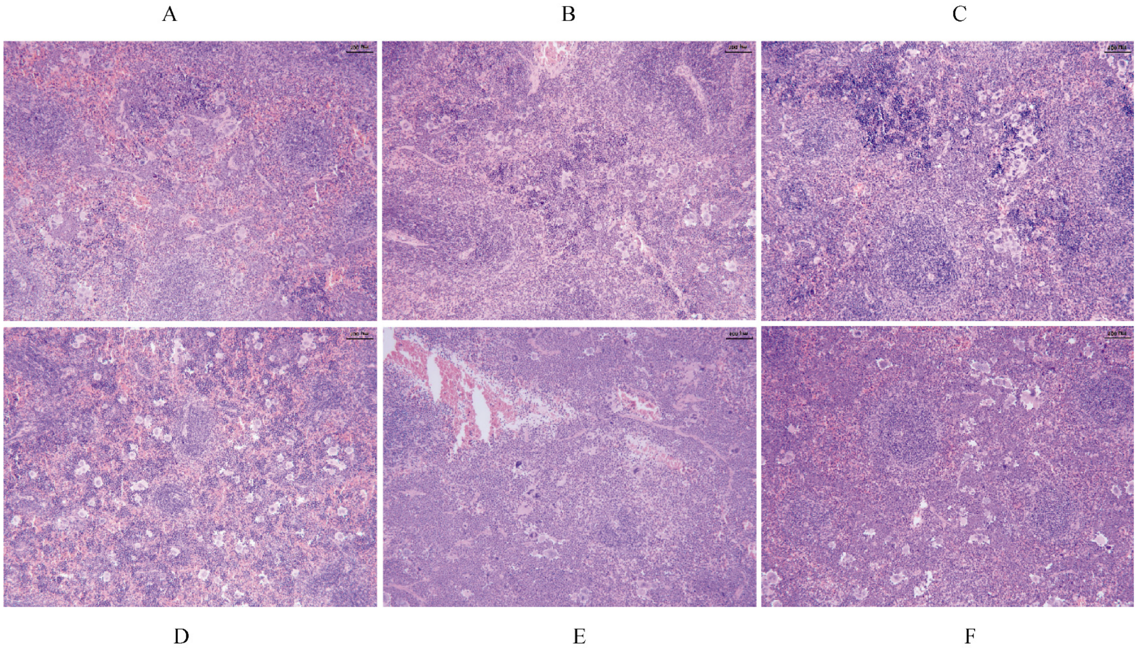

Fig. 8 Effects of SMF on spleen histology in mice with CTX-induced anemia (H&E staining × 100). A: NC mice; B: MC mice; C: SMF (2 g·kg^{-1}); D: SMF (4 g·kg^{-1}); E: SMF (8 g·kg^{-1}); F: CCA positive control group

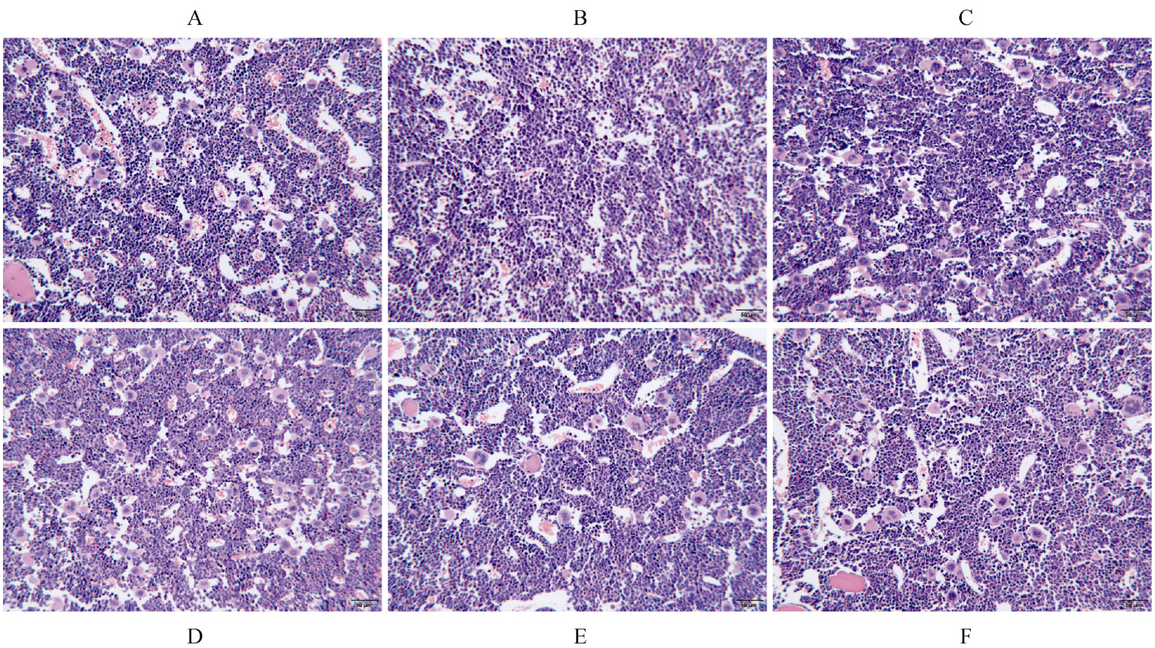

Fig. 9 Effects of SMF on the femur bone marrow histology in mice with CTX-induced anemia (H&E staining × 200). A: NC mice; B: MC mice; C: SMF (2 g·kg^{-1}); D: SMF (4 g·kg^{-1}); E: SMF (8 g·kg^{-1}); F: CCA positive control group

recovery of aplastic anemia in small molecules. Cyclophosphamide is a commonly used inducer of anemia animal models that mimics chronic aplastic anemia by inhibiting bone marrow hematopoietic hyperplasia. Therefore, this experiment used a cyclophosphamide-induced aplastic anemia mouse as a model animal. The protective effect of SMF on CTX-induced anemia was studied by monitoring hematopoietic activity [22].

SMF can directly improve peripheral blood, improve the symptoms caused by anemia by increasing RBC and PLT counts, Hb concentration and HCT. GM-CSF is a hematopoiesis-related cytokine that promotes the proliferation of early myeloid progenitor cells and stimulates myeloid progenitors to differentiate into neutrophils and monocytes [23]. This study also found SMF increased the secretion of GM-CSF in serum, and the improvement was dose-dependent. It can be speculated that one of the ways in which SMF can improve anemia is to increase the amount of GM-CSF, thereby improving hematopoiesis.

SMF can increase the spleen index, but it does not reverse the thymic atrophy of model mice. We all know that the spleen participates in the regulation of hemopoiesis [24]. There are still a small number of hematopoietic stem cells in the adult spleen. When the animal is severely ischemic or under certain pathological conditions, hematopoietic function can be restored, and red blood cells, granulocytes and platelets are produced. From the results, we know that SMF can significantly increase the spleen index and promote hematopoiesis.

In this study, it can be also seen from pathological sections that SMF increased the number of spleen cells and the proportion of megakaryocytes in the spleen, which promoted the recovery of spleen function after CTX injury. The bone marrow has hematopoiesis, immunity and defense functions. Bone marrow includes hematopoietic cells at different stages of maturity [17] and the bone marrow toxicity of CTX was reduced by SMF, which increased the number of bone marrow hematopoietic cells and promoted the recovery of function.

In addition, in the aspect of oxidative stress, CTX cytotoxicity includes free radical production and increased oxidative stress [25]. CTX damages the antioxidant system, decreases antioxidant enzymes including SOD, CAT, and GSH-PX activities, T-AOC level, and it augments MDA activity, which reflects the level of oxidative stress [26-27]. When the content of GSH and SOD in the body decreases, the oxidative damage of excessive free radicals generated by external stimulation or cell aging in the body cannot be effectively inhibited, and common diseases and various aging diseases are generated. Oral SMF increased T-AOC and antioxidant enzyme activity in the spleen and serum. This indicates that SMF can reduce the stimulation of cyclophosphamide and other chemotherapeutic drugs on the body while reduce the side effects, and restore hematopoiesis, thereby enhancing the body's ability to resist aplastic anemia caused by chemotherapy drugs.

All of these results suggested that SMF improved anemia by reversing the inhibition of antioxidant enzyme activity and promoting hematopoiesis following CTX administration.

Conclusion

Anemia has many causes including an imbalance of oxidative metabolism [28-29]. In this study, SMF increased antioxidant capacity and promoted recovery from CTX-induced anemia. It also improved hematological parameters, enhanced antioxidant profiles and reduced the pathological damage of the spleen and bone marrow, improving the anemia induced by CTX. The results showed that SMF has potential as an agent to protect against chemotherapy-induced anemia. It is hoped that this study will support the use of *P. Multiflori* small molecular fraction as an adjunct to chemotherapy to counteract its side effects and to provide a reference for further studies of efficacy, mechanism, and possible clinical applications.

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