

Compatibility effects of ginseng and Ligustrum lucidum Ait herb pair on hematopoietic recovery in mice with cyclophosphamide-induced myelosuppression and its material basis

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Research Article

Compatibility effects of ginseng and *Ligustrum lucidum* Ait herb pair on hematopoietic recovery in mice with cyclophosphamide-induced myelosuppression and its material basisJiahong Han[☆], Min Dai[☆], Yan Zhao, Enbo Cai^{*}, Lianxue Zhang^{**}, Xiaohuan Jia, Nian Sun, Xuan Fei, Hui Shu

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ABSTRACT

Background: Ginseng (G) and *Ligustrum lucidum* Ait (LLA) are core traditional Chinese medicines in treating myelosuppression formula. The present study was designed to profile effect of G and LLA herb pair (G-LLA) on myelosuppressed mice.

Methods: The mice myelosuppression model was established by intraperitoneal (i.p.) injection of cyclophosphamide (Cy). Hematopoietic function of bone marrow was measured by hemopoietic progenitor cell culture and peripheral blood count, and serum hemopoietic factors were tested by enzyme-linked immunosorbent assay. Bone marrow cell cycle was performed by flow cytometry. HPLC was used to measure 20 potential chemical components related to myelosuppression, including ginsenoside Rg₁, Re, Rb₁, Rc, Rb₂, Rb₃, Rd, Rk₃, Rh₄, 20 (S)-Rg₃, 20 (R)-Rg₃, Rk₁, Rg₅, salidroside, and so on.

Results: G, LLA, and G-LLA improved the amount of peripheral blood cells and bone marrow cells of myelosuppressed mice ($P < 0.01$). They significantly increased the colony quantity of colony-forming unit–granulocyte macrophage, burst-forming unit–erythroid, colony-forming unit–erythroid, and colony-forming unit–megakaryocyte and amount of G₂/M and S phase cells ($P < 0.01$). They also significantly decreased the amount of hematopoiesis-related cytokines ($P < 0.01$). The content of chemical components in G-LLA changed, and the change of rare saponin was the most obvious.

Conclusion: These results show that G-LLA herb pair might produce synergistic or complementary compatibility effects on bone marrow suppression after chemotherapy. It suggests that the substance basis of G-LLA for treating bone marrow suppression may be effective chemical components.

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1. Introduction

Cancer is a broad group of diseases involving unregulated cell growth. In cancers, cells divide and grow uncontrollably, forming malignant tumors, which may invade nearby parts of the body [1]. It is recognized internationally as one of the major diseases leading to human death, seriously threatening human life and social development. Chemotherapeutic drugs are essential tools in malignancy treatment [2]. Cy is one of the alkylating agents that are used most extensively [3]. However, its treatment is often accompanied by serious side effects [4]. Myelosuppression, for one, is

serious in that it may cause serious morbidity and mortality problems [5], making stimulation of hematopoiesis an important issue in clinical cancer treatments. After high doses of chemotherapy, the patients almost invariably suffer severe pancytopenia. The severe neutropenia resulted acquisition of life-threatening infections. Postchemotherapy-marked thrombocytopenia may lead to serious bleeding. Significant pancytopenia may also delay onset of the next cycle of chemotherapy [6,7].

Traditional Chinese Medicines (TCMs) have been used for thousands of years in China. Their less side effect, proven efficacy, and low cost have been considered valuable as complementary or

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alternative medicines for health care and treatment of diseases worldwide [8,9]. Chinese herbal formulas are the main form of TCM clinical application [10]. Herb pairs are the minimum units of TCM formulae, which are formed by only two herbs. It is not a random combination of two herbs, nor is the simple accumulation of efficacy, but the simple and delicate experience of ancient Chinese medicine practitioners. Essentially, herb pairs exert the role of reducing toxicity and enhancing efficacy by drug–drug interaction [11]. Owing to the functional characteristics of multicomponents and multitargets [12], studying the material basis of herb pair can help to analyze the theory of TCM compatibility and its pharmacological action mechanism [13].

Ginseng (G) has been used as a therapeutic medicine for thousands of years. Its traditional function is to replenish qi. Now, research shows that it may be useful for treating cardiovascular diseases, cancers, tumors, and chronic metabolic syndromes such as diabetes [14–16]. The most pharmaceutically active constituents found in G is ginsenoside [17]. *Ligustrum lucidum* Ait (LLA) is a Yin tonic. It has immunomodulatory and antitumor effects [18]. One of the main active ingredients is salidroside [19]. These two TCMs are widely used in prescriptions for treating myelosuppression, for example, Sijunzi decoction [20] and Buxue Shengbai decoction [21]. Antitumor Shengbai tablet is also involved in these two TCMs [22]. G and LLA herb pair can invigorate qi and supplement blood and nourishing yin, both qi and blood, Yin and Yang.

Based on the aforementioned fact, we inferred that the herb pair G and LLA (G-LLA) may result in an improved bone marrow hematopoietic function. To test our hypothesis, we studied the effects of G, LLA, and G-LLA on hematopoietic function of bone marrow, including peripheral blood and bone marrow nucleated cell (BMNC) counts, and the immune organs in Cy-induced myelosuppression mice. Meanwhile, we detected the proliferation and differentiation of hematopoietic progenitor cells, hematopoiesis-related cytokines factor content, and bone marrow cell cycle. In addition, the material basis of G-LLA was determined by HPLC.

2. Materials and methods

2.1. Chemicals and materials

Ginsenoside Rg₁, Re, Rf, Rb₁, Rg₂, Rc, Rb₂, Rb₃, Rd, Rk₃, F₂, Rh₄, 20 (S)-Rg₃, 20 (R)-Rg₃, Rk₁, Rg₅, Rh₂, compound K, Protopanaxadiol (PPD), Protopanaxatriol (PPT), salidroside, tyrosol, hydroxytyrosol, ligustroflavone, and specnuezhenide were obtained from Aladdin (Shanghai, China) or Yuanye (Shanghai, China).

2.2. Preparation of herb extracts

G-LLA (100 g) was soaked in 1 L of water for 60 min before boiling for 60 min. An aqueous extract was obtained by filtration. Water was added and cooked twice, and then, all filtrates were combined and concentrated. Single-herb extracts were prepared in the same way, and each concentrate was stored at 4°C until use. In this study, the leaching rates of G, LLA, and G-LLA were 52%, 20%, and 35.36% respectively.

2.3. Animals and groups

Male Balb/c mice (weighing 18–22 g) were obtained from the Laboratory Animal Quality Testing Center of Jilin Province (Certificate No. SCXK-2016-0003). All mice were adapted to the new laboratory 7 days before the experiment and maintained at controlled temperature (22 ± 2°C) and humidity (50 ± 10%) with a 12-h/12-h light/dark cycle. All efforts were made to minimize the pain of animals. The animal procedures were performed in accordance

with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of China Academy of Chinese Medical Sciences.

2.4. Treatment and experimental design

After one-week environmental adaptation period, mice were randomly divided into six groups: control, Cy, G (Ginseng), LLA (*Ligustrum lucidum* Ait), G-LLA (Ginseng and *Ligustrum lucidum* Ait), and positive (Zhenqi Fuzheng granules). All the groups except the control group were injected intraperitoneally with Cy (100 mg·kg⁻¹, 0.1 mL·10 g⁻¹) once daily for 3 continuous days. Positive, G, LLA, and G-LLA groups were daily administered intragastrically with equal volume Zhenqi Fuzheng granules (4.5 g·kg⁻¹), G (0.52 g·kg⁻¹), LLA (0.3 g·kg⁻¹), and G-LLA (0.884 g·kg⁻¹), respectively, at 9:00 AM for 12 days, whereas control and Cy groups received an equivalent normal saline (0.9% NaCl aq) for 12 days.

2.5. Index detection

2.5.1. Determination of peripheral blood cells

Twenty-four hours after the last administration, blood was collected on the day of sacrifice by retroorbital bleed into EDTA-K₂ tubes using an automatic blood analyzer to detect white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), and platelet (PLT).

2.5.2. Karyocyte count

Double femurs were removed under aseptic conditions, and bone marrow cells were flushed using sterile phosphate-buffered saline (PBS) to form a single-cell suspension. The suspension was placed into a centrifuge tube and centrifuged for 10 min at 1200 r·min⁻¹. Two milliliters of RBC lysis buffer was added, let undisturbed for 3 minutes after blending, and centrifuged for 10 min at 1200 r·min⁻¹. The bone marrow karyocytes were removed and rinsed twice with 1 mL of sterile PBS for 10 min at 1200 r·min⁻¹ and then resuspended in sterile PBS and counted using an inverted microscope.

2.5.3. Calculation of immune organ indices

Mice were sacrificed 24 hours after the last intragastric administration. Then, the thymus and spleen of mice of each group were removed and weighed, and the following index was calculated. The spleen and thymus indexes were calculated using the following formula:

$$\text{Spleen index (\%)} = (\text{spleen weight} / \text{body weight}) \times 100\%$$

$$\text{Thymus index (\%)} = (\text{thymus weight} / \text{body weight}) \times 100\%$$

2.5.4. Bone marrow culture of colony formation

After the BMNC count was adjusted to the desired concentration, the colony-forming unit–granulocyte macrophage (CFU-GM), the colony-forming unit–megakaryocyte (CFU-Meg), colony-forming unit–erythroid (CFU-E), and burst-forming unit–erythroid (BFU-E) were cultured in a semisolid culture system using mercapto alcohol, 3% L-glutamine, horse serum, recombinant murine granulocyte macrophage-colony stimulating factor (rmGM-CSF) (50°ng·mL⁻¹), recombinant human erythropoietin (rhEPO) (20°u·mL⁻¹), recombinant murine Interleukin-3 (rmIL-3) (20°ng·mL⁻¹), recombinant murine Thrombopoietin (rmTPO) (5°ng·mL⁻¹), BMNCs, Iscove's modified Dulbecco's medium (IMDM), and 3% agar. There were three parallel groups in each

group cultivated at 37°C, 5% CO₂, and saturated humidity. On the 4th day of culture, CFU-E cell colonies were counted under an inverted phase contrast microscope (8–32 cells were taken as one colony), and on the 8th day of culture, BFU-E cell colonies (50 cells were taken as one colony), CFU-GM cell colonies (50 cells were taken as one colony), and CFU-Meg cell colonies (3 cells were taken as one colony) were counted under an inverted phase contrast microscope.

2.5.5. Cytokine assay

The levels of cytokine GM-CSF, erythropoietin (EPO), and thrombopoietin (TPO) were determined by colorimetry according to the manufacturer-provided procedure. The colorimetry was read using an enzymatic reader using the mouse enzyme-linked immunosorbent assay kit according to the manufacturer's instructions.

2.5.6. Cell cycle

Single bone marrow cell suspension was centrifuged (1 000 r/min, 10 min). The supernatant was removed, and precipitated cells were washed twice with PBS, and then 2 mL of 70% cold ethanol was added to fix the cells overnight at 4°C. The cells were then dyed with propidium iodide staining solution (protection from light for 30 min). Then, cell cycle was detected by flow cytometry. Data of every cell cycle phase were calculated in percentage.

2.6. HPLC analysis: determination of main chemical components in G, LLA, and G-LLA

2.6.1. Sample preparation

Samples mentioned in section 2.2 were taken in a 100-ml conical flask and extracted with 50 ml of methanol at room temperature (30°C) once for 1 h. When the extraction was completed, the extract was filtrated and transferred into a 50-ml conical flask. Twenty-five milliliters of the extract was taken in an evaporating dish, and the extract was concentrated under reduced pressure of -0.09 MPa at 80°C. The residue was transferred to a 5-ml volumetric flask, diluted to the desired volume with methanol, and then filtered for HPLC analysis.

2.5.2. Establishment of chromatographic conditions

Ginsenoside analysis was carried out on a 26-201 liquid chromatograph (Alltech, US). The analytical column was an Agilent C₁₈ (State of California, USA), 5 µm, 250 mm × 4.6 mm.

The separation of ginsenosides was obtained by gradient elution [elutents were a mixture of water (A) and acetonitrile (B)]. The process was performed according to the following profiles: 0–40 min, 18–21% (B); 40–42 min, 21–26% (B); 42–46 min, 26–32% (B); 46–66 min, 32–33% (B); 66–68 min, 33–34% (B); 68–73 min, 34–38% (B); 73–80 min, 38–49% (B); 80–84 min, 49% (B); 84–85 min, 49–51% (B); 85–90 min, 51–60% (B); 90–92 min, 60–65% (B); 92–99 min, 65% (B); 99–104 min, 65–85% (B); 104–111 min, 85% (B); 111–115 min, 85–18% (B); 115–122 min, 18% (B). The flow rate was kept at 1.0 mL/min. The injection volume was 10 µL. The absorbance was measured at a wavelength of 203 nm, and the column temperature was maintained at 30°C.

The separation of salidroside, tyrosol, hydroxytyrosol, ligustroflavone, and specnuezhenide was obtained by gradient elution [elutents were a mixture of water (A) and acetonitrile (B)]. The process was performed according to the following profiles: 0–10 min, 8–15% (B); 10–30 min, 15–30% (B); 30–40 min, 30–8% (B); 40–42 min, 8% (B). The flow rate was kept at 1.0 mL/min. Detection was carried out at 220 nm. The injection volume was 10 µL.

2.7. Statistical analysis

All the statistical analyses were performed using the Statistical Package for Social Sciences (SPSS (IBM, USA)), version 17.0. The quantitative data are expressed as the means ± standard deviation. All statistical comparisons were performed using a one-way analysis of variance test. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Improves general condition of myelosuppressed mice

Cy administration for 3 days adversely affected the mice compared with the control mice. Control mice were active, sleek, and eating well, maintaining normal weight, whereas Cy mice were inactive, dull, lusterless, unresponsive to stimuli, and not eating well, with shrunken back. They also displayed weight loss and thin fur, whereas G-treated mice, LLA-treated mice, or G-LLA-treated mice displayed reduced adverse effects in their appearances, fur, and general conditions. The G-LLA group showed the most obvious performance.

3.2. Increased peripheral blood cells counts of myelosuppressed mice

These results indicated that the amount of WBC, RBC, HGB, and PLT in the Cy group was significantly less than that in the control group ($P < 0.01$ or $P < 0.05$). This confirmed the successful mice model of bone marrow suppression. G, LLA, and G-LLA therapy could significantly improve peripheral blood cell counts of myelosuppressed mice. There was no significant difference in the amounts of WBC, RBC, and HGB between the G-, LLA-, and G-LLA-treated mice. However, G-LLA significantly increased PLT ($P < 0.05$) (Fig. 1)

3.3. Increased the count of BMNCs of myelosuppressed mice

As shown in Fig. 2, the BMNCs were significantly reduced in Cy group ($P < 0.01$). After administration, G-LLA significantly increased BMNCs compared with the Cy group ($P < 0.01$), and its effect was superior to G and LLA ($P < 0.01$).

3.4. Improves thymus index and spleen index of myelosuppressed mice

As shown in Table 1, the mean thymus index in the Cy mice was significantly decreased and the spleen index in the Cy mice was significantly increased compared with that in the control group ($P < 0.01$). G, LLA, and G-LLA significantly increased the thymus index, compared with the Cy group ($P < 0.01$). G-LLA-treated group showed superior thymus index compared to G- and LLA-treated groups ($P < 0.01$) and showed better spleen index than G-treated group ($P < 0.01$).

3.5. Promotes proliferation of granulocytic, erythrocyte, and megakaryocyte progenitor cells of myelosuppressed mice

Table 2 showed that CFU-GM, BFU-E, CFU-E, and CFU-Meg colony formation decreased significantly in the Cy mice ($P < 0.01$ vs. normal mice). In response to G, LLA, and G-LLA therapy for 12 days, the CFU-GM, BFU-E, CFU-E, and CFU-Meg colony numbers significantly increased ($P < 0.01$ vs. Cy mice), and G-LLA was more significant than G and LLA ($P < 0.01$).

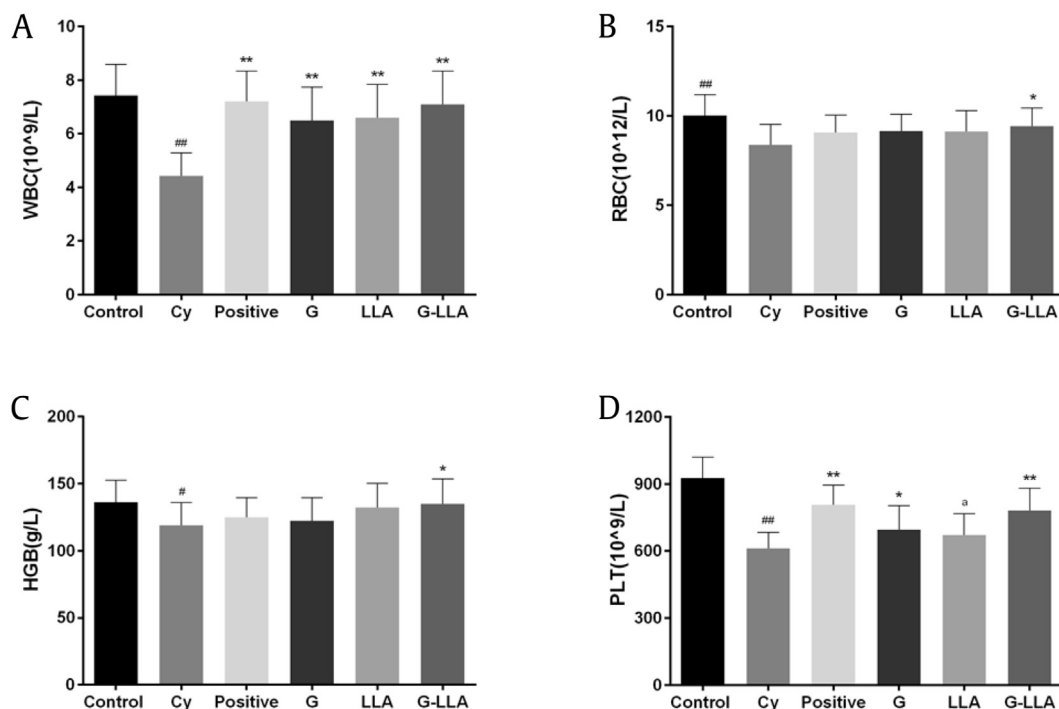


Fig. 1. Effect of G-LLA on peripheral blood cells in Cy-induced mice. (A) WBC, (B) RBC, (C) HGB, (D) PLT. Data are expressed as mean \pm SD. [#]P < 0.05, ^{##}P < 0.01, vs control; ^{*}P < 0.05, ^{**}P < 0.01, vs Cy; ^aP < 0.05 vs G-LLA. Cy, cyclophosphamide; G-LLA, ginseng *Ligustrum lucidum* Ait herb pair; HGB, hemoglobin; PLT, platelet; RBC, red blood cell; WBC, white blood cell.

3.6. Upregulates expression of hematopoiesis-related cytokines of myelosuppressed mice

To examine Cy-induced hematopoietic cytokine production, the content of cytokines in serum was estimated by enzyme-linked immunosorbent assay (Fig. 3). Cy treatment dramatically increased GM-CSF, EPO, and TPO expression in mice. When treated with G-LLA, the release of GM-CSF and TPO decreased remarkably ($P < 0.01$), whereas there was definite improvement in the expression of EPO ($P < 0.01$).

3.7. Promotes cell proliferation of myelosuppressed mice

The ratio of G₀/G₁ phase in the Cy group increased and the ratio of S and G₂/M phase decreased after Cy administration (Fig. 4).

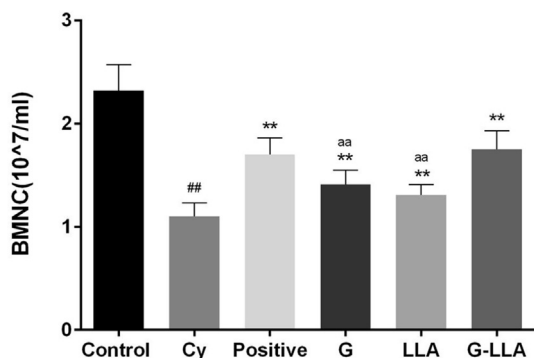


Fig. 2. Effect of G-LLA on the BMNC count in Cy-induced mice. Data are expressed as mean \pm SD. ^{##}P < 0.01, vs control; ^{**}P < 0.01, vs Cy; ^{aa}P < 0.01 vs G-LLA. BMNC, bone marrow nucleated cell; Cy, cyclophosphamide; G-LLA, ginseng *Ligustrum lucidum* Ait herb pair; SD, standard deviation.

Table 1

Effect of G-LLA on the thymus/spleen index in Cy-induced mice

Groups	Thymus index (mg/g)	Spleen index (mg/g)
Control	1.50 \pm 0.13	4.03 \pm 0.52
Cy	0.51 \pm 0.08 ^{##}	6.54 \pm 0.89 ^{##}
Positive	1.01 \pm 0.12 ^{**}	5.51 \pm 0.72 ^{**}
G	0.70 \pm 0.05 ^{**aa}	4.94 \pm 0.31 ^{**aa}
LLA	0.68 \pm 0.08 ^{**aa}	6.03 \pm 0.62
G-LLA	0.88 \pm 0.06 ^{**}	5.54 \pm 0.57 ^{**}

Data are expressed as mean \pm SD.

Cy, cyclophosphamide; G, ginseng; LLA, *Ligustrum lucidum* Ait; SD, standard deviation.

^{##}P < 0.01 vs control.

^{**}P < 0.01 vs Cy.

^{aa}P < 0.01 vs G-LLA.

Table 2

Effect of G-LLA on the yield of the hematopoietic stem/progenitor cells in *in vitro* colony

Groups	CFU-GM	CFU-E	BFU-E	CFU-Meg
Control	83.00 \pm 9.27	106.90 \pm 13.66	58.20 \pm 10.25	96.90 \pm 12.97
Cy	37.00 \pm 6.62 ^{##}	51.70 \pm 8.63 ^{##}	26.10 \pm 4.79 ^{##}	45.00 \pm 7.22 ^{##}
Positive	69.10 \pm 6.95 ^{**}	79.20 \pm 8.39 ^{**}	43.20 \pm 6.09 ^{**}	73.00 \pm 6.00 ^{**}
G	48.90 \pm 6.05 ^{**aa}	71.10 \pm 6.59 ^{**aa}	36.00 \pm 4.42 ^{**aa}	64.10 \pm 7.48 ^{**aa}
LLA	59.00 \pm 6.38 ^{**aa}	71.40 \pm 8.45 ^{**aa}	40.00 \pm 5.21 ^{**aa}	61.10 \pm 7.05 ^{**aa}
G-LLA	74.30 \pm 8.78 ^{**}	85.20 \pm 7.25 ^{**}	48.80 \pm 6.86 ^{**}	82.20 \pm 7.76 ^{**}

Data are expressed as mean \pm SD.

BFU-E, burst-forming unit–erythroid; CFU-E, colony-forming unit–erythroid; CFU-GM, colony-forming unit–granulocyte macrophage; CFU-Meg, colony-forming unit–megakaryocyte; Cy, cyclophosphamide; G, ginseng; LLA, *Ligustrum lucidum* Ait; SD, standard deviation.

^{##}P < 0.01 vs control.

^{**}P < 0.01 vs Cy.

^{aa}P < 0.01 vs G-LLA.

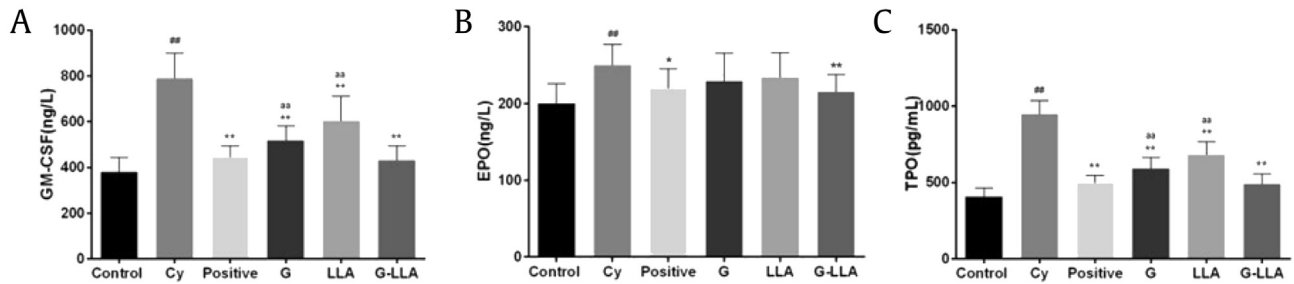


Fig. 3. Effect of G-LLA on the hematopoiesis-related cytokines. (A) GM-CSF, (B) EPO, (C) TPO. Data are expressed as mean \pm SD. ^{##}P < 0.01, vs control; ^{*}P < 0.05, ^{**}P < 0.01, vs Cy; ^{aa}P < 0.01 vs G-LLA. Cy, cyclophosphamide; EPO, erythropoietin; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-LLA, ginseng *Ligustrum lucidum* Ait herb pair; SD, standard deviation; TPO, thrombopoietin.

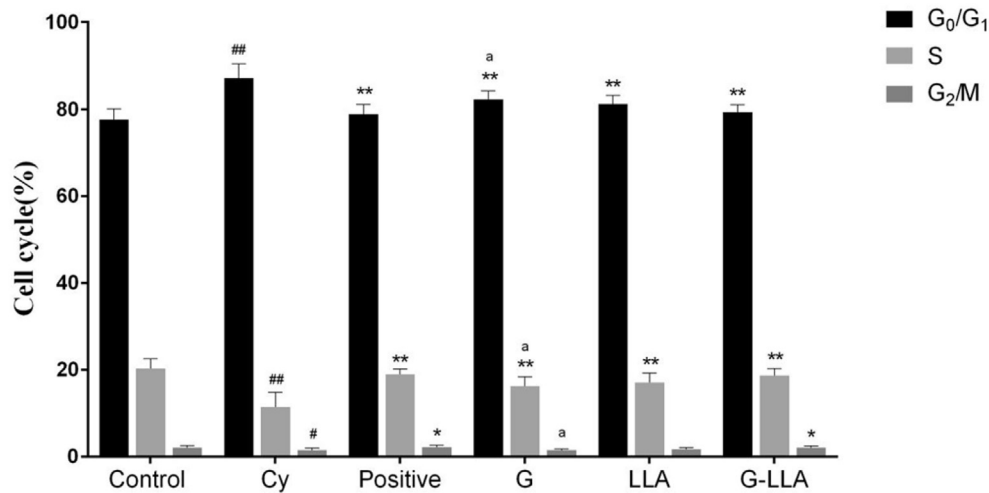


Fig. 4. Effect of G-LLA on cell cycle in Cy-induced mice. Data are expressed as mean \pm SD. [#]P < 0.05, ^{##}P < 0.01, vs control; ^{*}P < 0.05, ^{**}P < 0.01, vs Cy; ^aP < 0.05 vs G-LLA. Cy, cyclophosphamide; G-LLA, ginseng *Ligustrum lucidum* Ait herb pair; SD, standard deviation.

These changes in cell cycle phase were significant compared with the control group ($P < 0.05$ or $P < 0.01$). The percentage of G₀/G₁ phase cells in G, LLA, and G-LLA groups significantly decreased ($P < 0.01$) and S phase cells significantly increased ($P < 0.01$) compared with the Cy group. The G-LLA group showed better results than the G group ($P < 0.05$).

3.8. Changes in the content of active ingredients in G-LLA

Calibration curves were constructed from the measured peak areas (Table 3). The remarkable ginsenoside profile differences between G and G-LLA are shown in Fig. 5 and Fig. 6. As can be seen from the HPLC chromatogram, compared with G, the conventional saponins in G-LLA decreased and transformed into some rare ginsenosides. In these newly formed saponins, the content of Rg₅ is the highest, up to 1.544%, and that of 20(R)-Rg₃ increased from 0 to 0.144%.

Table 4 and Fig. 7 show the contents of the active ingredients of LLA and G-LLA. Compared with LLA, the contents of hydroxytyrosol and ligustroflavone in G-LLA were increased, whereas the contents of salidroside, tyrosol, and specnuezhenide were decreased.

4. Discussion

Modern research proves that most antitumor drugs have side effects that are diverse on the hematopoietic system. Some drugs have direct cytotoxicity to bone marrow stem cells or their

Table 3
Regression equation

Constituent	Regression equation	Linear range/(mg·mL ⁻¹)	R ²
Rg ₁	Y = 941151X+4400.5	0.00625–0.8	0.9995
Re	Y = 1319214X+6776.2	0.00625–0.8	0.9995
Rf	Y = 2128457X+10822	0.00625–0.8	0.9997
Rg ₂	Y = 2179465X+489.13	0.00625–0.8	0.9997
Rb ₁	Y = 1592585X-1499.8	0.00625–0.8	0.9995
Rc	Y = 1427674X+11390	0.00625–0.8	0.9991
Rb ₂	Y = 1406287X+6352.6	0.00625–0.8	0.9998
Rb ₃	Y = 1047403X+4769.1	0.00625–0.8	0.9998
Rd	Y = 1489587X+7119.6	0.00625–0.8	0.9998
Rk ₃	Y = 778048X+834.75	0.00625–0.8	0.9998
F ₂	Y = 1844627X+9341.1	0.00625–0.8	0.9997
Rh ₄	Y = 560149X+4632.2	0.00625–0.8	0.9990
20 (S)-Rg ₃	Y = 2165757X-3227.3	0.00625–0.8	0.9997
20 (R)-Rg ₃	Y = 2083251X+21551	0.00625–0.8	0.9991
PPT	Y = 2633345X+15096	0.00625–0.8	0.9995
Rk ₁	Y = 3056413X+1370.3	0.00625–0.8	0.9998
Rg ₅	Y = 475481X-2798.3	0.00625–0.8	0.9998
CK	Y = 2566303X+19093	0.00625–0.8	0.9994
Rh ₂	Y = 2225003X+10720	0.00625–0.8	0.9997
PPD	Y = 3330911X+45446	0.00625–0.8	0.9996
Salidroside	Y = 7771756X+6002.1	0.01560–0.25	0.9990
Tyrosol	Y = 16039794X+24909	0.01560–0.25	0.9991
Hydroxytyrosol	Y = 11133699X-30019	0.01560–0.25	0.9990
Ligustroflavone	Y = 9472074X+1134.3	0.00195–0.03	0.9992
Specnuezhenide	Y = 5801682X+44282	0.06250–1.0	0.9991

CK, compound K.

progenitor cells, whereas others indirectly affect hematopoietic function [23]. Upon most occasions, drugs that have direct effect can lead to the reduction of circulating blood cells and in bone marrow cellularity, and when their dose is very high, it may cause irreversible reduction of the proliferative potential of hematopoietic stem cells [24]. Related experiments show that this permanent stem cell injury may cause the hematopoietic bone marrow failing to produce a sufficient number of blood cells instead which leads to myelosuppression. Therefore, myelosuppression is the most common and serious side effect of cytotoxic chemotherapy.

Myelosuppression is a decrease in the activity of blood cells in the bone marrow. Both WBC and RBC in peripheral blood are

derived from stem cells in the bone marrow [25]. The research mechanism suggests that bone marrow is the main source of cells for the hematopoietic and immune systems [26]. Both the number of nucleated cells in the bone marrow and the blood cells in the peripheral blood represent the hematopoietic function of the bone marrow [25,27]. Cy could cause reductions in circulating blood cells and in bone marrow cellularity, and at very high doses, it may irreversibly decrease the proliferative potential of hematopoietic stem cells [24]. According to our study, the Cy group showed decreased peripheral blood and BMNC counts. After treatment in the G-LLA group and other drug groups, the mice showed different degrees of recovery of WBCs and BMNCs, and the effect in the G-

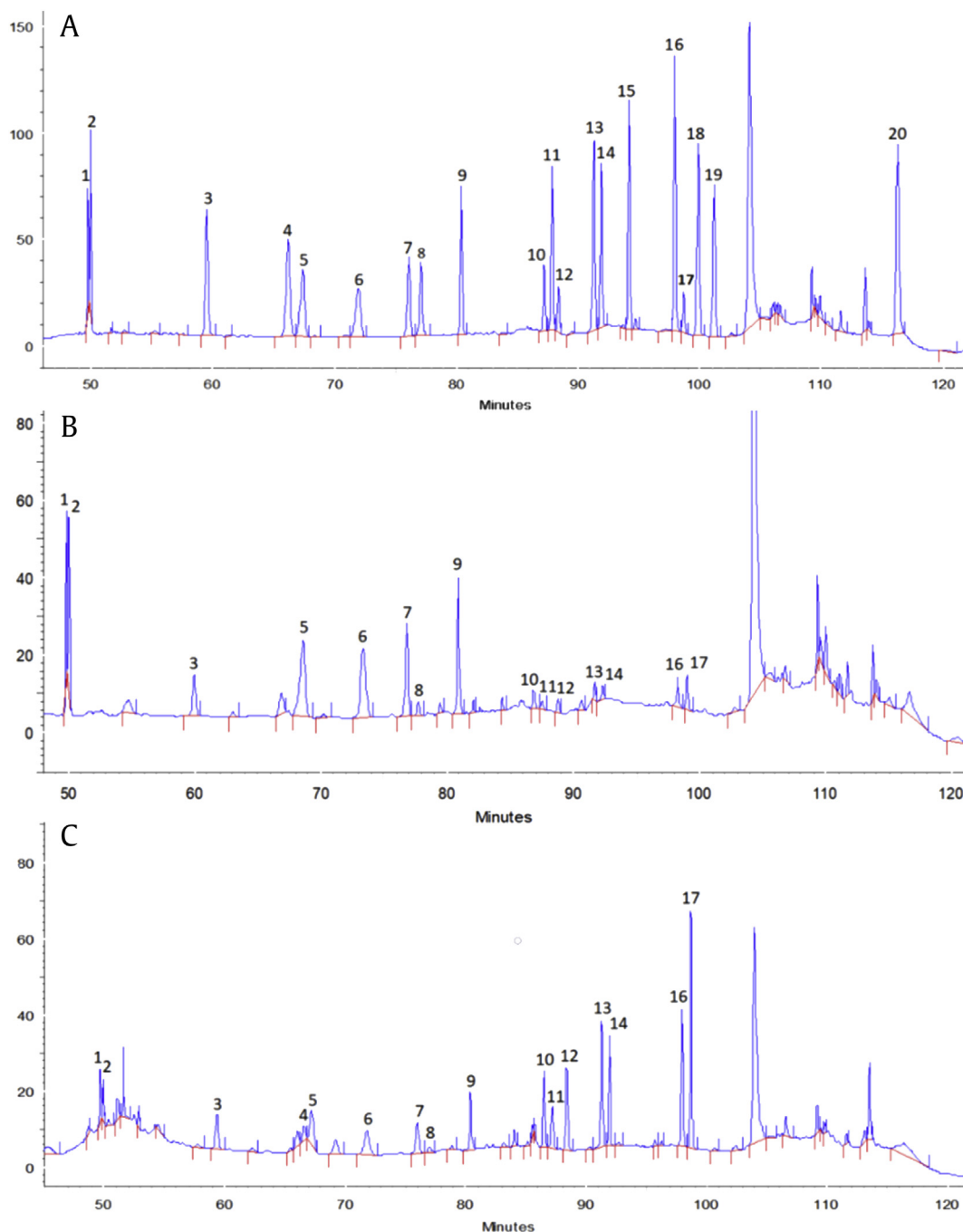


Fig. 5. The remarkable ginsenoside profile differences between G and G-LLA. (A) Calibration curve; (B) G; (C) G-LLA. Peak numbers: 1, Rg₁; 2, Re; 3, Rf; 4, Rg₂; 5, Rb₁; 6, Rc; 7, Rb₂; 8, Rb₃; 9, Rd; 10, Rk₃; 11, F₂; 12, Rh₄; 13, 20(S)-Rg₃; 14, 20(R)-Rg₃; 15, PPT; 16, Rk₁; 17, Rg₃; 18, compound K; 19, Rh₂; 20, PPD. G-LLA, ginseng *Ligustrum lucidum* Ait herb pair.

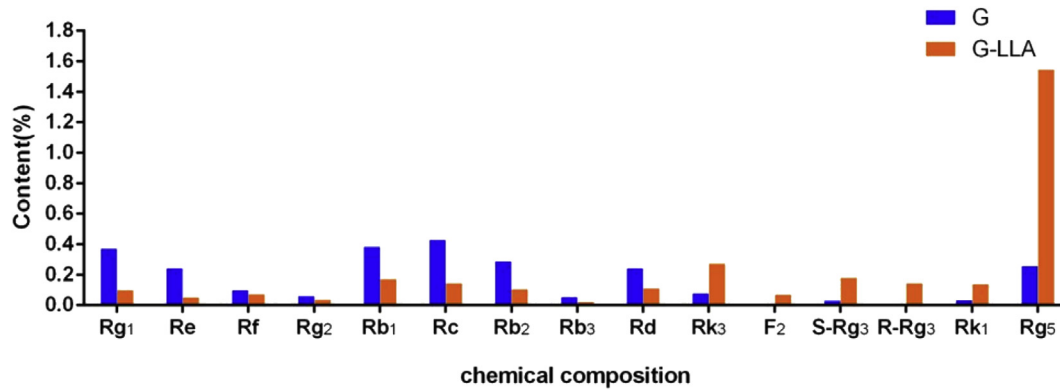


Fig. 6. The remarkable ginsenoside profile differences between G and G-LLA. G-LLA, ginseng *Ligustrum lucidum* Ait herb pair.

LLA group was more obvious, which was not obvious for RBC. This may be explained by the much longer lifespan of RBCs [28].

Thymus and spleen are two main immune organs that play important roles in cellular and humoral immunity. Some scholars advocated that the changes in the thymus index and spleen index can reflect the damage and recovery of the immune system [29]. Thymus and spleen functions can be inhibited by Cy treatment [30]. After treatment with Cy, there was impaired bone marrow hematopoietic function, thymus atrophy, and spleen compensatory enlargement. G-LLA group and other drug groups can have high thymus indices, and G-LLA and G can relieve splenomegaly and normalize the spleen. It suggested that G-LLA was more effective in promoting the recovery of the immune organs of Cy mice.

Hematopoietic progenitors are the product of differentiation of hematopoietic stem cells. Through their proliferation, granulocyte, erythrocyte, and megakaryocyte colonies are formed. The production of these colonies reflects the proliferation of bone marrow hematopoietic cells [31]. After the bone marrow-suppressed mouse model was prepared, G, LLA, and G-LLA were given for 12 days. Then, bone marrow cells were taken out for culture. Experimental results showed that the CFU-GM, BFU-E, CFU-E, and CFU-Meg colony generation rates in the model group were significantly lower than those in the normal group, indicating that the hematopoietic function of the bone marrow was significantly suppressed after model establishment. Compared with the Cy group, G, LLA and G-LLA can promote colony formation of CFU-GM, BFU-E, CFU-E, and CFU-Meg, suggesting that G, LLA, and G-LLA can increase the number of hematopoietic progenitor cells and further promote the recovery of bone marrow hematopoietic function after radiotherapy and chemotherapy injury.

Cytokines emerged as major regulators in health and disease and host-specific response to inflammation, immunity, and cancer [32]. Modern research indicates that hematopoietic cells require the presence and stimulation of specific growth factors for their proliferation and differentiation [33]. GM-CSF is an important growth factor, which can strengthen the production of bone marrow hematopoietic progenitor cells and mobilize them from

hematopoietic tissue into blood circulation [34]. And, it has the ability to stimulate the proliferation and maturation of granulocytes and macrophages [35]. TPO, a glycoprotein hormone produced by the liver and kidney, can modulate platelet production [36–38]. EPO is an important regulator of erythropoiesis in mammals and other organisms [39]. Normally, only a small amount of cytokines exists in serum. The amount of cytokines in serum should be increased by Cy. With the treatment of drugs, the amount of cytokines in the microenvironment will gradually fall back to normal. It can be seen from the effect on TPO that all groups can effectively balance the content of TPO. The effect on GM-CSF indicated that all groups had obvious positive regulation effect on GM-CSF and that G-LLA had better regulation effect. It suggested that G-LLA can promote the recovery of cytokines, especially TPO and GM-CSF.

There are two important monitoring points in the cell cycle regulation, located between the G_1 and S phases and the G_2 and M phases [40]. Bone marrow cells in the proliferative phase are most sensitive to chemoradiotherapy injury [41]. After radio-chemotherapy, DNA damage is caused in hematopoietic cells, the cell cycle checkpoint mechanism is activated, and the cell cycle is arrested in the G_1 phase to repair damaged DNA. For cells that cannot be repaired, an apoptotic program will be initiated to eliminate damaged cells [42]. The distribution of the various phases of the cell cycle can reflect the degree of damage or recovery of bone marrow cells [43]. In our study, the cell ratio of G_0/G_1 stage increased in the Cy group, whereas the cell ratio of S stage and G_2/M stage decreased significantly. It showed that the DNA damaged cells stagnated in the G_1 phase and led to S phase arrest; G-LLA could promote cells in the bone marrow from G_0/G_1 phase to enter S phase and then the next G_2/M phase. It is suggested that G-LLA could improve cells to pass the G_1/S phase checkpoint and successfully enter the cell cycle to facilitate the repair of damaged DNA and accelerate cell proliferation.

During the processing of ginseng samples, the ginsenosides will be transformed to form rare ginsenosides under the influence of temperature and pH [44]. For ginseng preparations, owing to the complexity of the prescription of TCM preparations, saponins are more likely to undergo structural transformation to form secondary saponins during the preparation process [45,46]. Secondary saponins are rare saponins. Recent scientific studies have shown that rare saponins have better pharmacological effects than conventional saponins in protecting apoptosis of cells [47], inhibition of tumor cells, and immune regulation [48]. According to our study, compatible with G and LLA, the conventional saponins were degraded and transformed into rare ginsenosides. The triol type of saponins Re was transformed into rare saponins Rk₃, and its

Table 4
Chemical contents analysis of LLA and G-LLA

Group	LLA (%)	G-LLA (%)
Salidroside	0.094	0.077
Tyrosol	0.069	0.062
Hydroxytyrosol	0.060	0.062
Ligustroflavone	0.004	0.008
Specnuezhenide	0.281	0.180

G-LLA, ginseng *Ligustrum lucidum* Ait herb pair; LLA, *Ligustrum lucidum* Ait.

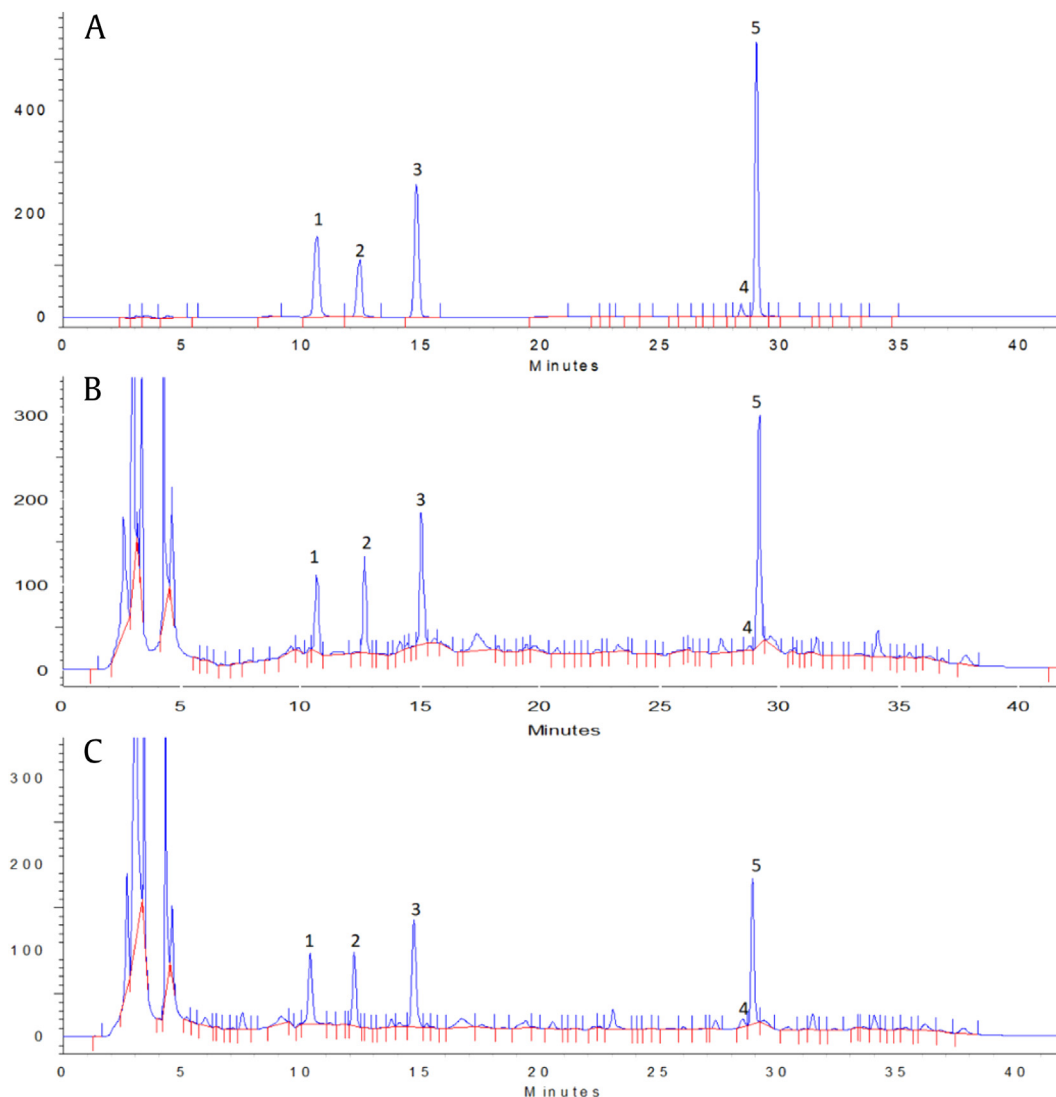


Fig. 7. HPLC chromatograms showing the chemical composition of LLA and G-LLA. (A) Calibration curve; (B) LLA; (C) G-LLA. Peak numbers: 1, hydroxytyrosol; 2, salidroside; 3, tyrosol; 4, ligustroflavone; 5, specnuezhenide. G-LLA, ginseng *Ligustrum lucidum* Ait herb pair.

content was 0.271%. The diol type of saponins Rb₁, Rc, Rb₂, Rb₃, and Rd also underwent transformation to form rare saponins Rg₅, Rk₁, and Rg₃. The content of Rg₅ increased the most, up to 1.544%; the content of Rg₃ is determined out of nothing; and the content of Rk₁ is 0.136%. The production and increase of these rare saponins make G-LLA get more absorbed, which enhances its tonic effect.

The main pharmacologically active substances of *Fructus Ligustrum lucidum* Ait include salidroside, tyrosol, hydroxytyrosol, ligustroflavone, specnuezhenide, and polysaccharide [18,49]. It is reported that salidroside can treat myelosuppression [50]. In our study, although the content of salidroside in G-LLA decreased, it was still very high. Meanwhile, the compatibility of the two drugs increased the dissolution of ligustroflavone. These active ingredients may contribute more to the total pharmacological activity of the herb pairs.

5. Conclusion

In conclusion, the aforementioned results show that G-LLA has ameliorative function against Cy-induced myelosuppression and affects hematopoiesis recovery via improvements of various

parameters such as WBC, BMNCs, spleen/thymus index, hematopoietic progenitor, hematopoiesis-related cytokines, and cell cycle. It is suggested that this improvement may be related to the material basis of G-LLA—the change of active ingredients. The findings may be of great importance for the development of G-LLA as a therapeutic agent for the prevention and treatment of human myelosuppression to further study the pharmacodynamic material basis of G-LLA herb pair and provide a new way of thinking for the clinical prescription of TCM.

Conflicts of interest

All the authors declare that there are no conflicts of interest.

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