

## Quality assessment of black ginseng materials utilizing chemometrics and modeling inflammation in Zebrafish

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### Abstract

Black ginseng is a novel variant of processed ginseng that is produced by subjecting ginseng to a series of nine steaming and drying cycles. However, the precise connection between the processing cycle and the quality of black ginseng remains uncertain, and further investigation is required to establish the association between quality markers and biological activity. In this research, high-performance liquid chromatography was employed to analyze the composition of 17 ginsenosides present in samples of black ginseng. The anti-inflammatory properties of black ginseng saponin extract were tested utilizing a zebrafish tail fin amputation model. The findings indicate that the concentrations of Rg3, Rg5, and Rk1 in black ginseng samples exhibited their highest levels following the seventh processing cycle, hence demonstrating the most effective regulatory impact on neutrophils and macrophages. This study posits that the correlation between these three ginsenoside and biological activities could potentially serve as a crucial factor in ensuring the quality control of black ginseng, thereby contributing to the advancement of quality control measures for health foods and dietary supplements that utilize black ginseng as their primary constituent.

**Keywords:** black ginseng; ginsenoside; immune response; inflammatory models; quality assessment

### Introduction

Ginseng, scientifically known as *Panax ginseng* C.A. Meyer, holds a prominent position as a well-recognized

and extensively utilized herb globally (He *et al.*, 2018). Its usage as an adaptogen, facilitating the recuperation, and prophylaxis of the human body, has been well-documented. Throughout the history of human

utilization of ginseng, it has been observed that the hue of steamed ginseng materials, such as red ginseng, undergoes alterations (He *et al.*, 2018). In addition, there exists sun-dried ginseng, which offers convenient preservation and acts as a preservative. Sugar ginseng is produced through maceration in honey. Fermented ginseng, on the other hand, undergoes a low-temperature fermentation procedure. Lastly, ginseng marinated in wine or vinegar is also a traditional way of processing. However, the process of steaming ginseng remains the most employed technique (In *et al.*, 2017; Kim *et al.*, 1985; Lim *et al.*, 2010; Paek *et al.*, 2006; Zhang and Xu, 2018). The steamed ginseng materials are deemed to be more conducive for preservation, and their physiological impacts have undergone alterations, particularly with the better modulation of the body's immune system (He *et al.*, 2018). Hence, the utilization of ginseng that undergoes steaming has consistently had significance as a drug in traditional medicine, while also serving as a crucial ingredient in numerous health foods and dietary supplements (Riaz *et al.*, 2019).

Black ginseng is a novel variant of processed ginseng that is produced by subjecting ginseng to a series of nine steaming and drying cycles, resulting in a distinct black hue (Nam *et al.*, 2012). The potential biological effectiveness of black ginseng, such as anti-tumor, anti-inflammatory, antioxidant, anti-aging, and anti-diabetic activities is believed to surpass that of ginseng products derived from other processing procedures (Huang *et al.*, 2023). However, it is worth noting that black ginseng may exhibit increased cytotoxicity in laboratory settings, which could be attributable to the unique properties of certain saponin components present in black ginseng (Park *et al.*, 2022). No apparent abnormalities were observed in the physiological indicators, blood biochemistry, and histopathology analyses following the administration of black ginseng extract, as determined through tests conducted on rat models (Park *et al.*, 2022). The chemical composition of black ginseng, particularly ginsenosides, shows significant alterations during nine steaming and drying cycles, resulting in corresponding modifications to the biological activity of black ginseng (Huang *et al.*, 2023). For instance, the repeated steaming and drying processing of black ginseng leads to an augmentation of its antioxidant properties (Kim *et al.*, 2011). However, the technique of nine cycles of steaming and drying is derived from the conventional preparation procedures employed for certain herbs, such as *Rehmannia glutinosa* Libosch, in Traditional Chinese medicine (TCM) (Sun *et al.*, 2018). At present, there exists a dearth of conclusive evidence about the correlation between the precise frequency of steaming and drying processes and the resultant quality and biological activity of black ginseng. In the production process, there is a deliberate reduction in the number of steaming and drying cycles for specific

black ginseng materials. This reduction is motivated by factors such as cost, time efficiency, and material conservation. Thus, the presence of significant diversity in the biological activity of black ginseng materials available in the market is evident. Hence, the investigation of the correlation between the cycles of steaming and drying processes applied to black ginseng, and its resultant quality and efficacy, has emerged as a crucial concern in the realm of black ginseng product quality control.

In this research, the identification of the 17 ginsenosides present in the black ginseng material, which underwent a process of different cycles of steaming and drying, was conducted. Subsequently, a zebrafish inflammatory model was employed to evaluate the bioactivity of total ginsenoside extracts derived from different black ginseng materials. Here, transgenic zebrafish lines *Tg* (mpx:GFP/ mpeg:mCherry) were employed as a model system to investigate the impact of ginsenoside extracts on the migration of innate immune cells, specifically neutrophils and macrophages, during inflammatory conditions. Finally, we summarized a relationship between processing cycles and the quality and anti-inflammatory efficacy of black ginseng. Our findings contribute to the understanding of quality control measures for black ginseng and offer novel research evidence for enhancing the overall quality of black ginseng products.

## Materials and Methods

### Preparation of black ginseng materials

*Raw materials:* The raw roots and rhizome of *Panax ginseng* C.A. Meyer (5-year-old) were obtained from the Beijing Institute of Traditional Chinese Medicine in Beijing, China. Before submitting to Changchun University of Chinese Medicine in Changchun, China, Prof. Dr. Li Li validated all samples. To prepare processed black ginseng, the raw materials were subjected to drying in an oven at a temperature of 50°C. Subsequently, the dried materials were steamed with water for 180 min at 100°C (Metwaly *et al.*, 2019). Following this, the processed materials were dried at the same temperature of 50°C for 24 h (Metwaly *et al.*, 2019). The production of the carcinogen benzo(a)pyrene is an inevitable consequence of the distinctive heat treatment procedure employed in the preparation of black ginseng. The concentration of this hazardous compound will increase rapidly with an increase in the drying temperature. Hence, to mitigate the excessive carbonization of black ginseng, a drying temperature of 50°C is employed (Jo *et al.*, 2009). This process was repeated nine times to obtain black ginseng materials corresponding to each cycle from one to nine, and the dried product of fresh ginseng was used as the control group.

## Preparation of ginsenoside extracts from black ginseng materials

The methodology employed for the extraction of ginsenoside from black ginseng samples, which have undergone zero to nine cycles of preparation, adheres to the guidelines outlined in the Pharmacopoeia of the People's Republic of China (2020) (Commission, 2020). In accordance with the specifications for experimental design, the extraction process was optimized as follows: the black ginseng samples' roots and rhizome were pulverized, and a precise measurement of approximately 2 g of black ginseng powder was obtained. This powder was then placed into a 50 mL volumetric flask, followed by the addition of 50 mL of chloroform. The mixture was allowed to stand overnight and subsequently subjected to ultrasonic degreasing for 1 h. The resulting chloroform liquid was discarded, and the solvent of the residual drug was evaporated. The remaining substance was transferred into a 100 mL Erlenmeyer flask along with filter paper. The experimental procedure involves the addition of precisely 50 mL of water-saturated n-butanol into a container. The container should be securely sealed and stored. Following an overnight period, the mixture should be subjected to sonication for 30 min, utilizing a power of 250 W and a frequency of 50 kHz. The primary filtrate should be filtered and discarded. Then, a precise volume of 25 mL of the continuous filtrate was transferred to a test tube. The test tube was subjected to a nitrogen blow drier to facilitate drying. The resulting residue in the test tube was dissolved immediately in methanol to achieve a test solution concentration of 40 mg/mL. The solution should be thoroughly shaken. The extraction solution underwent filtration using a 0.22 µm filter membrane and was thereafter introduced into the high-performance liquid chromatography (HPLC) system through direct injection.

## Ginsenoside reference standards preparation

The 17 reference samples were used as standards at different concentrations to quantify the amounts present in the processed samples. Monomeric ginsenoside Rb1 (3.008 mg/mL), Rb2 (1.508 mg/mL), Rb3 (2.561 mg/mL), Rc (2.012 mg/mL), Rd (1.308 mg/mL), Re (1.520 mg/mL), Rf (1.610 mg/mL), Rg1 (2.412 mg/mL), Rg2 (2.068 mg/mL), Rg3 (0.442 mg/mL), Rg5 (0.173 mg/mL), Rh1 (0.772 mg/mL), S-Rh2 (0.281 mg/mL), R-Rh2 (0.428 mg/mL), Rk1 (0.242 mg/mL), Ro (2.521 mg/mL), and F1 (1.825 mg/mL) were dissolved in methanol and diluted to obtain a series of mixed standard solutions of different concentrations. The above standards were acquired from Beijing Solarbio Science & Technology Co., Ltd, located in Beijing, China. The purity of all reference substances exceeded 98%.

## High-performance liquid chromatography analysis

The HPLC analysis was conducted utilizing an Agilent 1260 instrument (Agilent Technologies, Singapore International Pte. Ltd). The equipment was ensembled with a micro-vacuum degasser, quaternary pump, auto-sampler, thermostated column compartment, and diode array detector. The diode array detector was connected to an Agilent ChemStation. The experiment involved the utilization of an Agilent 5 µm-ZORBAX SB-C18 column (4.6 × 250 mm; USA) for conducting chromatographic analysis. The column was kept at a constant temperature of 40°C (Sun *et al.*, 2009). The wavelength of detection utilized in the experiment was 203 nm. The binary gradient elution system utilized a mixture of acetonitrile, water, and a 5% acetic acid aqueous solution (in a ratio of 10:85:5, v/v/v) as solvent A, and a mixture of acetonitrile and water (in a ratio of 80:20, v/v) as solvent B (Sun *et al.*, 2009). The separation was accomplished by implementing a gradient program consisting of the following conditions: at 0 min, the mobile phase composition of 0% B was employed; from 0 to 10 min, the mobile phase composition was adjusted to 30% B; from 10 to 25 min, the mobile phase composition was further modified to 50% B; from 25 to 40 min, a mobile phase composition of 100% B was utilized; from 40 to 50 min, the mobile phase composition remained at 100% B; from 50 to 53 min, the mobile phase composition was reverted to 0% B; and finally, from 53 to 60 min, a mobile phase composition of 0% B was maintained (Sun *et al.*, 2009). The flow rate was established at a rate of 1.5 mL per minute, whereas the sample injection volume was 10 mL. A methanol-based solution was created, consisting of 17 bioactive reference chemicals. The employed methodology for analyzing the chemical contents is widely recognized and has been thoroughly validated, demonstrating satisfactory levels of reproducibility and repeatability for each ingredient. The limit of detection (LOD), the limit of quantification (LOQ), and R<sup>2</sup> values are listed in Table S1.

## Zebrafish tail-fin amputation model for anti-inflammatory tests

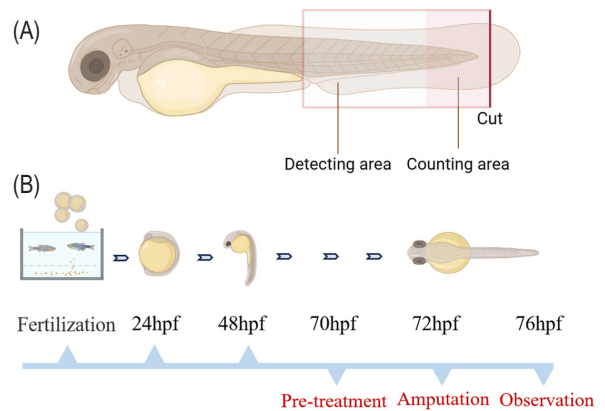
The induction of local inflammation in zebrafish can be achieved by performing tail-fin amputation on zebrafish larvae (He *et al.*, 2020). The present model can be utilized for the examination of the immunomodulatory response by investigating the migratory behavior of macrophages and neutrophils (Xie *et al.*, 2021). In this study, zebrafish larvae of double transgenic lines *Tg* (mpx:GFP/mpeg:mCherry) were used as the research object, and tail-fin amputation was exploited to explore the migration patterns of neutrophils and macrophages. To track the movement of neutrophils, the myeloid-specific peroxidase (mpx) was genetically modified to express a

green fluorescent protein (GFP). Similarly, the migration of macrophages was indicated by marking the macrophage-expressed gene (*mpeg*) with a red fluorescent mCherry protein.

**Study on identifying the suitable ginsenoside concentration:** The culture medium for zebrafish, known as egg water, is prepared by combining distilled water with a certain proportion of quick sea salt. Ginsenoside extracts were dissolved in egg water to prepare solutions with concentrations of 20 µg/mL, 50 µg/mL, and 100 µg/mL, respectively. Zebrafish larvae at the age of three days were immersed in a solution of egg water that included varying concentrations of ginsenoside extract for 96 h. During this time frame, the researchers conducted an observation of the fatal and teratogenic effects of ginsenoside extract on zebrafish at intervals of 24 h, 48 h, 72 h, and 96 h. The purpose of this inquiry is to provide further elucidation on the safe concentration of ginsenoside extract.

**Immunomodulatory study of ginsenoside on zebrafish:** Zebrafish larvae that were three days old were subjected to a pretreatment period of 2 h. During this period, they were exposed to either a solution containing ginsenoside extract at a concentration of 20 µg/mL or beclomethasone or egg water. This pretreatment was conducted before the amputation procedure (He *et al.*, 2020; Xiong *et al.*, 2022). Next, the larvae were relocated into a solution of egg water supplemented with 0.02% tricaine (Yuanye Bio-Technology Co., Ltd, Shanghai, China) to induce anesthesia. Following this, the larvae were arranged in Petri dishes that were coated with a layer of 2% agarose. This setup facilitated the subsequent process of partially amputating the tail-fin, as depicted in Figure 1A. The amputation procedure was performed utilizing a Leica M165C stereomicroscope, a micro-manipulator, and a 1 mm sapphire blade manufactured by World Precision Instruments (He *et al.*, 2020; Xiong *et al.*, 2022). Following that, the zebrafish larvae that had undergone amputation were then reintroduced into either a fresh solution of ginsenoside extract or egg water for 4 h, as depicted in Figure 1B. Zebrafish larvae were subjected to microscopic examination for real-time observation to facilitate the examination of macrophage and neutrophil migration.

The zebrafish used in this study were cared for and managed in accordance with the standards provided by the Zebrafish Model Organism Database (<http://zfn.org>) and in adherence to the regulations set forth by the local animal welfare council of Changchun University of Chinese Medicine. All animal procedures were conducted in strict adherence to the Guidelines for Care and Use of Laboratory Animals established by Changchun University of Chinese Medicine. The experiments were duly authorized by the Animal Ethics Committee of



**Figure 1.** The provided diagram illustrates the experimental setup for tail-fin amputation tests conducted on zebrafish. (A) The term “detecting area” refers to the visible area observed under a microscope, while the term “counting area” refers to the specific region utilized for counting migrating cells. The thick red line is used to show the place of amputation. (B) The time series of the experiment.

Changchun University of Chinese Medicine (Approval No. 2020233).

### Data processing and statistical analysis

The analysis involved the determination of the concentration of 17 components present in each extract of ginsenoside. The K-means clustering approach was employed to differentiate between the 17 chemicals present in ginsenoside extracts. This analysis was conducted using the tools included in the MetaboAnalyst 5.0 software program (Pang *et al.*, 2022), which can be accessed at <http://www.metaboanalyst.ca>. Subsequently, a two-tailed, unpaired Student’s t-test was conducted using SPSS version 23.0 (IBM, Armonk, NY) to compare the various ginsenoside extracts based on the quantities of each detected compound and the overall amounts of these 17 compounds, respectively. Statistical significance was determined at a significance level of  $p < 0.05$ .

The imaging of severed larvae was conducted utilizing a Leica MZ16FA fluorescence stereomicroscope, with support of LAS version 3.7 software. The quantification of migrating neutrophils with green color and macrophages with red color was performed on the wound site of a zebrafish larva, as depicted in Figure S1B. Subsequently, a one-way analysis of variance (ANOVA) was employed, followed by a post hoc analysis using the least significant difference (LSD) method, to compare the cell counts between the groups subjected to amputation and those treated with ginsenoside extract. Statistical significance was determined at a significance level of  $p < 0.05$ .

## Results

### Chemical analysis of ginsenoside extracts

The process of preparing black ginseng involves a repeated cycle of steaming and drying, which is performed nine times. Throughout each steaming and drying cycle, samples of the black ginseng are carefully preserved. The observation depicted in Figure 2A illustrates that with an increase in the processing cycle, there is a steady darkening of the color of the black ginseng sample. Similarly, Figure 2B demonstrates a corresponding darkening of the color of the ginsenoside extract.

To evaluate the chemical characteristics of ginsenoside extracts, the concentrations of the 17 main ginsenoside components were quantified in the extracts derived from black ginseng samples. These samples were taken at various cycles during the steaming and drying process, and three distinct batches of black ginseng samples were acquired during each processing cycle. Hence, a comprehensive set of 30 samples was identified for the particular analysis of 17 ginsenosides utilizing HPLC.

Upon consolidating the chemical data from three batches of black ginseng samples across each processing cycle,

it is evident from Figure 3A that the cumulative concentration of the 17 detected ginsenoside in the black ginseng samples generally exhibited a declining pattern as the processing cycle increased. Ginsenosides, which are triterpenoid glycosides, can be categorized into various types according to the structure of the aglycone. These types include protopanaxadiols (e.g., monomeric ginsenoside Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rg5, Rk1, and Rh2), protopanaxatriols (e.g., monomeric ginsenoside Re, Rg1, Rg2, Rf, F1, and Rh1), and oleanane (e.g., monomeric ginsenoside Ro) (Elshafay *et al.*, 2017; Wei *et al.*, 2020). The results depicted in Figure 3B, 3C, and 3D indicates a general decline in the levels of the three distinct configurations of ginsenosides as the processing cycle increases. However, it is worth noting that this decreasing trend does not hold for ginsenosides Rg3, Rg5, and Rk1. The levels of these three individual ginsenosides exhibited a notable increase as the processing cycle progressed, ultimately reaching their peak after the seventh processing cycle, followed by a subsequent decline (Figure 3E).

Subsequently, an unsupervised K-means clustering methodology (Likas *et al.*, 2003) was employed to analyze the chemical data, to visually represent the variations among distinct ginsenoside extracts derived from samples of black ginseng. The results of K-means clustering are

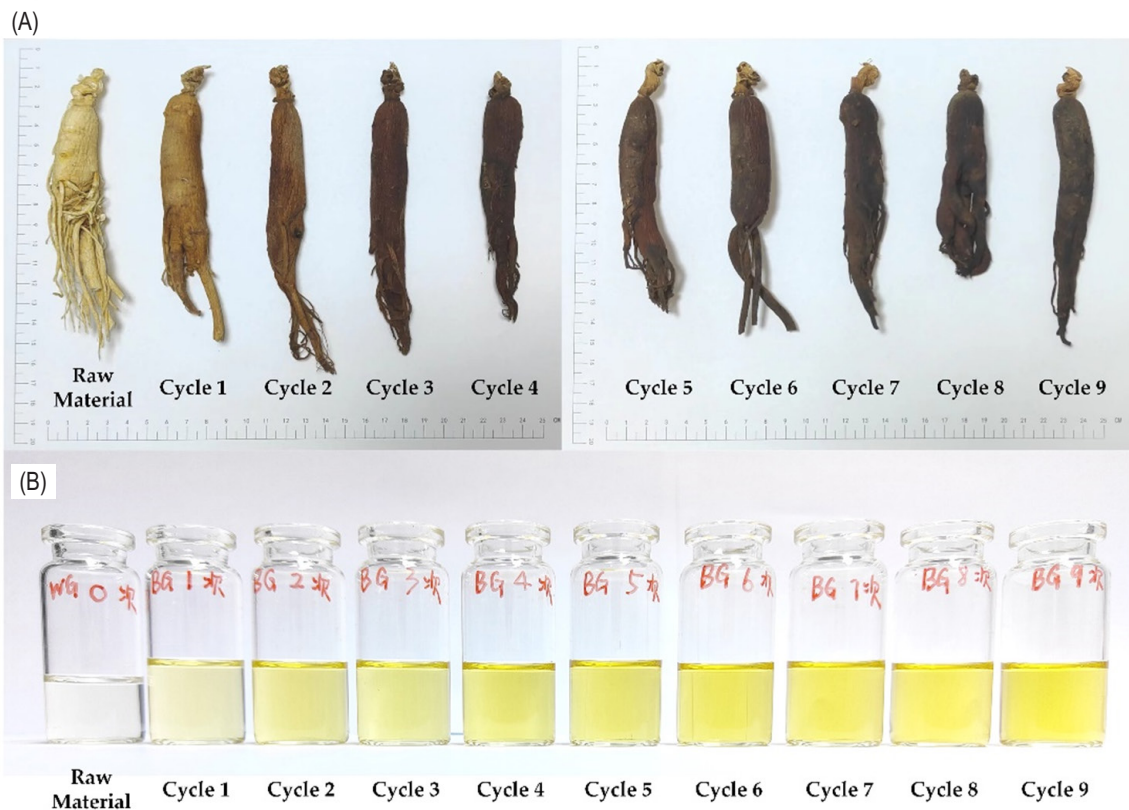
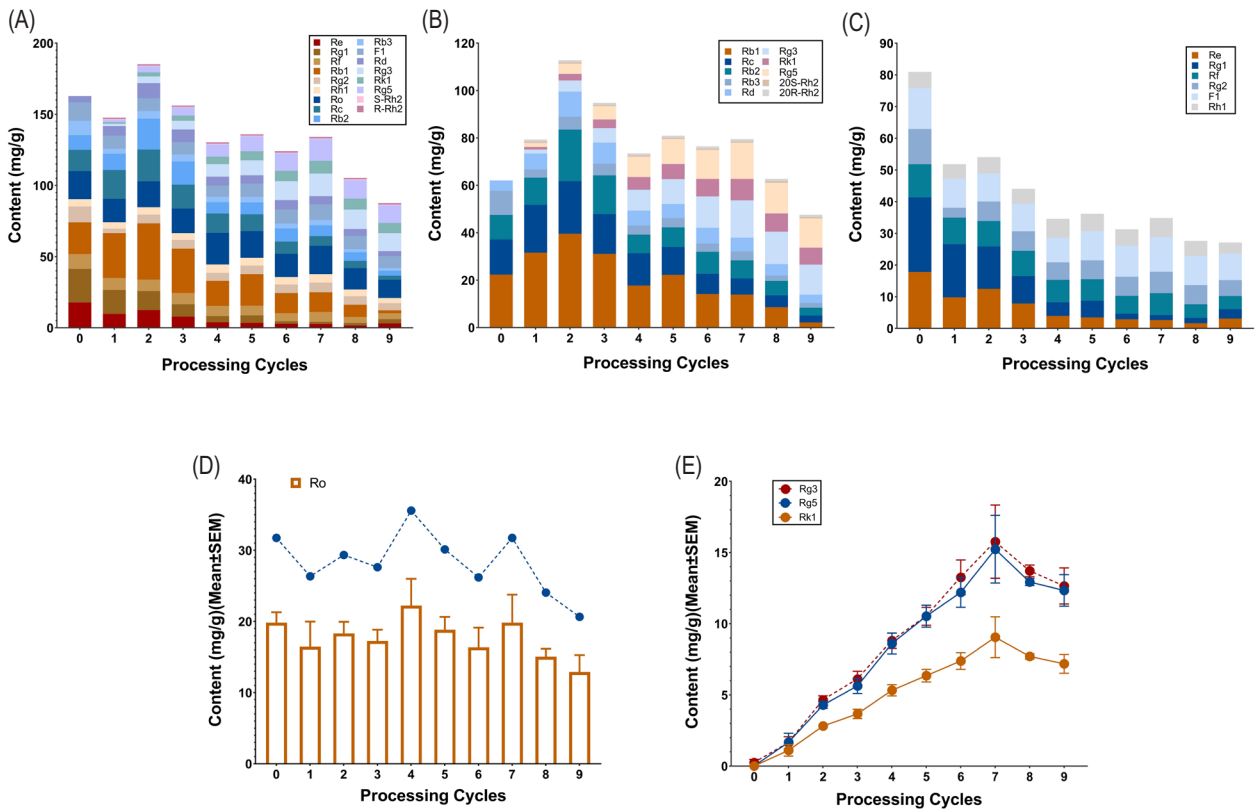


Figure 2. Samples of black ginseng and ginsenoside extract obtained from various processing cycles. (A) Raw and black ginseng samples. (B) Ginsenoside extract samples.



**Figure 3.** The dynamic variations in the composition of 17 ginsenosides over nine processing cycles. (A) The chemical profiles of the detected 17 ginsenosides. (B) The chemical profiles of the detected ginsenoside of protopanaxadiols type. (C) The chemical profiles of the detected ginsenoside of protopanaxatriols type. (D) The chemical profiles of the detected ginsenoside of oleanane type (e.g., monomeric ginsenoside Ro). (E) The dynamic variations in the composition of Rg3, Rg5, and Rk1. Note: Processing cycle 0 indicates raw dried ginseng materials.

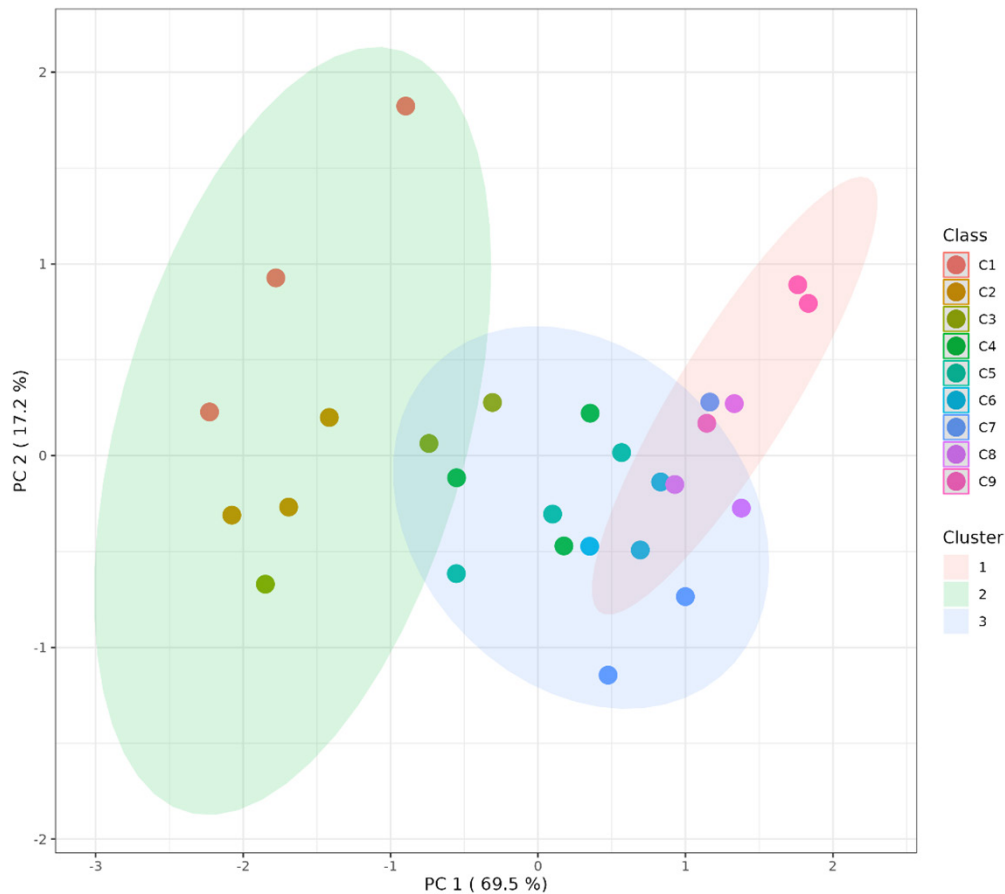
depicted in Figure 4. When the number of groups was set to three, all 27 samples of black ginseng were continuously allocated to distinct clusters. The details of K-means clustering are listed in Table 1. The black ginseng samples acquired following the third and seventh processing cycles serve as the demarcation points that delineate the three clusters. Hence, we choose to collect samples from the aforementioned two demarcation points, as well as distinct black ginseng samples within each cluster (namely, samples derived from cycles 1, 3, 4, 7, and 9) for further analysis.

Next, ANOVA was employed to assess the levels of 17 ginsenosides among samples of black ginseng that underwent different processing cycles, specifically cycles 1, 3, 4, 7, and 9. The findings indicated that there were notable variations in the levels of monomeric ginsenosides Rg1, Rb1, Rc, Re, Rf, Rg3, Rg5, and Rk1 among the various black ginseng samples (Figure 5). Noteworthy are the ginsenosides Rg3, Rg5, and Rk1. The contents of these three chemicals in the samples following the first and third processing cycles exhibited notably lower values compared to the outcomes seen in the remaining

processing cycles ( $p < 0.05$ ) (Figure 5). Furthermore, whereas the black ginseng sample following the seventh processing cycle exhibits the highest concentration of these three components, there is no discernible difference when compared to the samples acquired during the fourth and ninth processing cycles (Figure 5).

**Anti-inflammatory activity tests**

In this study, three different concentrations of ginsenoside extract obtained from black ginseng samples were administered to zebrafish larvae at the age of 3 days. The ginsenoside extract was derived from three separate samples collected during processing cycles 1, 3, 4, 7, and 9, respectively. The administration of the extract was done by introducing it into the egg water of the cultured zebrafish larvae. The exposure duration lasted for 96 h. During the specified time frame, the replacement of new egg water, which contained ginsenoside extract at a consistent dosage, occurred every 24 h. The survival rate of 30 zebrafish larvae was subsequently recorded for each experimental group. The experimental findings indicate that zebrafish larvae exhibited 100% survival when



**Figure 4.** K-means clustering results based on the content of 17 ginsenosides in black ginseng samples collected from nine distinct processing cycles. C means processing cycle.

**Table 1.** The detailed results of K-means clustering analysis.

Cluster	Members
Cluster 1	C7-S2; C8-S1; C8-S2; C8-S3; C9-S1; C9-S2; C9-S3
Cluster 2	C1-S1; C1-S2; C1-S3; C2-S1; C2-S2; C2-S3; C3-S1; C3-S2
Cluster 3	C3-S3; C4-S1; C4-S2; C4-S3; C5-S1; C5-S2; C5-S3; C6-S1; C6-S2; C6-S3; C7-S1; C7-S3

Note: C – processing cycle; S – specific black ginseng sample.

exposed to ginsenoside extracts at a dosage of 20  $\mu\text{g}/\text{mL}$  (Figure 6A). However, it was observed that as the concentration of ginsenoside extract was augmented, there was a notable rise in the death rate of zebrafish larvae, particularly at a concentration of 100  $\mu\text{g}/\text{mL}$  (Figure 6C). Hence, a concentration of 20  $\mu\text{g}/\text{mL}$  of ginsenoside extract was chosen to evaluate the anti-inflammatory efficacy of black ginseng.

In order to assess the anti-inflammatory properties of ginsenoside extracts, a conventional zebrafish tail-fin amputation paradigm was employed. The ginsenoside extracts from three independent black ginseng samples

obtained during each processing cycle were mixed, and afterward, the ginsenoside extracts (20  $\mu\text{g}/\text{mL}$ ) were examined for their anti-inflammatory activity based on the regulating effect of neutrophils and macrophages during the formation of inflammatory lesions in the tail of zebrafish larvae (Figure S1 A). The findings indicate that following injury to the tail-fin of zebrafish larvae, there was a substantial increase in the population of neutrophils and macrophages in the tail, indicating a pronounced inflammatory response (Figure 7). Furthermore, the administration of the positive drug (beclomethasone) demonstrated efficacy in reducing the abundance of neutrophils and macrophages in the tail (Figure 7).

The ginsenoside extract derived from black ginseng samples that were processed in the first cycle did not exhibit a statistically significant impact on neutrophil count (Figure 7A). Conversely, the ginsenoside extract derived from black ginseng samples that were processed in the third cycle demonstrated a notable down-regulating effect on neutrophil count ( $p < 0.05$ ) (Figure 7A). Additionally, the ginsenoside extract derived from black ginseng samples that were processed in the fourth, seventh, and ninth cycles displayed a significant down-regulating effect

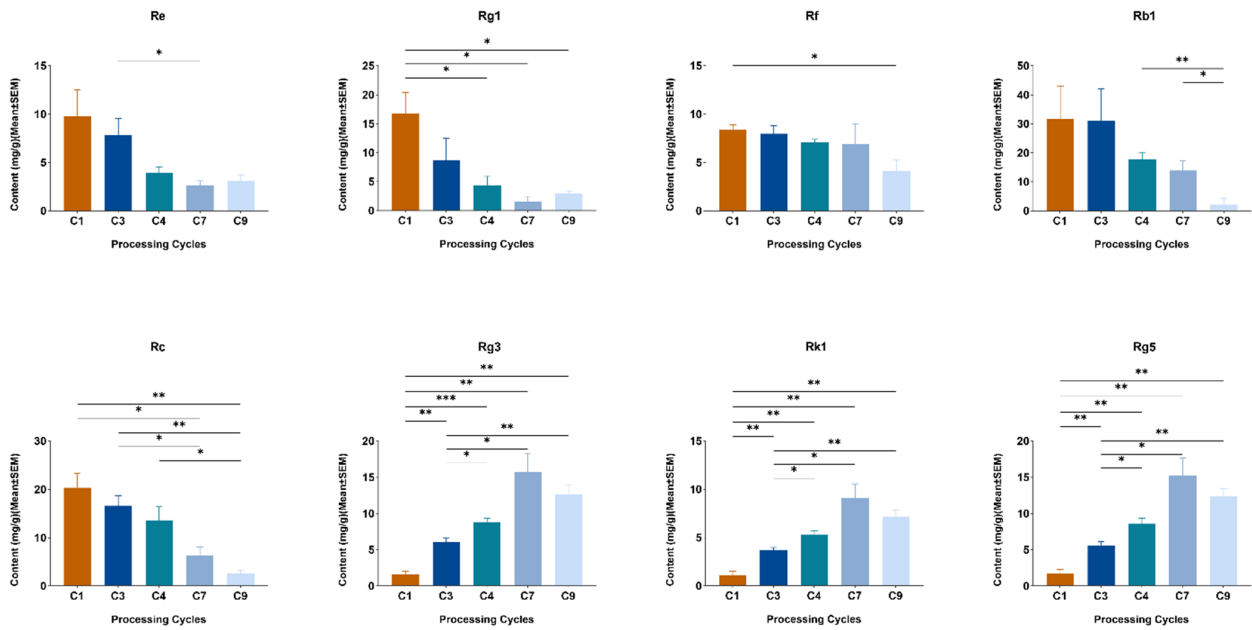


Figure 5. The histograms compare the levels of certain ginsenoside compounds across different cycles, including cycles 1, 3, 4, 7, and 9. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

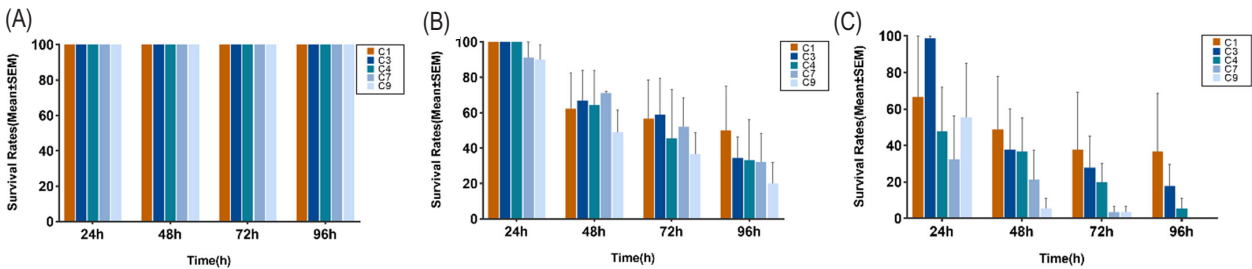


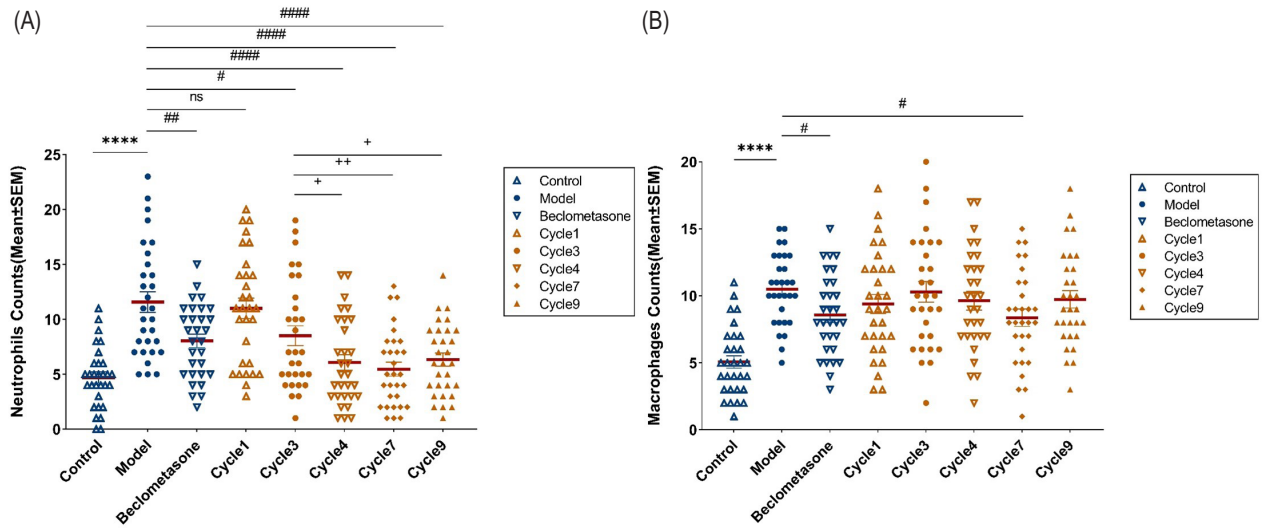
Figure 6. Safety evaluation results of ginsenoside extract. (A) The concentration of ginsenoside extract is 20  $\mu\text{g/mL}$ . (B) The concentration of ginsenoside extract is 50  $\mu\text{g/mL}$ . (C) The concentration of ginsenoside extract is 100  $\mu\text{g/mL}$ .

on neutrophil count ( $p < 0.05$ ), surpassing the down-regulating effect observed in the samples derived from the third processed cycle ( $p < 0.05$ ), even superior efficacy compared to the positive drug (Figure 7A). Moreover, it is noteworthy that alone the ginsenoside extract subjected to the seventh processing cycle exhibited a substantial down-regulatory impact on macrophage count ( $p < 0.05$ ) (Figure 7B). This effect closely resembled that of the positive drug. The other ginsenoside extracts did not have a discernible modulatory impact on the accumulation of macrophages at inflammatory locations (Figure 7B). In summary, the saponins derived from black ginseng, when obtained during the seventh processing cycle, exhibit a heightened capacity to modulate immune cells more completely.

## Discussion

The initial preparation of black ginseng took place in South Korea, after which its usage spread extensively to

China and many Southeast Asian nations (Metwaly *et al.*, 2019). Throughout this study, individuals employed a multitude of processing techniques (Metwaly *et al.*, 2019). The utilization of the nine-time steaming and drying method in this study is based on its historical significance as a traditional technique for processing herbs in TCM (Wang *et al.*, 2009). However, regardless of the type of processing technique employed, the temperature and duration of steaming and drying are the most crucial factors to consider (Jo *et al.*, 2014). The ginsenoside profile of ginseng underwent significant alterations as a result of processing aimed at achieving a black coloration, both externally and internally, as evidenced by chemical analysis (Nam *et al.*, 2012). The level of browning in ginseng increases as the intensity of steaming treatment rises, primarily due to the reduction in moisture content and the subsequent carbonization resulting from repeated cycles of steaming and drying. Simultaneously, the hydrolysis and decomposition of sucrose occurs, resulting in the formation of glucose and fructose. During successive steaming



**Figure 7.** The enumeration of migrating macrophages and neutrophils. (A) Neutrophils. (B) Macrophages. \*Control versus Model, \* $p < 0.05$ , \*\*\*\* $p < 0.0001$ ; # Model versus Treatment, #  $p < 0.05$ , ##  $p < 0.01$ , ####  $p < 0.0001$ ; + Comparison of different cycles, + $p < 0.05$ , ++ $p < 0.01$ ; ns, no significant difference.

cycles, the interaction between glucose, fructose, and free amino acids leads to a chemical reaction known as the “Maillard reaction” (Jin *et al.*, 2015; Kim *et al.*, 2013; Oh *et al.*, 2021). This reaction occurs when the carbonyl group of the reducing sugar reacts with the free amino group of the amino acid, resulting in browning. Nevertheless, the elevation in temperature and the frequency of processing instances lead to the occurrence of a carbonization reaction in ginseng, resulting in the generation of the hazardous compound known as benzo(a)pyrene (Jo *et al.*, 2009). Hence, the examination of black ginseng holds significance not only in relation to its biological efficacy but also necessitates comprehensive investigation into its safety aspects.

However, quantitative testing on benzopyrene was not conducted in this study for the following reasons: One aspect of our study primarily centered on investigating the impact of steaming and drying cycles on the immunomodulatory properties of black ginseng. On the other hand, drawing from prior research, the steaming and drying temperatures utilized in our experiment resulted in a lower level of benzopyrene, falling well within the established safety thresholds (Jo *et al.*, 2009; Lee and Shim, 2007). Moreover, when considering the practical utilization of ginseng in the context of healthy food, it is advised to consume 2 to 3 g of dried plant material or 3 to 9 g of tea dosage daily (Yennurajalingam *et al.*, 2015). However, it is worth noting that there is a dearth of comparable evaluations regarding the safe dosage of black ginseng, and the potential impacts of benzopyrene at this dosage remain uncertain. This implies that the comprehensive assessment of the functional properties of active constituents and the safety concerns associated with toxic constituents following the processing of black

ginseng remains challenging at present. Hence, further research is warranted to do a comprehensive and thorough examination of safety in the future.

Ginsenosides are the primary biologically active compounds found in ginseng (Ratan *et al.*, 2021). Several pharmacopeias in various nations and regions have designated certain markers, such as Rg1, Rb1, and Re, as indicators of high content and quality in ginseng (Commission, 2016; Commission, 2020; Committee, 2011; Committee, 2013; Convention, 2000). However, our analysis reveals that the levels of those monomeric ginsenosides in black ginseng exhibit a notable drop as the processing cycle increases. In contrast, the concentrations of Rg3, Rg5, and Rk1 experience a considerable increase, which is consistent with prior research findings (Jo *et al.*, 2014). Following the application of heat treatment to ginseng, an internal denaturation reaction is expected to take place (Chen *et al.*, 2020). The malonyl residues present in the molecular structure of protopanaxadiol saponins Rb1, Rc, Rb2, and Rd exhibit thermal instability and are susceptible to hydrolysis upon exposure to steam. This hydrolysis process leads to the conversion of these malonyl residues into Rg3. Following the process of dehydration, a conversion occurs resulting in the formation of Rg5 and Rk1 from Rg3 (Lee *et al.*, 2015). The initial concentration of typical saponins found in ginseng tends to diminish after successive processing, while the proportion of infrequent saponins formed because of events such as demalonylation, deglycosylation, and hydrolysis tends to rise (Piao *et al.*, 2020). This implies that future investigations should thoroughly examine the potential impacts of Rg3, Rg5, Rk1, and other relevant factors on the quality assurance of black ginseng.

Recently, scientists have put forth the notion that the therapeutic regulation of herbs and their extracts ought to encompass more than just the identification of key compounds. They argue that it is equally important to assess their biological activities and develop a quality evaluation framework capable of elucidating the correlation between indicators and activities (Kang *et al.*, 2019). The primary biological activity of ginseng lies in its capacity to modulate the immune system (He *et al.*, 2018). Consequently, an anti-inflammatory assessment was conducted using the zebrafish tail-fin amputation model to investigate the impact of black ginseng saponins on the functionality of innate immune cells. This is to get a deeper understanding of the variances in biological activity among black ginseng samples derived from various processing cycles. Furthermore, the utilization of transgenic zebrafish has proven to be a potent methodology for investigating the anti-inflammatory and immunomodulatory properties of ginsenoside extracts and monomeric ginsenosides, allowing rapid and visual evaluation (Sun *et al.*, 2020). Based on the chemical analysis and bioactivity analysis conducted in this study, it can be inferred that there is a positive correlation between the duration of the processing cycle and the levels of Rg3, Rg5, and Rk1. Once the concentrations of the three components have reached a specific threshold, namely at the completion of the fourth cycle, the discernible disparity in the regulatory efficacy of black ginseng saponin extracts on neutrophils becomes less apparent. However, it is only when the concentrations of Rg3, Rg5, and Rk1 reach their maximum levels, specifically after the completion of the seventh cycle, that the saponin extract derived from black ginseng will exert a notable influence on the activity of macrophages. This observation suggests that there may be a positive relationship between the combined levels of Rg3, Rg5, and Rk1 present in the extract and its capacity to modulate the immune system. While it is acknowledged that other ginsenoside components may also influence the immunomodulatory activity of the samples (He *et al.*, 2020), our research demonstrates the correlation between the processing cycle and the content and biological activity of the three aforementioned ginsenosides. Furthermore, many reports have revealed that Rg3, Rg5, and Rk1 exert an influence on neutrophils or macrophages during the anti-inflammatory process (Heo *et al.*, 2023; Kang *et al.*, 2018; Kim *et al.*, 2012; Lee *et al.*, 2013b; Shen *et al.*, 2017; Xiong *et al.*, 2022; Yu *et al.*, 2017).

Rg3, Rg5, and Rk1 exhibit diverse pharmacological properties, encompassing anti-tumor effects (Choi *et al.*, 2015), immune regulatory capabilities (Yoo *et al.*, 2021), and the potential to ameliorate kidney injury through the modulation of inflammation and apoptosis (Park *et al.*, 2015). Prior research has indicated that the potential mechanism underlying the whitening effects of ginsenosides Rg5 and Rk1 could include the activation

of the MEK-ERK signaling pathway (Jin *et al.*, 2018). Furthermore, it has been observed that the inhibition of TGF- $\beta$ 1-induced epithelial-mesenchymal transition and the promotion of stem-like characteristics are achieved through the downregulation of Smad2/3, NF- $\kappa$ B, ERK, p38 MAPK, and JNK signaling pathways in non-small cell lung cancer by Rk1 and Rg5 (Kim *et al.*, 2021). The administration of Rg3 resulted in a reduction of the upregulation of pro-inflammatory markers, including tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , and cyclooxygenase (COX-2), within the hippocampus (Lee *et al.*, 2013a). Apoptosis was observed to be produced by Rg3, which was found to activate caspase-3, caspase-8, and caspase-9, while also modulating the expression of Bcl-2 and Bax (Park *et al.*, 2014). In addition, the administration of Rg5 resulted in the inhibition of TNF- $\alpha$  and IL-1 $\beta$  production, as well as a decrease in the expression of NF- $\kappa$ B, p65, and COX-2 in mice with renal inflammation induced by cisplatin (Li *et al.*, 2016). Similarly, the administration of Rk1 demonstrated a considerable inhibitory effect on the excessive production of TNF- $\alpha$  and IL-1 $\beta$ , as well as a reduction in the expression of inducible nitric oxide synthase and COX-2 in hepatic tissues of mice (Hu *et al.*, 2019). Furthermore, it should be noted that Rk1 exhibits inhibitory effects on NMDA receptors, thus presenting itself as a potentially valuable therapeutic intervention for pathological conditions associated with neuronal excitotoxicity (Ryoo *et al.*, 2020). Pharmacokinetic studies reveal a notable finding, indicating that Rg3, Rg5, and Rk1 exhibit superior rates of absorption compared to Rb1, Rb2, and Rc (Yoo *et al.*, 2021). This presents compelling evidence that Rg3, Rg5, and Rk1 exert their effects on pathways associated with inflammation and the immune response, underscoring the significant biological impact of these ginsenoside molecules. Hence, it is postulated that the Rg3, Rg5, and Rk1 could serve as significant indicators in the realm of black ginseng quality control, particularly in enhancing comprehension of the quality assessment pertaining to the immunomodulatory efficacy of black ginseng products.

Extensive studies have been conducted on the processing technology of black ginseng, with a primary emphasis on the identification and alteration patterns of chemical constituents (Metwaly *et al.*, 2019). The observed alterations in the compositions of Rg3, Rk1, and Rg5, as demonstrated in this investigation, align with the findings reported in prior investigations (Yoo *et al.*, 2021). Prior research has also assessed the efficacy of black ginseng, nevertheless, the scope of that research was restricted to the examination of the biological activity of black ginseng samples using certain processing techniques (Lee *et al.*, 2021). The investigation of the correlation between various processing cycles and the biological activity of black ginseng remains largely unexplored. Consequently,

we have incorporated a study of alterations in chemical composition and biological activity to comprehend the comprehensive effects of processing on both aspects within this framework. Additionally, we seek to investigate the relationship between changes in composition and activity. It is imperative to highlight that the findings of this study do not provide support for the necessity of implementing fixed cycles in the processing of black ginseng. Instead, they underscore the significance of effectively managing the steaming and drying process to evaluate the quality of black ginseng. The findings of this study offer novel perspectives on the significant scientific matters about the exploration of the correlation between black ginseng processing and quality control. Consequently, this study contributes to the advancement of research in quality control for black ginseng, as well as medicinal and health products that incorporate black ginseng as a primary constituent.

## Conclusions

The present work conducted a complete analysis of the chemical properties and biological activities of black ginseng saponin extracts, with a focus on evaluating their quality. The evaluation was carried out by comparing extracts prepared using various steaming and drying cycles. It has been observed that the extract's capacity to modulate neutrophils and macrophages achieves its maximum efficacy when the cumulative concentration of Rg3, Rg5, and Rk1 is elevated. The optimal adjustment in our investigation was observed in black ginseng samples following the completion of the seventh processing cycle. It is important to highlight that this does not imply that the processing of black ginseng necessitates the implementation of a specific procedure. The findings of this study serve as a catalyst for further investigation into the correlation between the processing of black ginseng and quality control, with a focus on the composition and biological effectiveness of certain ginsenosides. Further optimization of this proof-of-concept study is necessary, including augmenting the sample size and expanding the detection range of saponin components and biological activities. Furthermore, the safety evaluation of consuming black ginseng remains a significant area of interest for future investigations. To gain a deeper understanding of the benzopyrene generated during the processing of black ginseng, it is recommended to perform thorough research employing mammalian models that exhibit a more reliable correlation with human dose conversion. Subsequent research endeavors may delve into a more focused examination of the biological properties exhibited by individual saponins, namely Rg3, Rg5, and Rk1, which undergo an augmentation in black ginseng because of processing. In addition, the application of omics technology enables the investigation of

the regulatory mechanism behind the anti-inflammatory and immunomodulatory activity of black ginseng. This approach facilitates a comprehensive exploration of the associated action pathways and the potential existence of synergistic interactions among its constituent monomers. Overall, these enhancements will enable a more extensive exploration of the quality control measures for black ginseng. This work offers novel insights into enhancing the quality of black ginseng products, thereby contributing to the advancement of quality control measures for health foods and dietary supplements that utilize black ginseng as their primary constituent.

## Author contributions

Mengmeng Sun and Min He designed the study. Lulu Wang, Meijie Yu, and Yao Fu conducted a chemical analysis. Lulu Wang and Meijie Yu conducted bioactivity tests and statistical analysis. Li Li prepared black ginseng materials. Xianghe Meng provided modeling inflammation in zebrafish. Lulu Wang drafted the manuscript. Jian Yang contributed to revisions of the manuscript. All authors read and approved the final manuscript.

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## Data availability statement

The datasets used in this study are available from corresponding authors upon reasonable request.

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## Conflicts of interest

Authors declare no conflicts of interest.

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## Supplementary

Table S1: LOD, LOQ, and R<sup>2</sup> value of identified 17 ginsenosides.

Compound name	R <sup>2</sup>	LOD (mg/mL)	LOQ (mg/mL)
Ginsenoside Re	0.99039	0.06357	0.19264
Ginsenoside Rg1	0.99381	0.05605	0.16985
Ginsenoside Rf	0.99211	0.13511	0.40943
Ginsenoside Rb1	0.99229	0.40068	1.21419
Ginsenoside Rg2	0.99416	0.20324	0.61588
Ginsenoside Rh1	0.99747	0.17181	0.52064
Ginsenoside Ro	0.98506	0.07132	0.21613
Ginsenoside Rc	0.99610	0.26075	0.79015
Ginsenoside Rb2	0.99227	0.21201	0.64244
Ginsenoside Rb3	0.99313	0.21329	0.64635
Ginsenoside F1	0.99214	0.35095	1.06348
Ginsenoside Rd	0.99089	0.11467	0.34747
Ginsenoside Rg3	0.99063	0.09544	0.28920
Ginsenoside Rk1	0.99319	0.03139	0.09513
Ginsenoside Rg5	0.99408	0.03575	0.10834
Ginsenoside S-Rh2	0.99227	0.04320	0.13089
Ginsenoside R-Rh2	0.99403	0.06038	0.18297

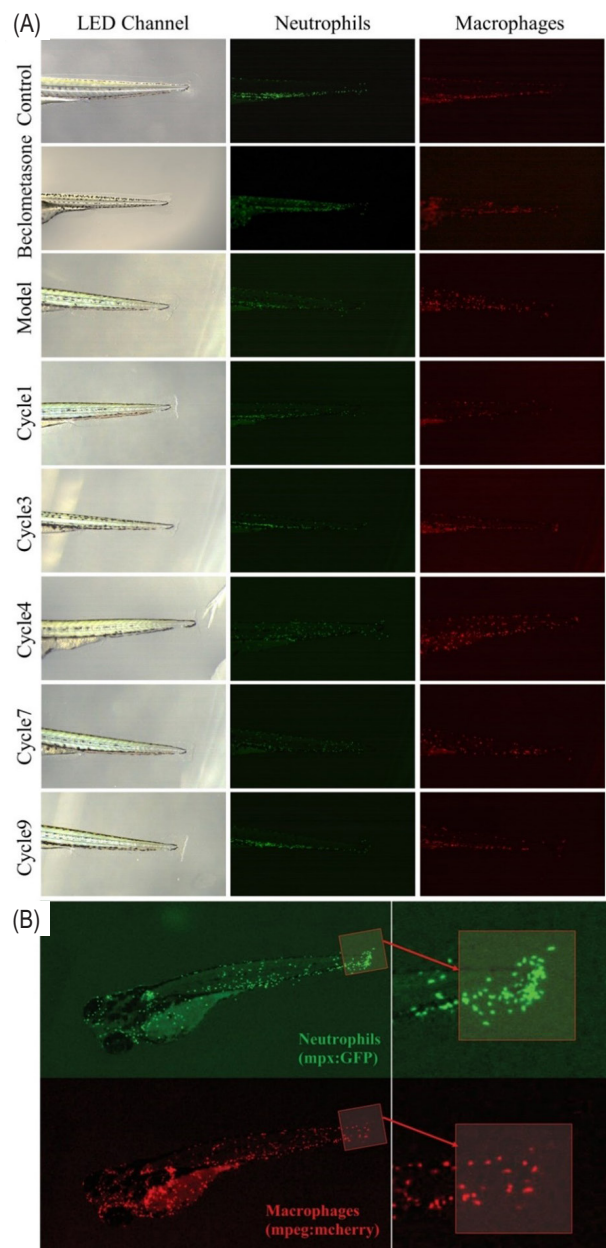


Figure S1. (A) Schematic diagram of observing neutrophils and macrophages in the tail of zebrafish larvae. (B) The neutrophils that have migrated can be observed as a green hue surrounding the wound region, whereas the migrating macrophages are discernible as a red shade in the vicinity of the wound.