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EXPERIMENTAL RESEARCH

Study on the anti-inflammatory activity of chaga mushroom aqueous extract-alcohol precipitate

ZHONG Xiuli, WEI Chanming[1], CHEN Jiageng[2], ZHOU Shuyuan[3], CAO Qiuyang[4], HUANG Xiaoting[3], LIU Ningzhi[1], WANG Chengxi[1,4*], MEI Wenjie[1,4]

[1]*School of Pharmacy, Guangdong Pharmaceutical University, Guangzhou 510006, China;*

[2]*School of Life Science and Biopharmacology, Guangdong Pharmaceutical University, Guangzhou 510006, China;*

[3]*Guangzhou Ruby Biotechnology Co. Ltd, Guangzhou 510006, China;*

[4]*Guangdong Molecular Probe and Biomedical Imaging Engineering Technique Research Center, Guangzhou 510006, China*

[Abstract] **Objective** To extract the active ingredient from chaga mushroom (aqueous extract-alcohol precipitates) to study its safety and anti-inflammatory activity. **Methods** The aqueous extraction-alcohol precipitation method was used to extract the active ingredient of chaga mushroom for mass fraction analysis of polysaccharides. Zebrafish embryos were used for the acute toxicity study and the median lethal dose (LD_{50}) was used to assess the safety of the aqueous extract-alcohol precipitates. Physical methods were used to amputate the caudal fins of zebrafish and induce inflammation, and this model was used to study the anti-inflammatory activity. **Results** The LD_{50} of chaga mushroom aqueous extract-alcohol precipitate measured in the acute toxicity study was 6.888 mg/mL. The anti-inflammatory experimental results show that chaga mushroom aqueous extract-alcohol precipitate inhibit the aggregation of neutrophils and macrophages at inflammation sites, differences were statistically significant ($P < 0.05$). **Conclusion** Chaga mushroom aqueous extract-alcohol precipitates exhibits good anti-inflammatory effects within the safe concentrations.

[Key words] Chaga mushroom; Inonotus obliquus; extraction; polysaccharides; safe dose; anti-inflammatory activity

1 Introduction

Inflammation is a defense response to invading pathogens, cell damage, or external stimuli[1]. Usually, inflammation protects the body from bacteria or virus invasion through the secretion of pro-inflammatory cytokines[2]. However, prolonged inflammation is harmful to the body, to a great extent, causing tissue injury, inducing organ failure[3-4], or even leading to cancer[5-6]. Dexamethasone is a widely used anti-inflammatory drug in clinical practice, but long-term treatment can cause various side effects, such as substance metabolism and water-salt metabolic disturbances, thereby inducing hypertension and atherosclerosis.

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[*Corresponding author] Email: gdpuwcx@163.com

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Therefore, there is still a need to find or develop new anti-inflammatory drugs.

Chaga mushroom (Inonotus obliquus (Fr.) Pilat) is also known as clinker polypore and birch conk, and is a medicinal fungi that is parasitic on the trunk of Siberian elm, Southern silky oak, and Japanese white birch[7]. Chaga mushrooms have a diameter of 7 ~ 36 cm with a hard surface and appear yellowish-brown to black. They are rich in polysaccharides, triterpenoids, inotodiol, flavonoids, and saponins, and have a broad range of pharmacological activities[8]. According to the literature, triterpenoids in chaga mushrooms have functions, such as antineoplastic, blood glucose-lowering, blood lipid-lowering, antioxidant, immune-boosting, and so on[9]. Additionally, polysaccharides in chaga mushrooms can improve immunity and have pharmacological effects, such as anticoagulant, anti-aging, and antineoplastic effects[10-11]. However, there are few studies on the anti-inflammatory activity of chaga mushrooms. In this paper, aqueous extraction and alcohol precipitation was used to extract the active ingredients in chaga mushroom, and its safety and anti-inflammatory activity were examined.

2 Equipment and materials

2.1 Equipment

B-220 thermostatic water bath and RE-52AA rotatory evaporator were purchased from Shanghai Yarong Biochemistry Instrument Factory. TE214S electronic analytical balance was purchased from Sartorius Scientific Instruments (Beijing) Co. Ltd. SZ680 zoom-stereo microscope was purchased from Chongqing Optec Instrument Co. Ltd. ZXSD-B1090 biochemical incubator was purchased from Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd. Pipettes were purchased from Sartorius AG (Germany). Z-A-S5 zebrafish breeding system was purchased from Shanghai Haisheng Biotech Co. Ltd. DMi8 fluorescence microscope was purchased from Leica Microsystems GmBH (Germany).

2.2 Materials

Chaga mushroom (place of origin: Russia); absolute ethanol (analytical grade) was purchased from Tianjin Zhiyuan Chemical Reagent Co. Ltd.; phenol (analytical grade) and concentrated sulfuric acid (chemically pure) was purchased from Guangzhou Chemical Reagent Factory; glucose (analytical grade) was purchased from Damao Chemical Reagent Factory; tricaine (mass fraction > 97.0%) was purchased from TCI (Shanghai) Development Co. Ltd.; 3,4-Dichloroaniline (mass fraction: 99%) was purchased from ScienMax Inc.; dexamethasone (mass fraction: 98%) was purchased from Macklin Inc.; distilled water was used for the experiment; the culture solution was self-made (0.54-g sea salt + 100 μL methylene blue + 9 L pure water)[12].

2.3 Animals

AB wild-type and Tg transgenic zebrafish (colora: EGFP) were purchased from the China Zebrafish Resource Center. Zebrafishes were housed in a circulating aquaculture system, where the water temperature was controlled at 28.5 ± 1.0 ℃; pH was 6.9 ~ 7.5, the light-dark cycle was 14h/10h; and they were fed with brine shrimp twice a day in the morning and afternoon.

2.4 Data processing

The Graphpad Prism 8.0.2 software was used for the statistical analysis of the data. A difference of $P < 0.05$ was considered statistically significant. The Bliss method was used to calculate LD_{50}.

3 Methods and results

3.1 Preparation of chaga mushroom aqueous extract-alcohol precipitate

The most commonly used extraction method

for polysaccharides of chaga mushrooms is aqueous extraction and alcohol precipitation[13-14]. This method is simple, consumes less energy, and does not disrupt diverse structures.

The extraction method of Li et al.[15] was used as a reference for this paper: 20 g of dried chaga mushroom was crushed, and 200 mL distilled water was used for extraction at 95 °C. The extraction was conducted four times[15], at an interval of two hours. Following that, vacuum filtration was conducted, and the filtrates were combined and concentrated to 90 mL. Following that, 4 X volumes of absolute ethanol was slowly added with constant vigorous stirring until it became 80% concentrated. The solution was left to stand until complete precipitation occurred. The next day, extraction filtration was conducted. The filtrate was discarded and the filter cake was washed thrice with absolute ethanol after which it was dried at 50 °C for 12 h. Finally, 3.5 g of dried chaga mushroom solid extract was obtained and the extraction rate was 17.5%.

3.2 Measurement of chaga mushroom polysaccharide mass fraction

According to the literature[16], the main components of chaga mushroom aqueous extract-alcohol precipitates are polysaccharides, reducing sugars, proteins, ash content, etc, and polysaccharides had the highest mass fraction. Therefore, phenol-sulfuric acid spectrophotometry was employed to measure the mass fraction of chaga mushroom aqueous extract-alcohol precipitates in this paper.

3.2.1 Plotting of standard curves

The experimental protocol of Li et al.[15] was used as a reference. 100 mg glucose was weighed, and distilled water was added to a volume of 100 mL. The mass concentration of glucose was 1 mg/mL. 0.0, 0.5, 1.0, 1.5, 2.0, and 2.5 mL of reference solution was precisely measured and top up to 25 mL with distilled water. Following that, 2.0 mL was added to a test tube, and 1.0 mL of 6% phenol was added. The test tube was oscillated before 5.0 mL of H_2SO_4 was added, after which the test tube was rapidly oscillated to mix evenly. The test tube was then heated in a water bath for 15 min before cooling. Absorbance was measured at 490 nm. The regression analysis was conducted with absorbance being the y-axis and glucose mass concentration being the x-axis to obtain the regression formula $Y = 13.921X - 0.026\ 6$, $R^2 = 0.997\ 4$. See Fig. 1 for the standard curve.

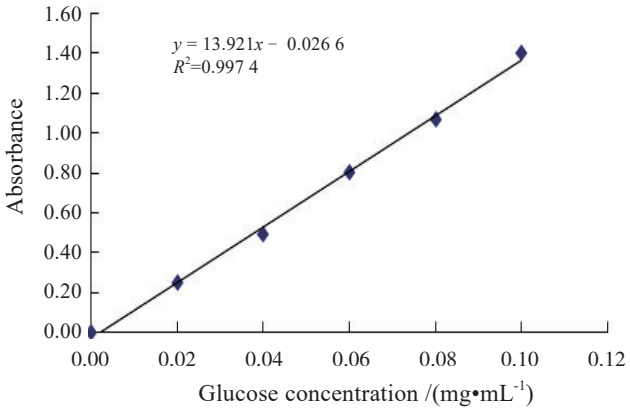

Fig. 1 Standard curve of glucose solution

3.2.2 Preparation of chaga mushroom polysaccharides test solution

10.0 mg of chaga mushroom aqueous extract-alcohol precipitate from section 3.1 was precisely weighed, and distilled water was added to top it up to 100 mL. The solution was mixed evenly.

3.2.3 Measurement of chaga mushroom polysaccharide mass concentration

1.0 mL of the aforementioned sample solution was precisely pipetted into a stoppered test tube. Distilled water was added to 2.0 mL, and the absorbance measurement was conducted based on the steps in section 3.2.1. Based on the calculation of the regression formula, the mass concentration of polysaccharides in chaga mushroom aqueous extract-alcohol precipitate was 18.9%, which was consistent with the literature[17].

3.3 Harvesting of zebrafish embryos

Sexually mature zebrafish [AB wild-type zebrafish or Tg (colora: EGFP) transgenic zebrafish] were placed in a mating tank with a divider in a female: male ratio of 1∶1 or 1∶2. The following morning, the divider was removed, and light stimulation was conducted. After zebrafishes had mated naturally for 1.5 h, zebrafish embryos were harvested and placed in a 28 ± 0.5 ℃ thermostatic incubator for incubation[12].

3.4 The acute toxicity study of chaga mushroom aqueous extract-alcohol precipitate in zebrafishes

Based on the preliminary experiment results, the mortality rate of 2.5 and 10 mg/mL chaga mushroom aqueous extract-alcohol precipitates in 96 hours post-fertilization (hpf) was 0% and 100%; therefore, five concentration groups were set up from 2.5 to 10 mg/mL, which were 2.5, 3.536, 5, 7.071, and 10 mg/mL (prepared using culture solution as the solvent). Additionally, a positive control group (4 mg/L 3,4-Dichloroaniline)[18] and a blank control group (culture solution) were set up.

The 6-8 hpf healthy AB zebrafish embryos were selected under a stereo microscope and added to a 6-well culture plate, with 20 embryos per well. After removing the culture solution from the wells, 5.0 mL of sample solution was added to every well, and the plate was incubated in a 28 ± 0.5 ℃ thermostatic incubator. Parallel triplicates were set up for the experiment.

The stereo microscope was used to observe the zebrafish embryos' developmental status at 24, 48, 72, and 96 hpf. The number of survived embryos, embryos with malformations, and the hatching status were recorded. In addition, the cumulative survival rate, cumulative hatching rate, and cumulative malformation rate were recorded. Results are shown in Fig. 2 to 5 and Tables 1 to 3.

The positive control group (4 mg/mL 3.4-dichloroaniline) showed significant malformation toxicity at 96 hpf, and the malformation rate was 100.0% ($P < 0.001$), where the malformations included pericardial edema, yolk sac edema, and spinal curvature.

At 96 hpf, 10 mg/mL chaga mushroom alcohol sedimentation solution was a significantly lethal to all embryos, and the lethality rate was 100.0% ($P < 0.001$); 7.071 mg/mL chaga mushroom alcohol sedimentation solution was significantly lethal to most embryos, and the lethality rate was 61.7% ($P < 0.001$); 5 mg/mL chaga mushroom alcohol sedimentation solution was significant lethal to some embryos, and the lethality rate was 11.7% ($P < 0.05$), which inhibited hatching, with a hatching rate of 0% ($P <0.001$); 3.536 mg/mL chaga mushroom alcohol sedimentation solution had significantly inhibited hatching, where the hatching rate was 0% ($P < 0.001$).

From the start of embryo development to 96 hpf, there was no significant difference ($P > 0.05$) in malformation rate and lethality rate of zebrafish embryos from the 2.5 mg/mL chaga mushroom alcohol sedimentation solution compared with that of the control group, indicating that the safe concentration may be 2.5 mg/mL or lower. In this paper, the Bliss method was used to calculate the LD_{50} of chaga mushroom alcohol sedimentation solution to be 6.688 mg/mL (Fig. 6).

The results of the acute toxicity experiment of chaga mushroom aqueous extract-alcohol precipitate in zebrafishes demonstrated that the safe concentration of chaga mushroom aqueous extract-alcohol precipitate $\leqslant$ 2.5 mg/mL with an LD_{50} of 6.688 mg/mL.

3.5 Impact of chaga mushroom aqueous extract-alcohol precipitate on inflammatory cells

One night before the experiment, fluorescent Tg (colora:EGFP) transgenic zebrafishes were selected under a fluorescence microscope as

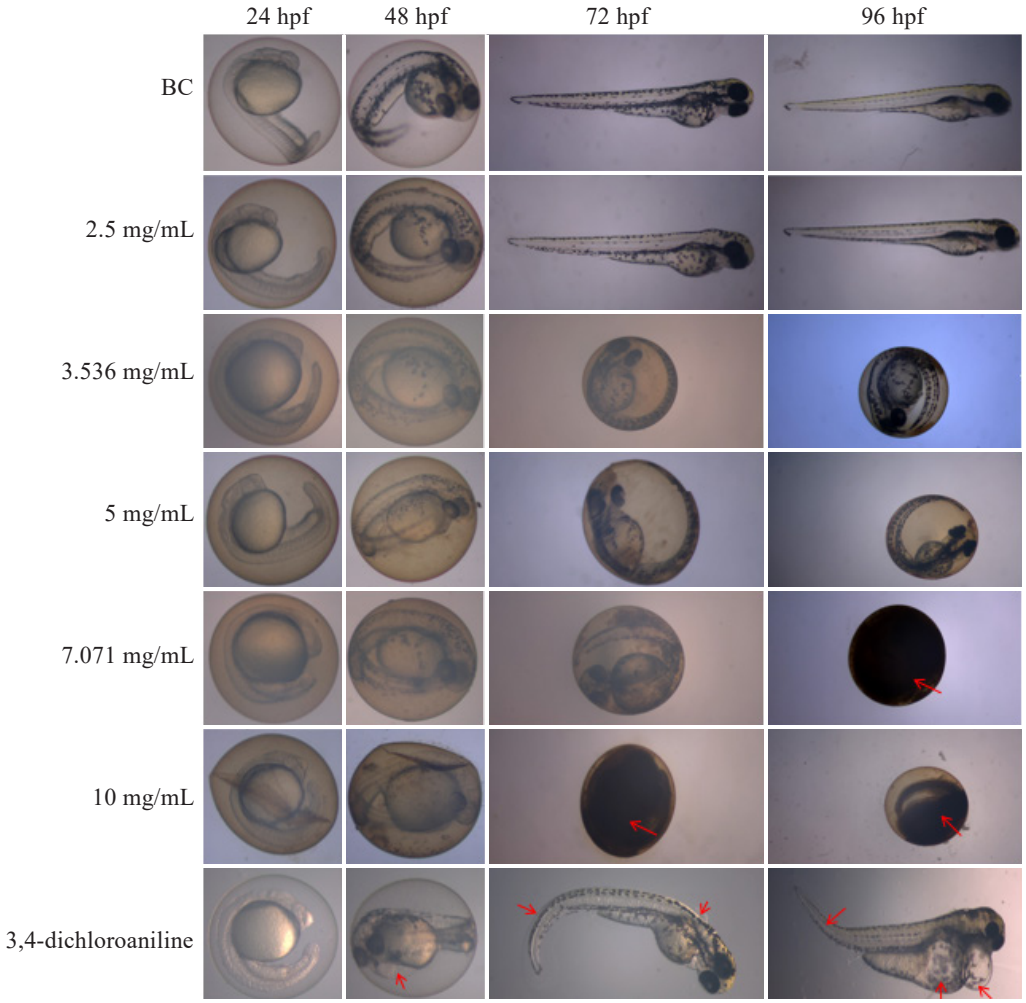

Fig. 2 Impacts of various mass concentrations of chaga mushroom aqueous extract-alcohol precipitate on the development of zebrafish embryo (magnification: 30X)

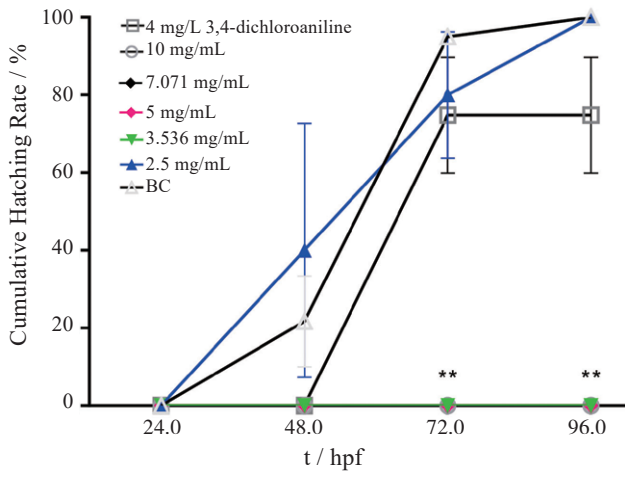

Fig. 3 Impacts of various mass concentrations of chaga mushroom aqueous extract-alcohol precipitate on cumulative hatching rate of zebrafish

Hatching rate = number of hatched embryos / number of surviving embryos ×100%; Data was expressed as $\bar{X} \pm$ SEM; $^{**}P < 0.01$

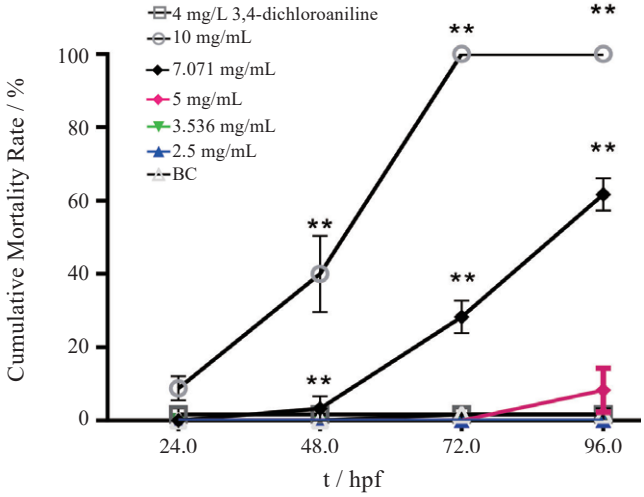

Fig. 4 Mortality rate at various concentrations of chaga mushroom aqueous extract-alcohol precipitate

Mortality rate = (number of dead embryos / N ×100%; Data was expressed as $\bar{X} \pm$ SEM; $^{**}P < 0.01$

a biological model for the inflammation experiment. An anti-inflammatory activity study was conducted according to the experimental protocol of Elks[19].

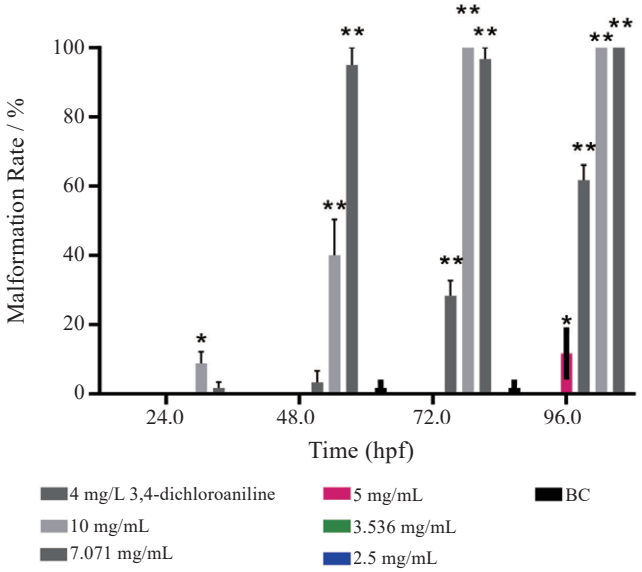

Fig. 5 Malformation rate at various concentrations of chaga mushroom aqueous extract-alcohol precipitate

Malformation rate = (number of dead embryos + number of malformed embryos) / N ×100. Data was expressed as $\bar{X}$ ± SEM; $^{**}P < 0.01$

3.5.1 Inhibitory effects of chaga mushroom aqueous extract-alcohol precipitate on inflammatory cell aggregation

Based on the acute toxicity study results,

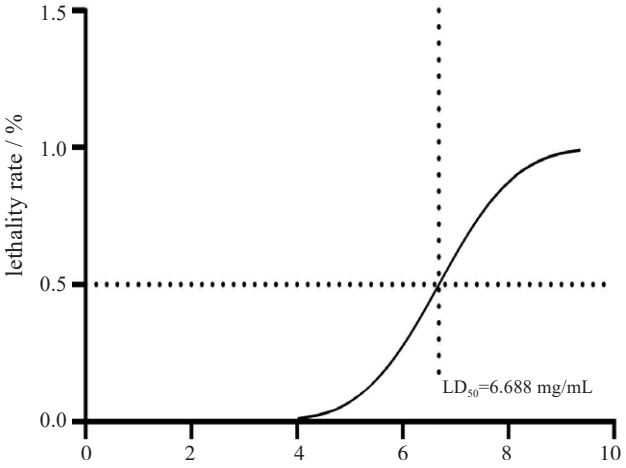

Fig. 6 LD_{50} of chaga mushroom aqueous extract-alcohol precipitate

Table 1 Cumulative hatching rate of zebrafishes under different mass concentrations of chaga mushroom aqueous extract-alcohol precipitate $\bar{X}$ ± SEM, %

	BC	2.5 mg/mL	3.536 mg/mL	5 mg/mL	7.071 mg/mL	10 mg/mL	4 mg/L 3,4-dichloroaniline
24 hpf	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
48 hpf	21.7 ± 11.7	40.0 ± 32.7$^{\#\#}$	0.0 ± 0.0**	0.0 ± 0.0**	0.0 ± 0.0**	0.0 ± 0.0**	0.0 ± 0.0**
72 hpf	95.0 ± 0.0	80.0 ± 16.3*	0.0 ± 0.0***	0.0 ± 0.0***	0.0 ± 0.0***	0.0 ± 0.0***	74.8 ± 14.9*
96 hpf	100.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0***	0.0 ± 0.0***	0.0 ± 0.0***	0.0 ± 0.0***	74.8 ± 14.9*

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with the control group.

Table 2 Mortality rate of different mass concentrations of chaga mushroom aqueous extract-alcohol precipitates $\bar{X}$ ± SEM, %

	BC	3.536 mg/mL	5 mg/mL	7.071 mg/mL	10 mg/mL	4 mg/L 3,4-dichloroaniline
24 hpf	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	8.8 ± 3.3	1.7 ± 1.7
48 hpf	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.3 ± 3.3	40.0 ± 10.4***	1.7 ± 1.7
72 hpf	1.7 ± 1.7	0.0 ± 0.0	0.0 ± 0.0	38.7 ± 4.4***	100.0 ± 0.0***	1.7 ± 1.7
96 hpf	1.7 ± 1.7	0.0 ± 0.0	8.3 ± 6.0	61.7 ± 4.4***	100.0 ± 0.0***	1.7 ± 1.7

$^{**}P < 0.01$; *** P 0.001 when compared with the control group.

Table 3 Malformation rate of different mass concentrations of chaga mushroom aqueous extract-alcohol precipitates $\bar{X}$ ± SEM, %

	BC	3.536 mg/mL	5 mg/mL	7.071 mg/mL	10 mg/mL	4 mg/L 3,4-dichloroaniline
24 hpf	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	8.8 ± 3.3	1.7 ± 1.7
48 hpf	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.3 ± 3.3	40.0 ± 10.4***	95.0 ± 5.0***
72 hpf	1.7 ± 1.7	0.0 ± 0.0	0.0 ± 0.0	38.7 ± 4.4***	100.0 ± 0.0***	96.7 ± 3.3***
96 hpf	1.7 ± 1.7	0.0 ± 0.0	11.7 ± 5.0*	61.7 ± 4.4***	100.0 ± 0.0***	100.0 ± 0.0***

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with the control group.

different mass concentrations of chaga mushroom aqueous extract-alcohol precipitate group (0.625, 1.25, 2.5 mg/mL), a negative control group (culture solution without tail amputation), a model control group (culture solution with tail amputation), and a positive control group (250 μmol/L and tail amputation) were set up. 72-hpf Tg (colora:EGFP) transgenic zebrafishes were pretreated in the various solution groups for an hour.

Following that, tricaine solution was used for anesthesia, and a sharp surgical blade was used to amputate half the caudal fin of zebrafishes (except for the negative control group) to construct the inflammation model of zebrafish. The constructed zebrafish models were randomly added to 5 mL control group and different mass concentrations of chaga mushroom aqueous extract-alcohol precipitate solution for six hours of treatment. After exposure, macrophage and neutrophil aggregation were observed using a fluorescence microscope, and photographs were taken.

Compared with the negative control group, the inflammatory cell counts of the other groups were significantly increased, indicating that the zebrafish inflammation model was successfully constructed.

Compared with the model control group, the number of neutrophils and macrophages that aggregated at the tail wound after treatment with chaga mushroom aqueous extract-alcohol precipitate was decreased at the early stage of inflammation, and this difference was statistically significant ($P< 0.05$). The results of the chaga mushroom aqueous extract-alcohol precipitate groups and the dexamethasone positive control group were identical, demonstrating that chaga mushroom aqueous extract-alcohol precipitate can inhibit the aggregation of neutrophil and macrophages (Fig.7).

3.5.2 Clearance effects of chaga mushroom aqueous extract-alcohol precipitate on inflammatory cells

Tricaine solution was used for anesthesia, and a sharp surgical blade was used to amputate half the caudal fin of zebrafishes (except for the negative control group) to construct the zebrafish inflammation model.

Based on acute toxicity study, different mass concentrations of chaga mushroom alcohol sedimentation groups (0.625, 1.25, 2.5 mg/mL), a negative control group (culture solution without tail amputation), a model control group (culture

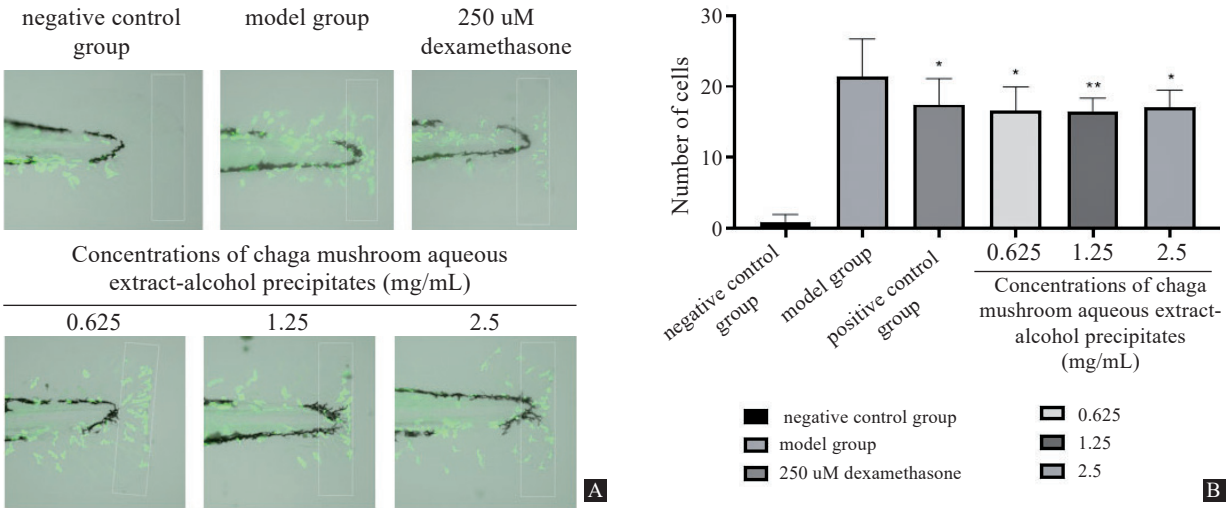

Fig. 7 Impact of chaga mushroom aqueous extract-alcohol precipitate on neutrophil and macrophage aggregation

A. Inflammatory cell aggregation in the caudal fin of various groups; B. Quantitation of inflammatory cell aggregation in the caudal fin * $P < 0.05$, ** P 0.01 when compared with the model control group.

solution with tail amputation), and a positive control group (250 μmol/L and tail amputation) had been set up. After the model was constructed for 6h, 72 hpf zebrafishes were placed in various groups for treatment. The clearance of macrophage and neutrophil (reverse migration) was observed using a fluorescence microscope, and photographs were taken.

At the middle and late stages of inflammation, there was no significant difference in neutrophils and macrophages at the tail wound of zebrafishes between the chaga mushroom aqueous extract-alcohol precipitates and dexamethasone positive control groups when compared with that of the model control group ($P > 0.05$), demonstrating that chaga mushroom aqueous extract-alcohol precipitates and dexamethasone does not promote clearance of inflammatory cells (Fig. 8).

4 Discussion

Dexamethasone is a commonly used anti-inflammatory glucocorticoid that inhibits the extravasation and aggregation of neutrophils and macrophages at inflammation sites, thereby inhibiting the activity of these cells to conduct anti-inflammatory effects. However, its use is limited by side effects, such as gastrointestinal irritation, nausea, vomiting, myasthenia, amyotrophy, and hypokalemia. People are increasingly hoping for safer and more effective drugs, and medicinal fungi stand out due to its broad bioactivities and safety. Examples of those include Ophiocordyceps sinensis, Tremella fuciformis, and Ganoderma lingzhi. Chaga mushroom is a medicinal and edible fungi. Studies have shown that it has antineoplastic, antiviral, antioxidant, hepatoprotective, blood glucose-lowering, and blood lipid-lowering effects[20-24]. However, there are few studies on its anti-inflammatory effects.

In this study, the aqueous extraction and alcohol precipitation method were used to extract the active ingredient of chaga mushroom, and the extraction rate was 17.5%. The polysaccharide mass fraction of the extracted active ingredient was measured to be 18.9%, which was consistent with the value reported in the literature[17]. This shows that the aqueous extraction and alcohol precipitation can be used to extract the active ingredient in chaga mushrooms. According to reports, the main components of chaga mushroom aqueous extract-alcohol precipitate also include reducing sugars, proteins, and ash content in addition to polysaccharides (with highest mass fraction). This study did not further confirm the

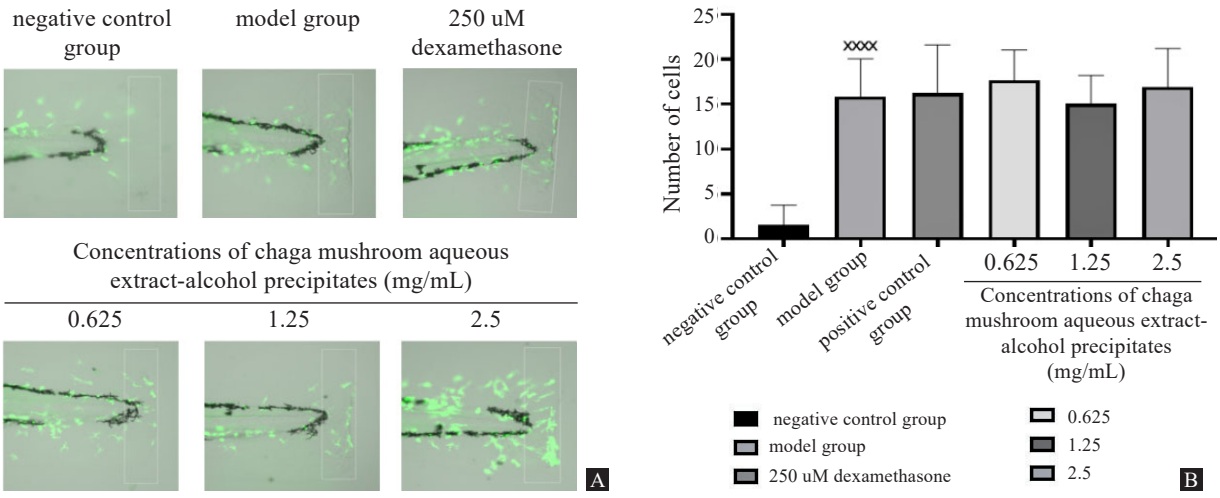

Fig. 8 Impact of chaga mushroom aqueous extract-alcohol precipitate on neutrophil and macrophage clearance

A. Inflammatory cell clearance in the caudal fin of various groups; B. Quantitation of inflammatory cell clearance in caudal fin

mass fractions of other components, which should be further studied.

Zebrafish's inflammation models mainly include the injury-induced inflammation model, bacterial infection model, and viral infection model[25]. One of the traumatic inflammation models was constructed by cutting off the ventral or caudal fins of transgenic zebrafish[25] and so on to induce an inflammatory immune response in the organism. The main characteristic of inflammation is the recruitment and aggregation of inflammatory cells to inflammation sites. Six hours after injury, neutrophil and macrophage counts at the site reach a peak in zebrafishes. 12 h after injury, neutrophil, and macrophage counts start to decrease[25]. In this study, a zebrafish inflammation model was constructed to examine the anti-inflammatory activity of chaga mushroom aqueous extract-alcohol precipitate. Results showed that chaga mushroom aqueous extract-alcohol precipitate and dexamethasone can both inhibit aggregation of neutrophils and macrophages at inflammation sites and exhibit anti-inflammatory effects.

The study subject of this paper was only the chaga mushroom aqueous extract-alcohol precipitate, and further studies must examine specific components and the activity of single components after purification.

5 Conclusion

Medicinal fungi contain substances with therapeutic effects in human diseases and are safer than clinical chemotherapy. This study showed that chaga mushroom aqueous extract-alcohol precipitates can effectively inhibit aggregation of inflammatory cells at inflammation sites *in vivo*, and exhibit significant anti-inflammatory effects, which have some research value.

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