Study on the oxidative stress mechanism mediated by lead acetate in Pinelliaternata based on zebrafish model

Abstract: Pinelliaternata, as a toxic traditional Chinese medicine, contains various active ingredients such as alkaloids, which can inhibit oxidative stress mechanisms. The experiment used zebrafish models and other methods to study the changes and effects of oxidative stress levels in organisms, and explored the mechanism of Pinelliaternata in inhibiting oxidative stress, laying the foundation for the development of antioxidants using Pinelliaternata as raw material.
Keywords: Pinelliaternata; Oxidative stress; Zebrafish model; fluorescent probe

Introduction

As a toxic traditional Chinese medicine, Pinelliaternata (Thunb.) Breit. is a dried tuber of the Araceae plant Pinelliaternata (Thunb.) Breit. It contains various chemical components, including alkaloids, organic acids, volatile oils, flavonoids, steroids, and sugars. It has the effects of reducing inflammation, inhibiting heart rate, temporarily lowering blood pressure, inhibiting the central nervous system, and anti-tumor.

Oxidative stress refers to a state in which there is an imbalance between oxidation and antioxidant activity in the body, with a tendency towards oxidation. The presence of oxygen free radicals and other active oxidants in the intracellular and extracellular environment can cause damage to cells, leading to stress reactions and the production of large amounts of oxidative intermediates, which can damage and destroy various tissues in the body and trigger a series of diseases.

This project takes Pinelliaternata as the starting point, adopts the zebrafish model, and sets evaluation indicators for each stage and level of oxidative stress mechanism to explore its inhibitory effect on oxidative stress mechanism [5].

1 Materials and Instruments

1.1 Materials

Pinelliaternata concentrated powder (20:1, purchased by Henan Longteng Bioengineering Company); Lead acetate trihydrate and copper sulfate pentahydrate (quality purity>98%, purchased from Chengdu Cologne Chemical Reagent Factory); Sudan Black B staining solution (quality purity>98%, purchased from Shanghai Aladdin Reagent Company); DCFH-DA fluorescent probe (purchased by Shanghai Koyizhe Laboratory); DPPP fluorescent probe (purchased by Shanghai Aladdin Reagent Company); AO fluorescent probe (purchased by Zhenjiang Baikeses Company);

1.2 Instruments

Conventional biological microscope (Shanghai Optoelectronic Technology Co., Ltd.); Inverted fluorescence microscope (Olympus Corporation);

2 Experimental section

2.1 Embryo toxicity experiment

Select AB zebrafish that develop normally 7-9 hpf after fertilization under a microscope, and randomly transfer them to a 12 well plate containing different concentrations of Pinelliaternata solution, with 30 fish per well and 3 replicates set up. Incubate in a constant temperature incubator at 28 ℃, replace with a new solution every 24 hours after administration, and incubate until 72 hpf. Calculate the minimum lethal concentration (LC100) and maximum non

lethal concentration (LC0) of Pinelliaternata.

Six drug concentration groups were set up within the LC100 and LC0 range of Pinelliaternata for toxicity experiments, with concentrations of 5, 5.5, 6, 6.5, 7, 7.5, and 8ug/ml, respectively. Embryo water without any drugs was used as the blank group. Three replicates were set for each concentration, with 30 embryos per replicate. After administration, the embryo water containing the corresponding drug dose concentration was replaced every 24 hours. The hatching of zebrafish embryos was observed and recorded, and the experiment was repeated three times to calculate the mortality rate within 72 hours.

Draw the dose toxicity curve using GraphPad Prism 6.0 software and calculate the LC50 and LC20 of Pinelliaternata solution separately.

2.2 The effect of Pinelliaternata on the heartbeat of zebrafish embryos

Transfer 7-9 hpf zebrafish embryos to a 24 well plate cultured in 1ml of medium without any treatment in the normal group. In the model control group, the final concentration of (CH3COO) 2Pb · 3H2O was 1ug/ml. Pinelliaternata solution (LC50, LC10) and 1ug/ml (CH3COO) 2Pb · 3H2O were co treated for 1 hour for membrane detachment. Then, anesthesia with freshly prepared 0.02% fish tranquilizer was performed, and the heart rate of each embryo within 3 minutes was recorded under a microscope. The average heart rate per minute was then calculated.

2.3 Grouping and Modeling

Fish eggs were randomly divided into a blank control group (NC) and a lead acetate group (CH3COO) 2Pb. Select 10 normally developing embryos from each group. Place normally developing 7-9 hpf zebrafish embryos in a 24 well plate filled with artificial seawater. Each module was treated with the corresponding drug, and the final concentration of the (CH3COO) 2Pb group was 1.6 * 10-6ug/ml, induced for 2 hours. The NC group consists of artificial seawater without any additives. Each group has three repetitions.

After induction, 6 samples were selected from each group and subjected to membrane detachment with 1mg/ml trypsin. They were stained with DCFH-DA for 40 minutes and incubated together for 1 hour for microscopic examination to evaluate the modeling effect.

After 2 hours of induction, the culture medium for each group of embryos was replaced with fresh artificial seawater without any other additives. Change the culture medium daily. Observe and record the hatching rate of fish eggs at 24h and 48h, and record the activity status of juvenile fish.

2.4 Determination of oxidative stress intensity

According to the results of Experiment 2.2.1, Pinelliaternata was divided into low (LC20) and high (LC50) dose concentration groups, with lead acetate (1.6 * 10-6ug/ml) as the model control group and embryo water as the blank group.

Select AB embryo zebrafish that develop normally 7-9 hpf after fertilization and soak them in solutions of different concentrations (LC20, LC50) of Pinelliaternata for 10 hours. Then transfer the zebrafish embryos to a 6-well cell culture plate with added lead acetate and incubate at 28 ℃ for 72 hours. At the same time, set up a blank group and a model group, with 30 embryos in each group, and set up 3 parallel groups.

Observe the development of zebrafish embryos in each group, randomly select 5 zebrafish fry from each group, and incubate them with DCFH-DA (20ug/ml) staining solution in the dark at 28 ℃ for 1 hour, DPPP (25ug/ml) staining solution in the dark at 28 ℃ for 1 hour, and AO (7ug/ml)

staining solution in the dark at 28 ℃ for 30 minutes.

After staining, the zebrafish were anesthetized with fish tranquilizer and transferred to a culture dish containing a 3% sodium methylcellulose aqueous solution for observation under a fluorescence microscope. The relative fluorescence intensity was analyzed and statistically analyzed using ImageJ software.

ROS generation rate, lipid peroxidation degree, or apoptosis rate=fluorescence intensity of the treatment group/fluorescence intensity of the blank group × 100%.

2.5 Determination of reactive oxygen species levels

2.5.1 Free radical scavenging

After synchronously culturing 1-dpf zebrafish embryos with different concentrations of Pinelliaternata solution (5ug/ml, 10ug/ml) for 10 hours, a blank group, a model group, and a positive group (simvastatin) were set up with 10 embryos in each group. Three replicates were set up and placed in a 12 well plate. After membrane detachment, DCFH-DA was used for fluorescence staining. The zebrafish were incubated in a 28 ℃ incubator for 24 hours and observed under an inverted fluorescence microscope. Data statistics were performed using Iamge J.

Free radical scavenging rate (%)=(intervention group fluorescence points - model group fluorescence points)/(blank group fluorescence points - model group fluorescence points) × 100

2.5.2 Inhibition of reactive oxygen species clusters

After synchronously culturing 3dpf zebrafish with different concentrations of Pinelliaternata solution (10ug/ml, 20ug/ml) for 10 hours, a blank group, a model group, and a positive group (simvastatin) were set up with 10 fish in each group. Three replicates were set up and placed in a 12 well plate for fluorescence staining with DCFH-DA. The zebrafish were incubated in a 28 ℃ incubator for 72 hours and observed under an inverted fluorescence microscope. Data statistics were performed using Iamge J.

2.6 Zebrafish in vitro stress test

2.6.1 Oxidative stress

After synchronously culturing 6dpf zebrafish with different concentrations of Pinelliaternata solution (10ug/ml, 30ug/ml) for 10 hours, a blank group and a model group were set up, with 10 fish in each group and 3 replicates. They were transferred to a culture dish containing 0.03% H2O2 and incubated at 28 ℃. The mortality of zebrafish was recorded every 30 minutes until complete death.

2.6.2 Heat stress

After synchronously culturing 6dpf zebrafish with different concentrations of Pinelliaternata solution (10ug/ml, 30ug/ml) for 10 hours, a blank group and a model group were set up, with 10 fish in each group and 3 replicates. The zebrafish were incubated in a 35 ℃ incubator, and the mortality of zebrafish was recorded every 30 minutes until complete death.

2.7 Data Analysis

Perform statistical analysis using GraphPad Prism 8.0.1, with $\bar{X} \pm S$. The comparison between groups was conducted using one-way analysis of variance, with $P<0.05$ indicating statistically significant differences.

3 Analysis

The construction of zebrafish oxidative emergency model using lead acetate trihydrate belongs to non-invasive modeling, which disrupts the redox balance in the body, damages cells, disrupts the regulation of reactive oxygen species, and affects the homeostasis of the internal environment. It is suitable for zebrafish with a large number of eggs and normal development, and can be used in experiments in a short period of time.

Constructing a zebrafish oxidative stress model and studying the mechanism of oxidative stress in vivo after administration of Pinelliaternata. Conduct embryotoxicity experiments (Figure 1-2) and obtain the minimum lethal concentration of 8-9 hpf zebrafish embryos at 8ug/ml and the maximum non lethal concentration at 5ug/ml. Based on this, expand the concentration gradient, draw the toxicity curve, and use mortality rate as the evaluation indicator. It is known that LC50 corresponds to a mass concentration of 6.5ug/ml, and LC20 corresponds to a mass concentration of 5.5ug/ml. At doses of 7ug/ml or above, more than half or even all zebrafish die.

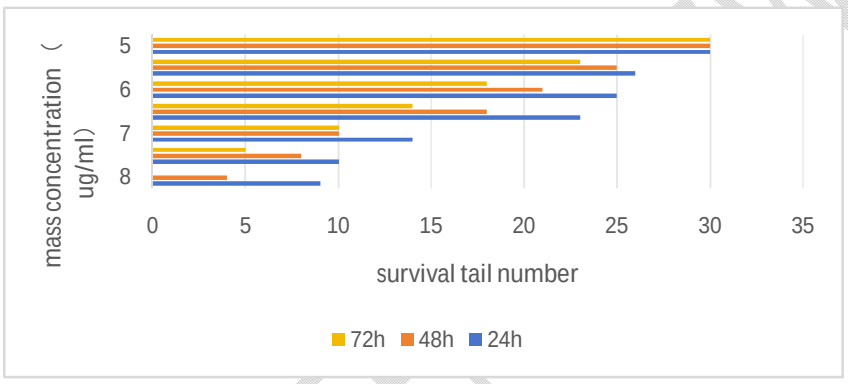


Figure 1 Survival numbers of zebrafish embryos in each group

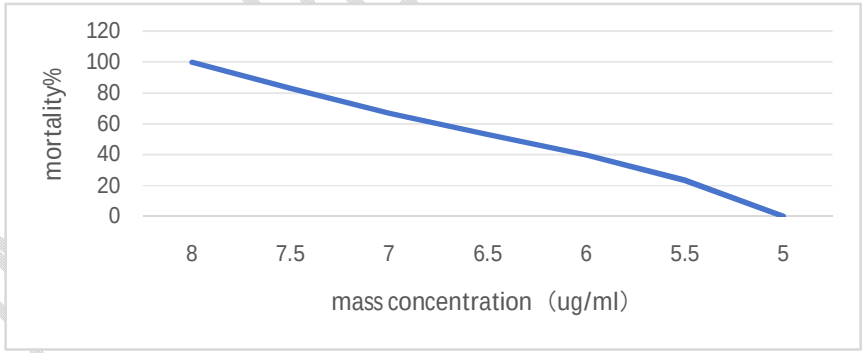


Figure 2: Death of zebrafish embryos in each group

Conduct zebrafish embryo heartbeat experiment (Figure 3) and record the heartbeat rate per minute. After hatching from embryos to juveniles, zebrafish's heartbeat can be clearly observed under a microscope, and the heart rate can be recorded through software. As shown in Figure 3 the heart rate of the model group reached 119, which was 16.67% higher than the heart rate of zebrafish embryos in the normal group., And through observation, it was found that about 43% of the embryos in the model group had abnormal enlargement or even malformation of the heart.

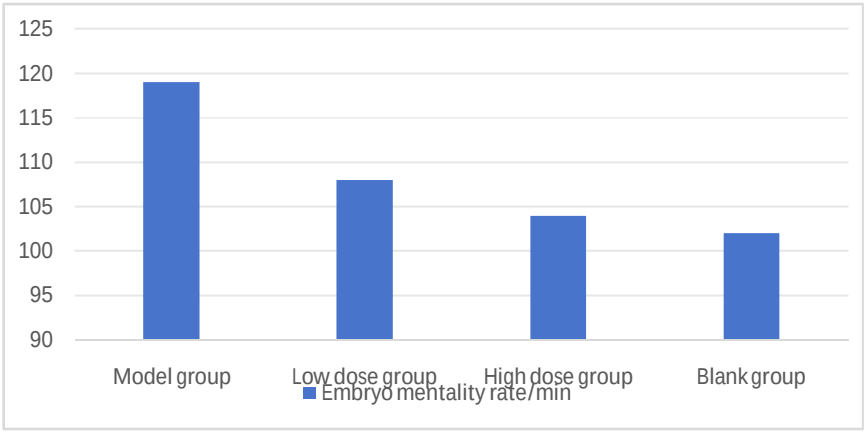


Figure 3: Heart rate of zebrafish embryos in each group

Constructing an oxidative stress model, grouping and modeling, three types of fluorescent probes were used for detection. The fluorescence intensity ratios of the three model groups to the blank group were 2.21, 1.85, and 2.85, respectively, indicating that the average fluorescence intensity of the model group was higher than that of the blank group ($p<0.01$), indicating successful modeling. During the process, the hatching rate of the blank group was 91.70%, while the hatching rate of the model group was 83.30%.

The intensity of oxidative stress was measured (Figure 4), with the levels of reactive oxygen species, lipid peroxidation, and cell apoptosis as the main research indicators. The intervention degree of Pinelliaternata treatment group was determined by the corresponding mass concentrations of LC50 and LC20. The inhibition rates of Pinelliaternata group I were 26.96%, 25.75%, and 30.13%, respectively ($P<0.01$), and the inhibition rates of Pinelliaternata group II were 46.30%, 47.73%, and 57.97%, respectively ($P<0.01$).

In the free radical scavenging experiment, compared with the model group, all groups showed a decrease ($P<0.01$), with 852 points in the high-dose group being lower than the positive group (1103). In the reactive oxygen species inhibition experiment, after administration, the inhibition rates of the low and high dose groups were 8.28% and 10.96% ($P<0.01$), which were lower than those of the positive group ($P<0.01$).

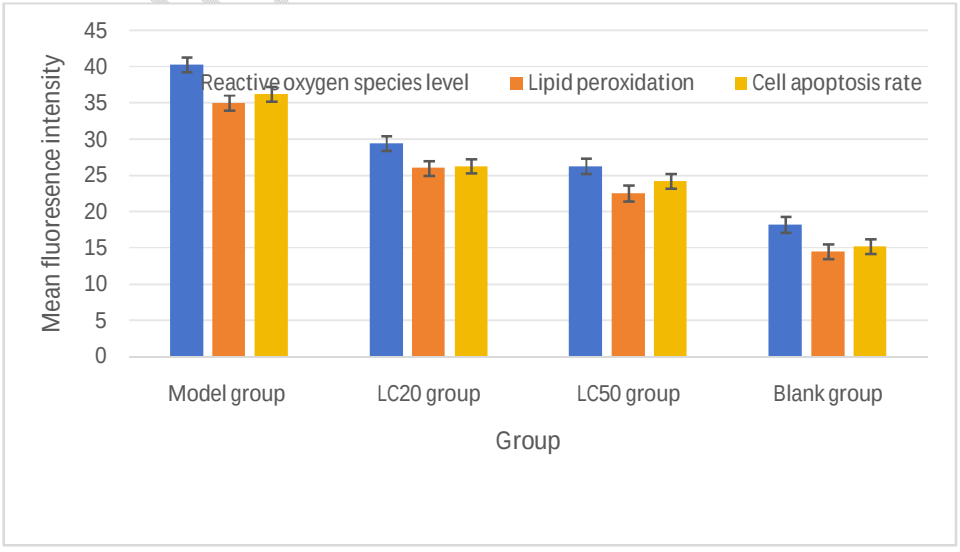
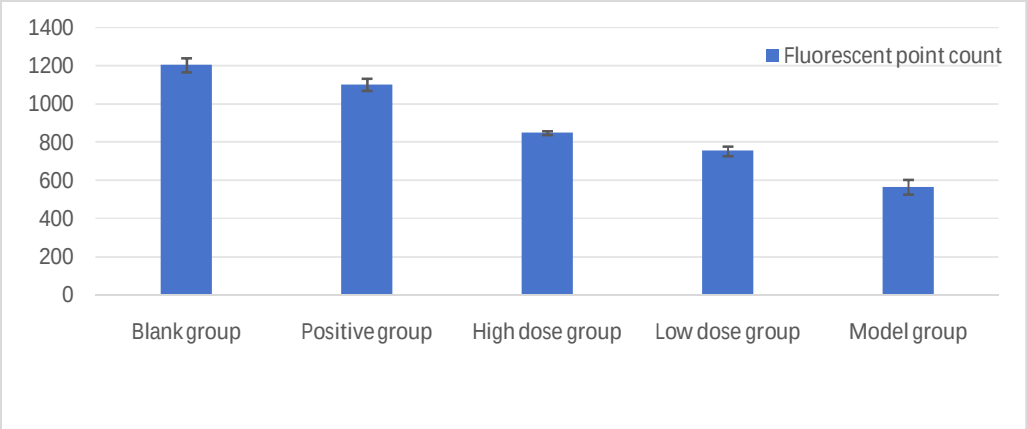
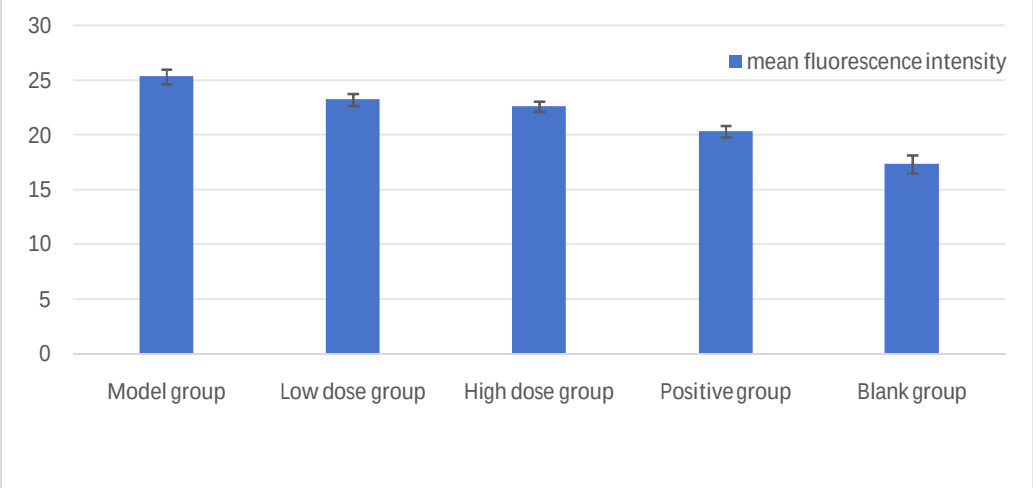


Figure 4 Oxidative stress intensity of zebrafish in each group



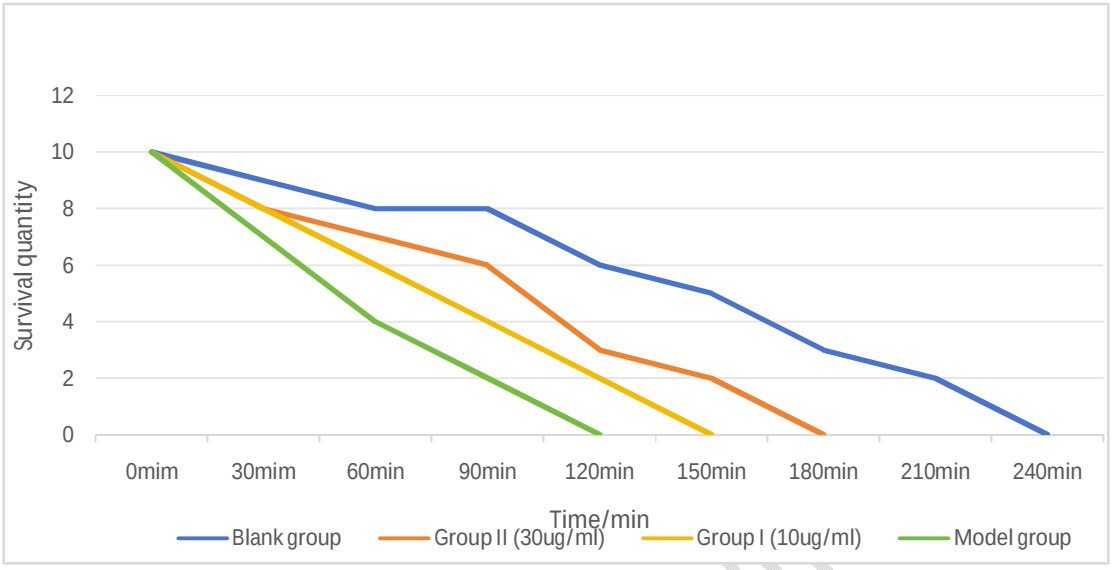
(a) Free radical scavenging



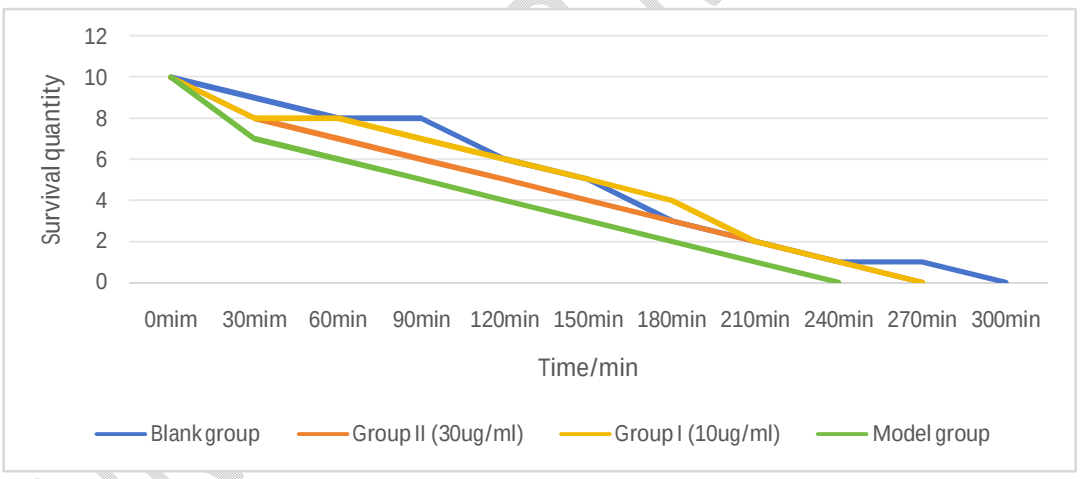
(b) Inhibition of reactive oxygen species clusters

Figure 5 Determination of reactive oxygen species levels in zebrafish of each group

Conducting an in vitro stress experiment on zebrafish, consisting of two parts: oxidative stress and heat stress, is an examination of the antioxidant capacity of zebrafish after administration. Oxidative stress experiments were conducted on 6dpf zebrafish using 0.03% H2O2, and it was demonstrated that all mice in the 120 minute model group died. As the dosage increased, the survival time of Group II significantly increased. A heat stress experiment was conducted, and all participants in the treatment group died at 270 minutes, which was longer than the model group.



(a) Oxidative stress experiment



(b) Heat stress experiment

Figure 6 In vitro stress of zebrafish in each group

4 Discussion

Pinelliaternata is a toxic traditional Chinese medicine, mainly composed of alkaloids such as choline and atropine. It can bind to receptors in the nervous system, inhibit the central nervous system, and achieve regulatory effects on the body. Mainly manifested in influencing the behavioral activities of organisms, inhibiting the loss of control of oxidative stress mechanisms, maintaining internal environmental homeostasis, and normal functioning of the body.

Oxidative stress is typically described as the disruption of the oxidative and antioxidant systems

within an organism, typically caused by the accumulation of ROS. ROS is an active molecule derived from molecular oxygen, mainly including superoxide anions and hydrogen peroxide [8]. As a part of the antioxidant system, ROS directly participates in cell proliferation and differentiation at the basal level, which is crucial for the normal development of the human body. It not only participates in the normal proliferation of human cells, but also plays an important role in the signaling pathways of various human diseases, such as tumors and inflammation [9].

The construction of zebrafish oxidative stress model uses lead acetate trihydrate as an inducer to induce oxidative damage in zebrafish, disrupt the redox balance, and cause cell damage. The experiment mainly analyzes the levels of reactive oxygen species, lipid peroxidation, and cell apoptosis, using fluorescent probe technology to focus on multiple evaluations of oxidative stress levels in the body after administration [10].

1,3-Bis (diphenylphosphine) propane (DPPP) is used in biological research to detect the content of lipid peroxides. The principle is that it reacts with peroxides to generate fluorescent substances, which produce 380nm fluorescence under 325nm excitation light. Its molecular structure contains an oxidation sensitive parent molecule, which is oxidized into another fluorescent signaling molecule in lipid peroxidation reactions, thereby achieving the detection of lipid peroxidation levels [11].

Kou Ding Orange (AO) is a fluorescent dye with cell membrane permeability, which can penetrate live cell membranes and stain nuclear DNA and RNA. There is a difference in the binding amount between AO and DNA and RNA in cells, and the complex can emit fluorescence of different colors. When AO binds to dsDNA, it emits green fluorescence, while when it binds to ssDNA and RNA, it emits red fluorescence. When bound to DNA, it is very similar in spectrum to fluorescein, with an excitation maximum of 502nm and an emission maximum of 525nm (green). When it binds to RNA, the excitation maximum shifts to 460nm (blue) and the emission maximum shifts to 650nm (red). This can detect the state of apoptotic cells and is a commonly used staining method in morphological studies of cell apoptosis [12].

The intervention level of Pinelliaternata treatment group was determined by the corresponding mass concentrations of LC50 and LC20. After administration, the inhibition rates of Pinelliaternata Group II were 46.30%, 47.73%, and 57.97%, respectively ($P<0.01$), indicating a positive correlation between reactive oxygen species levels and lipid peroxidation, but lower than the apoptosis rate. Pinelliaternata is highly likely to directly inhibit the normal growth state of cells, damage the overall cell structure, and exert its effect [13].

The level of reactive oxygen species in zebrafish was measured from two aspects: free radical scavenging and inhibition of reactive oxygen species clusters. DCFH-DA fluorescent probe was used for staining. DCFH-DA itself does not have fluorescence and can freely penetrate the cell membrane. After entering the cell, it is hydrolyzed by intracellular esterase to generate DCFH, which is loaded into the cell [14]. The intracellular reactive oxygen species can oxidize non fluorescent DCFH to generate fluorescent DCF, thereby achieving the detection of intracellular reactive oxygen species based on fluorescence numerical analysis to characterize the degree of cell damage.

In the free radical scavenging experiment, cells involved in oxidative stress in zebrafish were labeled with fluorescent probes, resulting in the appearance of fluorescent dots. After administration, the number of fluorescent dots significantly decreased. Compared with the model group, there was a decrease in both groups ($P<0.01$), with a decrease rate of 29.42% and

44.44% in the dose group points (P<0.01). In the inhibition experiment of reactive oxygen species, reactive oxygen species (ROS) is a ubiquitous expression of ROS during the growth and development of zebrafish. After administration, the inhibition rates of the low and high dose groups were 8.28% and 10.96%, respectively, which were lower than those of the positive group (P<0.01). This indicates that at different growth and development cycles, Pinelliaternata can affect the expression of reactive oxygen species in zebrafish, and the effect shows a dose-dependent characteristic with increasing dose [15].

In vitro stress experiments are divided into oxidative stress experiments and heat stress experiments. Induced with 0.03% H2O2, all mice in the model group died at 120 minutes, and all mice in the 150 minute treatment group began to die. As the dosage increased, the survival time of Group II significantly increased, indicating that the survival time was dose-dependent [16]. A heat stress experiment was conducted, and all participants in the treatment group died at 270 minutes, which was longer than the model group. This indicates the intervention and disruption of the oxidative stress mechanism in the body by external heat sources, but can be inhibited through the administration of Pinelliaternata [17].

Meanwhile, Pinelliaternata not only inhibits oxidative stress mechanisms, but also has toxic effects. It can affect the hatching rate and heartbeat of embryos. After administration, the hatching rate of embryos decreased (P<0.01) and the embryonic atria showed abnormal enlargement.

From this, it can be concluded that by combining the zebrafish model and conducting practical validation experiments to analyze changes in biological parameters, Pinelliaternata has a good effect in inhibiting oxidative stress mechanisms [18], and its own toxicity can also be controlled; To lay the foundation for further development of antioxidants using Pinelliaternata as raw material [19].

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