

# Insecticidal activity of *Ageratina adenophora* (Asteraceae) extract against *Limax maximus* (Mollusca, Limacidae) at different development stages and its chemical constituent analysis

Haojun Li

Guizhou University

Runa Zhao

Guizhou University

Yingna Pan

Guizhou University

Hui Tian

Guizhou University

Wenlong Chen

wlchen@gzu.edu.cn

Guizhou University

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## Research Article

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# Abstract

The great grey slug, *Limax maximus* is one of the common agricultural pests, which infesting the growing period of vegetables by making holes or lacerations in the leaves of vegetables, especially seedlings and tender leaves. To evaluate the insecticidal activity of *Ageratina adenophora* extract against slugs, the fecundity, growth, hatching rate, offspring survival rate, protective enzyme and detoxifying enzyme activity of slugs in different periods exposed to LC<sub>50</sub> of the extract for two different time intervals (i.e., 24 h, 48 h), and extracts phytochemical variability were studied. The LC<sub>50</sub> values of the *A. adenophora* extract against *L. maximus* was 35.9 mg/ml. This extract significantly reduced the hatching rate of eggs and the survival rate of offspring hatched from exposed eggs compared to the control, which exposed for 48 hours was lowest. The survival, growth, protective enzyme and detoxification enzyme activity of newly hatched and 40-day-old slugs decreased. In the *A. adenophora* extracts, tannins, flavonoids and saponins were identified, which may be beneficial in their biological effects. These results suggest that *A. adenophora* extract can be used as an alternative drug to kill slugs to effectively control the species.

## 1. Introduction

The terrestrial slug *Limax maximus*(Limaicidae)is one of the most important agricultural pests in China because of its wide distribution and many host species. Wild oysters and larvae can harm crops, especially Solanaceae, Cruciferae and Leguminosae crops. White mucus traces and sticky feces are often left after crawling, and the excreted feces are easy to breed bacteria. After crop damage, leaves, stems and fruits are bitten into gaps and holes, which seriously affect the quality of agricultural products and crop safety [1]. Slugs are not only harmful to crops, but also closely related to plant pathogens and the spread of parasites in humans, livestock and wild mammals, which pose a great threat to human health.

At present, the control of *Limax maximus* is mainly by spraying chemical pesticides such as tetrameric acetaldehyde [2],Long-term use of slugs is very easy to make slugs resistant and affect the control effect. in addition, the substance will also do harm to the environment, especially to water pollution [3].

The species *Ageratina adenophora* (Asteraceae) is a perennial herb of Compositae, native to South America [4]. It was introduced into Lincang, Yunnan, China from Myanmar in the 1940s and widely distributed in Yunnan, Guizhou, Sichuan, Guangxi and Chongqing. It was listed as an alien invasive species in China in 2003. It is often born in tidal wetlands or hillside roads. It has strong fruiting ability and spreads very fast. Eupatorium adenophorum *Ageratina adenophora* has strong vitality and fecundity, and has a wide adaptability[5]. Invading farmland, gardens, forests and other habitats, forming dominant communities, excluding other plant growth and breaking plant diversity has been rampant in southwest China [6]. Studies have shown that this plant shows active principles in its chemical constituents, such as flavonoids, saponins and tannins, which have biocidal activities, including killing molluscs [1, 7–9]. These results demonstrate the importance of this plant in controlling mollusk species.

The purpose of this study was to confirm the existence of flavonoids, tannins and saponins in the extract of *Ageratina adenophora*, and to analyze and identify their contents in the extract. The effects of LC<sub>50</sub> on egg, larva and adult slugs were calculated. To investigate the effects of 24h and 48h exposure to semi-lethal concentrations on different life stages of slugs.

## 2. Materials and Methods

### 2.1. Slugs and Plants

The eggs, newly hatched slugs and 40-day-old slugs used in this study were obtained from the ecological experimental field of Professor Chen, Institute of Entomology, Guizhou University (latitude: 26 °25 '39.62"N; longitude: 106 °40 '5.81"E; 1090m altitude). According to the method by [10], slugs were reared. Briefly, *L. maximus* were reared in plastic containers with holes of 9 cm in diameter and 6 cm in length, which contained 50 g of moist culture soil (120 ° 1h) and fresh vegetables and protein-rich feed mass (3:1) for feeding. The top of the box was wrapped with plastic films with small holes for ventilation. The environmental conditions were 25 ± 1 °C, 70 ± 5% r.h. and 16:8 h (L:D) in an environmental chamber.

The leaves of *Ageratina adenophora* were collected from the campuses of Guizhou University (latitude: 26 °42 '69.93"N; longitude: 106 °66 '95.34"E; 1083 m altitude) in Guiyang in December 2022. The leaves were washed and dried in an oven at 60 °C for 24 h, then pulverized into powder using a mechanical grinder. The plant extract was obtained by: infusing 100 g of the leaves powder in 1000 mL distilled water for 72 hours, and then filtering the obtained suspension solution, and tested with aqueous solution.

The sublethal concentration (LC<sub>50</sub>) of adult slugs was calculated for use in subsequent hatching and survival experiments.

### 2.2. Phytochemical analysis of aqueous extract of *Ageratina adenophora*

In order to determine the presence of various compounds like flavonoids, saponins, tannins, 5.0 g dried powder were boiled in distilled water for 10 min, filtered after the solution being cooled, and the supernatant was used for detection. The existence of flavonoids in the solution was proved by 10% sodium hydroxide colorimetric analysis [11]. The identification of saponins and the determination of foaming index in water extract were realized according to [12, 13]. In order to identify the presence of tannins in *Ageratina adenophora* extract, Agar solution was prepared in distilled water at a concentration of 2.5%. In order to distinguish the types of tannins (condensed or hydrolyzed), colorimetric tests were carried out with ferric chloride (FeCl<sub>3</sub>) at 2% concentration. In order to quantify condensed tannins, the Stiasny method [14] was used and repeated three times, and the existence of tannins was confirmed by the turbidity of the water extract.

## 2.3. Determination of flavonoids, saponins and tannins in *Ageratina adenophora* aqueous extract

The extract of LC<sub>50</sub> *Ageratina adenophora* was weighed as 1000 mL to make sample solution.

The content of flavonoids was determined according to the method of [15], with rutin as standard control. Briefly, 2 mg rutin was dissolved in ethanol solution by ultrasonic to make standard solution of 200 µg mL<sup>-1</sup>. The standard solution 0,0.1,0.2,0.3,0.3,0.4,0.5 and 0.6 mL were precisely taken and added with water to 2 mL, numbered 1 to 7, 100 µL respectively, and the corresponding reagent without rutin was used as blank reference. The absorbance (OD) value was read at 410 nm by full-wavelength microplate reader [16], and the standard curve was calculated. The sample solution 1.0 mL was used for color operation, and the OD value was determined by full-wavelength enzyme labeling instrument at 410 nm wavelength, repeated 6 times, and the data were recorded and analyzed.

The content of saponins was determined following the modified method of [17]. To 20 µL of aqueous extract of *A. adenophora* after the ethanol was evaporating, 5 mL of 72% sulfuric acid and 0.5 mL of 8% vanillin ethanol solution were added. The solution was mixed gently and OD was determined at 544 nm using full-wavelength microplate reader. Standard curve of ginsenoside Re (concentration range: 0–µg/mL) were used for the content of saponins, and results were expressed as. The experiment was repeated 6 times.

The total tannin content was estimated using the procedure of [18]. To 1 mL of extract, 5.0 mL water, 1.0 mL sodium tungstate-sodium molybdate mixed solution and 3.0 mL sodium carbonate solution was added successively for color reaction. The OD value was determined by full-wavelength microplate reader at 765 nm. Standard curve of gallic acid (concentration range: 0 µg/mL) were used for the content of tannin, and results were expressed as. The experiment was repeated 6 times for statistical analysis.

The contents of flavonoids, saponins and tannins in *Ageratina adenophora* extract were calculated ([19] according to the formula:

$$\% = \left( \frac{m \times V_2}{V_1 \times M \times 1000} \right) \times 100\%$$

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Where m is the mass of flavonoids, saponins and tannins,  $V_1$  is the sampling volume of the sample solution,  $V_2$  is the constant volume volume of the solution, and M is the sample mass.

## 2.4. Analysis of hatching rate of eggs exposed for 24 hours and 48 hours

LC<sub>50</sub> of *A. adenophora* aqueous extract were taken for bioassay against the eggs of great grey slugs. Ten eggs of one-day old were placed in a Petri plate (diameter 9 cm). The eggs were sprayed with 20 mL extract. For the control, eggs were treated with distilled water only. After 24 h and 48 h respectively, the eggs were transferred to plastic containers. Hatching of eggs was observed daily for 30 d. Each treatment was replicated four times. The experiment was carried out at 21–24°C, 84% relative air humidity). The hatching rate (%) is determined by the following formula [20]: hatching rate (%) = (number of slugs hatched / total number of eggs) x100

## **2.5. Survival rate of offspring hatched by eggs exposed to 24 hours and 48 hours**

The hatched slug larvae were kept under the same conditions mentioned above, fed with fresh vegetable leaves and protein-rich feed groups (3:1), and wet the incubator with distilled water every two days. Mortality and maturity were assessed every three days for 120 days, and the dead individuals were removed from the incubator. Survival rate (%) = (number of unviable embryos / total number of embryos) x100 [21].

## **2.6. Effects of LC<sub>50</sub> on the growth, survival and reproduction of newly hatched larvae and 40-day-old *Limax maximus***

In this experiment, 60 newly hatched slug larvae and 40-day-old slugs were selected, with an average size of 6.62 ± 0.57 mm (newly hatched) and 38.48 ± 2.14 mm (40-day-old), respectively. The *Limax maximus* were separated in groups (10 in each group, repeated 4 times) and placed in a plastic perforated box with pre-sterilized culture soil. The pre-calculated LC<sub>50</sub> *Ageratina adenophora* extract was 20 mL sprayed in the box. The control group consisting of the same number of *Limax maximus* was only treated with distilled water and sealed with cotton cloth and elastic rubber to prevent slugs from escaping. After the end of the exposure period, transfer the slugs to another container and cultivate it according to the above method.

Growth is determined by using Vernier calipers to measure body length [22]. To assess the mortality rate, the analysis was conducted every three days for 120 days, and the dead individuals were removed from the incubator by looking directly at the *Limax maximus*. Sexual maturation is assessed at the same frequency and determined by the presence of eggs in the womb [23].

## **2.7. Effects of LC<sub>50</sub> on the activities of protective and detoxifying enzymes in newly hatched larvae and 40-day-old slugs**

Protection enzymes, i.e., SOD, POD, CAT, and detoxification enzymes, i.e., CYP450, Glutathione-S-Transferase (GST), Acetylcholinesterase (AChE) inhibition activities, were completed as per commercial ELISA kit [24]. The newly hatched slug larvae and adults alive respectively treated by LC<sub>50</sub> of *A. adenophora* aqueous extract or distilled water after 24 and 48 h were collected for enzyme assay. The treated slugs were stored in a refrigerator at 80°C.

## 2.8. Statistical analysis

The regression equations of the standard samples for the determination of flavonoids, saponins and tannins were calculated and analyzed by Microsoft Excel 2021 software. LC<sub>50</sub> value was computed using Probit analysis in IBM SPSS Statistics 25 software[9]. The concentration-response [25] curve was established by the same test. The hatchability, mortality, growth and development of *Limax maximus* exposed to LC<sub>50</sub> were compared by Kruskal- Wallismethod and Student-NewmanKeuls method ( $p < 0.05$ ). The hatching rate, mortality and growth of slugs exposed to LC<sub>50</sub> were compared with BioEstat 5.0 software. The data of protective enzyme and detoxification enzyme activity were statistically analyzed by IBM SPSS Statistics 25, the difference between treatments was analyzed by single factor analysis of variance, and the significant difference was tested by LSD method. Use the Origin 2021 version of the software to draw curves.

## 3. Results

### 3.1. Phytochemical analysis of *Ageratina adenophora* extract

After the addition of sodium hydroxide solution, the color of *A. adenophora* extract changed from light yellow to red, which is a typical color of chalcone and gold ketone, thus confirming the existence of flavonoids in water extract. The existence of tannin was confirmed by Agar solution test, and the moss green confirmed the existence of condensed tannin after adding ferric chloride, which was also confirmed by Stiasny test. The results showed that there were saponins and the foam index was 100.

### 3.2. Determination of flavonoids, saponins and tannins in extract

#### 3.2.1. Flavonoids content

The regression equation of rutin reference substance was  $y = 0.1744x + 0.0007$ ,  $R^2 = 0.9946$ . The reference substance of rutin showed a good linear relationship in the range of 0.00-0.10 ug. The standard curve was drawn with the mass ( $\mu\text{g}$ ) as the X axis and the OD value as the Y axis as shown in Fig. 1.

After repeated testing for 6 times, the content of flavonoids in *A. adenophora* extract was 4.72% – 5.02%, as shown in Table 1.

Table 1  
Determination of flavonoids in *Ageratina adenophora* extract after 6 times of detection.

| Serial Number | OD    | Flavonoid Content(%) | Average Content(%) | Standard Deviation | RSD(%) |
|---------------|-------|----------------------|--------------------|--------------------|--------|
| 1             | 0.287 | 5.02                 | 4.87               | 0.1119             | 2.30   |
| 2             | 0.267 | 4.72                 |                    |                    |        |
| 3             | 0.277 | 4.91                 |                    |                    |        |
| 4             | 0.273 | 4.83                 |                    |                    |        |
| 5             | 0.282 | 4.96                 |                    |                    |        |
| 6             | 0.271 | 4.79                 |                    |                    |        |

### 3.2.2. Saponins content

The regression equation of ginsenoside Re reference substance was  $y = 0.0427x + 0.0119$ ,  $R^2 = 0.9965$ . The reference substance of ginsenoside Re showed a good linear relationship in the range of 0.00–10.00 ug. The standard curve was drawn with the mass ( $\mu\text{g}$ ) as the X axis and the OD value as the Y axis as shown in Fig. 2.

After repeated testing for 6 times, the content of saponins in *A. adenophora* extract was 1.79% – 1.89%, as shown in Table 2.

Table 2  
Determination of saponins in *Ageratina adenophora* extract after 6 times of detection.

| Serial Number | OD    | Saponins Content(%) | Average Content (%) | Standard Deviation | RSD (%) |
|---------------|-------|---------------------|---------------------|--------------------|---------|
| 1             | 0.159 | 1.87                | 1.85                | 0.041              | 2.21    |
| 2             | 0.141 | 1.79                |                     |                    |         |
| 3             | 0.162 | 1.88                |                     |                    |         |
| 4             | 0.149 | 1.83                |                     |                    |         |
| 5             | 0.145 | 1.81                |                     |                    |         |
| 6             | 0.165 | 1.89                |                     |                    |         |

### 3.2.3. Tannins content

The regression equation of gallic acid reference substance was  $y = 0.2082x + 0.0038$ ,  $R^2 = 0.9968$ . The reference substance of gallic acid showed a good linear relationship in the range of 0.00-0.60 ug. The standard curve was drawn with the mass ( $\mu\text{g}$ ) as the X axis and the OD value as the Y axis as shown in Fig. 3.

After repeated testing for 6 times, the content of tannins in *A. adenophora* extract was 1.31% – 1.40%, as shown in Table 3.

Table 3  
Determination of tannins in *Ageratina adenophora* extract after 6 times of detection.

| Serial Number | OD    | Tannin Content (%) | Average Content(%) | Standard Deviation | RSD(%) |
|---------------|-------|--------------------|--------------------|--------------------|--------|
| 1             | 0.049 | 1.40               | 1.35               | 0.0348             | 2.57   |
| 2             | 0.046 | 1.34               |                    |                    |        |
| 3             | 0.045 | 1.32               |                    |                    |        |
| 4             | 0.044 | 1.31               |                    |                    |        |
| 5             | 0.047 | 1.36               |                    |                    |        |
| 6             | 0.048 | 1.38               |                    |                    |        |

### 3.3. Calculation of lethal median concentration (LC<sub>50</sub>) of *Ageratina adenophora* aqueous extract

Using the statistical data of IBM SPSS Statistics 25 software and using Probit to analyze and calculate LC<sub>50</sub>, it is calculated that LC<sub>50</sub> is 35.9 mg/mL. The ratio of concentration to response is shown in Fig. 4.

### 3.4. Analysis of hatching rate after 24 and 48 hours of egg exposure

The results showed that *A. adenophora* extract had adverse effects on the hatching rate of eggs exposed for 24 h (H = 11.3742; p = 0.0018) and 48 h (H = 20.7134; p = 0.0002). The longer the exposure time, the lower the hatching rate (H = 4.127; p = 0.0319). There was no significant difference in the average hatching value of the control group between the two periods (H = 0.0556; p = 0.5319). The hatching rates of 24 h and 48 h exposure were 97.9% and 94.2%, respectively (Table 4).

Table 4

The hatching rate of *A. adenophora* aqueous extract was observed after exposure for 24 hours and 48 hours. (Average value, standard deviation, range of variation and percentage of hatchability).

| Groups  |         | Hatchability<br>$X \pm SD$ | Range of<br>variation | Hatchability<br>Percentage(%) |
|---------|---------|----------------------------|-----------------------|-------------------------------|
| Control | 24hours | $39.16 \pm 0.41^a$         | (38–40)               | 97.9                          |
|         | 48hours | $37.68 \pm 1.09^a$         | (36–39)               | 94.2                          |
| Exposed | 24hours | $22.64 \pm 3.10^b$         | (19–26)               | 56.6                          |
|         | 48hours | $7.48 \pm 1.65^c$          | (5–9)                 | 18.7                          |

<sup>a,b,c</sup> = means followed by different letters are significantly different according to the Kruskal-Wallis test ( $p < 0.05$ ).

### 3.5. Survival rate of offspring hatched from exposed eggs

Compared with the control group, the survival rate of hatched offspring of eggs exposed to the extract was significantly decreased (24 h:  $H = 9.9698$ ,  $p = 0.0037$ ; 48 h:  $H = 12.9856$ ,  $p = 0.0031$ ) (Fig. 5). And the survival rate of hatched offspring of eggs exposed for 24 h was also higher than that for 48 h ( $H = 7.5397$ ,  $p = 0.0036$ ). All the slugs in the 48 h exposure of extracts died at 87 days of observation. There was no significant difference in the survival rate of hatched offspring of eggs in the control group between the two periods (24 h: 90.3%, 48 h: 87.8%;  $H = 7.4856$ ,  $p = 0.2468$ ) (Fig. 5). During the observation period, the slugs of exposure group and the control group did not show sexual maturity.

### 3.6. Effects of $LC_{50}$ on the growth, survival and reproduction of newly hatched *Limax maximus* larvae exposed for 24 h and 48 h

The growth analysis of the exposed group showed that at 60 days, there was no significant difference in the average size between the exposed 24 h group and the control group ( $H = 0.5677$ ;  $p = 0.4772$ ). The exposure time had an effect on the growth rate, the longer the exposure time, the slower the growth rate, and the average size of the 48 h exposure group was lower than that of the control group.  $H = 8.6414$ ,  $p = 0.0036$ . The average size of 24 h and 48 h in the control group was  $52.21 \pm 3.94$  mm and  $47.66 \pm 2.48$  mm, and that in the exposure group was  $51.06 \pm 3.13$  mm and  $36.75 \pm 2.24$  mm, respectively.

The survival rate of newly hatched slugs larvae exposed to *Ageratina adenophora* extract was lower than that of the control group (24h:  $H = 5.7587$ ;  $p = 0.0221$ , 48h:  $H = 5.9039$ ,  $p = 0.0221$ ). The longer the exposure time, the lower the survival rate of slugs larvae  $H = 5.0128$ ,  $p = 0.0453$ . The duration of exposure had no

significant effect on the survival rate of the control group  $H = 0.29$   $p = 0.5273$ . All the rats in the 24-hour exposure group died at the end of 108 days, and those in the 48-hour exposure group died at the end of 81 days. The result is shown in Fig. 6.

At the end of the 75-day observation, the normal mature time of the *Limax maximus* was consistent with the normal mature time of the slugs. Only 28% of the slugs survived in the 24-hour exposure group reached sexual maturity, which was about  $68 \pm 7.1$  days, while the average mature time of the control group in the same period was  $55 \pm 4.5$  days. Only 11% of the slugs survived in the 48 h exposure group reached sexual maturity, and the average maturation time was  $73 \pm 1.8$  days, while that in the control group was  $58 \pm 4.9$  days.

### 3.7. Effects of $LC_{50}$ on the growth, survival and reproduction of 40-day-old *Limax maximus*

The growth analysis of the exposed group showed that the mortality rate of the exposed group was higher than that of the control group at 30 days, and there was a significant difference in the average size of slugs between the exposed group and the control group (24h:  $H = 11.0795$ ;  $p = 0.0004$  48h:  $H = 14.8655$   $p = 0.0001$ ). The average size of 24 h and 48 h exposure group was  $44.96 \pm 2.57$  mm and  $40.18 \pm 2.34$  mm respectively, while that of control group was  $51.74 \pm 3.05$  mm and  $50.83 \pm 2.99$  mm, respectively.

The *Limax maximus* in the exposed group did not die on the first day, and the first death occurred in the 48h exposure group on the second day. All the slugs in the 24 h exposure group died at the end of 117 days, and all the slugs in the 48 h exposure group died at the end of 99 days. The survival rate of exposure group was significantly lower than that of control group (24h:  $H = 5.4988$ ;  $p = 0.0212$  48h:  $H = 5.8271$   $p = 0.0351$ ). There was no significant difference in the survival rate between the two periods in the control group  $H = 0.0286$   $p = 0.9463$ . The survival rate of slugs exposed to drugs for 48 hours was higher in the first three weeks, then decreased gradually, and decreased rapidly from 30 days to 75 days. The results showed that *Ageratina adenophora* extract remained in *Limax maximus*. The survival rate of slugs decreased linearly in the 24-hour exposure group. The result is shown in Fig. 7. In the 24 hour exposure group, only 19% of the surviving slugs reached sexual maturity, and the mature period was about  $64 \pm 5.4$  days. No sexual maturity was observed in the surviving slugs in the 48 h exposure group. In the control group, 54% and 46.2% of the slugs survived for 24 hours and 48 hours reached sexual maturity, respectively, and the average maturity time was  $53 \pm 3.8$  days and  $55 \pm 2.4$  days, respectively.

### 3.8. Effects of $LC_{50}$ on the activities of protective and detoxifying enzymes in newly hatched larvae and 40-day-old slugs

The effects of  $LC_{50}$  on the activities of protective enzymes in slugs treated with *Ageratina adenophora* extract for 24 h and 48 h were significantly lower than those of the control, indicating that *A. adenophora* extract could inhibit the activities of SOD, POD and CAT. After treated with *A. adenophora* extract for 24 hours, the activities of SOD, POD and CAT protective enzymes of slug adults decreased significantly,

which indicated that *A. adenophora* extract could inhibit the protective enzymes of slug adults, and the slug adults were treated with *A. adenophora* extract for 48 hours. The results showed that the activities of protective enzymes in slugs were affected after treatment with *A. adenophora* extract.

The effect of LC<sub>50</sub> on the activity of detoxifying enzyme in slug treated with *A. adenophora* extract for 24 h and 48 h, the activity of detoxifying enzyme AchE in slug larva and adult was significantly higher than that in control, indicating that slug larva and adult AchE were significantly activated. After treated with *A. adenophora* extract for 24 h and 48 h, the activities of detoxification enzymes CYP450 and GSTs of slug were significantly lower than those of the control, indicating that CYP450 and GSTs enzymes of slug adults and larvae were significantly inhibited after treatment with *A. adenophora* extract. The effect of LC<sub>50</sub> on the activity of detoxifying enzyme in slug treated with *A. adenophora* extract for 24 h and 48 h, the activity of detoxifying enzyme AchE in slug larva and adult was significantly higher than that in control, indicating that slug larva and adult AchE were significantly activated. After treated with *A. adenophora* extract for 24 h and 48 h, the activities of detoxification enzymes CYP450 and GSTs of slug were significantly lower than those of the control, indicating that CYP450 and GSTs enzymes of slug adults and larvae were significantly inhibited after treatment with *A. adenophora* extract.

## 4. Discussion

In this study, by *A. adenophora* aqueous extracts acting on the slug, the extracts were found to have insecticidal activity against slugs of different life cycles (stages). The insecticidal activities exhibited by the extract can be explained by the presence of flavonoids, saponins and tannins, which are secondary metabolites in the extract.

*A. adenophora* extract had an effect on the hatching rate of *L. maximus*, indicating that the molecular structure of the active ingredient of the plant extract is simple to make it easier to penetrate into the membrane pores involving embryos. However, according to previous studies, the high molecular weight of the egg membrane hinders the passage of the drug [26]. Since the mortality rate of eggs exposed to the extract for 48 h was higher than that of 24 h, indicating that a longer exposure time will have a greater effect on the hatching rate of slug eggs.

Temperature may be a factor affecting egg hatching rate and slug growth and development [27]. The high or low temperature will reduce the vitality of molluscs, which the phenomenon of drowsiness occurs when the environmental conditions are inappropriate. In addition, The high temperature increased the oxygen consumption of molluscs, thus accelerating the rate of energy consumption in vivo [28]. The temperature in this study was controlled at 21–24°C.

Souza et al confirmed that newly hatched snails were more sensitive to the shell of the cashew nut extracts [29]. Gohar et al reported the molluscicidal activity of *Callistemon viminalis* (Sol. ex Gaertner) G. Don ex Loudon against newly hatched *Biomphalaria alexandrina* [30]. However, in this study, the adult *L. maximus* exposed for 24 h and 48 h showed a significant reduction in food intake, movement slowing, darker body surface color and somnolence after 45 days, which led to some differences in growth and

development and sexual maturity between the control group. The larvae of *L. maximus* were less sensitive to extracts than adults, which may be due to the fact that pedal glands of larvae secrete more mucus than that of adults [31]. Slugs respond to exogenous toxic substances by reducing their exposure to exogenous substances through mucus secreted by foot glands [32, 33].

Under stress, superoxide anion radicals, hydroxyl radicals, hydrogen peroxide and other reactive oxygen species accumulate in insects, which will cause damage to organisms. However, there are protective enzymes composed of SOD, POD and CAT in insects, which can scavenge excess free radicals. The three enzymes coordinate with each other to maintain the dynamic balance of free radical metabolism in the body, so as to protect the insect from injury or reduce injury [34]. Studies have shown that *Nilaparvata lugens* and *Sogatella furcifera* significantly increased the activities of SOD, POD and CAT in rice plants infected with rice black-streaked dwarf disease or southern rice black-streaked dwarf disease [35]. The results showed that the semi-lethal concentration ( $LC_{50}$ ) of *A. adenophora* extract showed an inhibitory effect on SOD activity in slug, an inhibition-activation-inhibition effect on POD activity, and an activation-inhibition effect on CAT activity.

CYP450, GSTs and AchE play an important role in decomposing exogenous toxicants and maintaining normal physiological metabolism [36]. Among them, GST can catalyze the sulfhydryl coupling between the electrophilic groups of toxic substances and reduced glutathione, and increase its hydrophobicity so that it can be easily excreted from the body [37]. It was found that the AchE activity of the 2nd instar larvae of *Lymantria dispar* was significantly inhibited by methylvitamin salt, and the GSTs activity was activated after 24 hours of treatment [38]. Carbendazim can inhibit the whole AchE of earthworm (Earthworm) and activate the whole GSTs. It can induce the activation of CYP450 at low concentration and inhibit it at high concentration [39]. Under the stress of sublethal concentration of tetrameric acetaldehyde, the activity of AchE increased at first and then decreased in the gill and abdominal foot of *Pomacea canaliculata*, while the activity of liver and intestine decreased at first and then increased [40]. The results showed that after treated with sublethal concentration of *A. adenophora* ( $LC_{50}$ ), the activities of CYP450 and GSTs in slugs were inhibited in varying degrees, but the activities of AchE were increased. Because the content of flavonoids in the extract was the highest, acetylcholinesterase was released in slugs, resulting in the cause of dehydration and death.

In this study, in the  $LC_{50}$  extract of *A. adenophora*, the content of flavonoids was the most, with an average content of 4.87%, followed by saponins and tannins, 1.85% and 1.35%, respectively. It can be inferred that flavonoids played a major role in the extract, but it can not be denied that the compounds with less content did not have an effect. If the flavonoids in *Ageratina adenophora* extract can be separated and extracted separately, whether it will have a more significant killing effect on slugs remains to be further confirmed in follow-up studies.

Flavonoids may play a role by inhibiting the detoxification system of snails. Some studies have found that the CYP450 enzyme of the land snail *Cantareus aspersus* changed after contact with tobacco leaves [41, 42], in this study, the activity of CYP450 enzyme decreased after *L. maximus* contact with *Ageratina*

*adenophora* extract. This enzyme is part of a family of proteins that play a role in detoxification and can degrade various foreign substances [43]. In addition, some studies have shown that flavonoids can activate AchE [44], release a large amount of acetylcholinesterase in slugs, destroy the special mucus produced by slugs, and lead to rapid dehydration of slugs, destruction of body surface cells, and death of a large amount of body fluids [45]. In previous studies, it is concluded that saponins have hemolytic toxicity, which can destroy the cell membrane of red blood cells and cause cytoplasmic extravasation, leading to the disintegration of red blood cells [46, 47]. It can be inferred that the killing effect of the aqueous extract of *R. roxburghii* on *L. maximus* may be related to the hemolytic type of saponins. Tannin is a kind of polyphenol which contains several hydroxyl groups, which can complex with protein and cause precipitation, thus inactivating it [48, 49].

Slugs exposed to toxic substances or gases cause physiological stress, resulting in reduced carbohydrate and glycogen reserves [50], which begins to deplete the body's protein as a supplementary energy source [51]. Studies have shown that the hemolymph protein and uric acid levels of molluscs exposed to molluscs increase, indicating that the protein as an energy source is degraded [50]. However, the protein may precipitate after complexation and cannot be used as an energy substance [52], resulting in energy imbalance and death in the slug.

At present, chemical agents are still mainly used in the control of molluscs in agriculture in China ([3]. The disadvantages of chemical control in agriculture are becoming more and more obvious. In order to control this species more effectively, it is important that plant extracts are effective in all stages of life of the target organism. In addition, in order to be better applied to agriculture, factory production, preparation and treatment of extracts, selectivity at low concentrations, and harm to humans, the environment and crops need to be considered. more comprehensive studies on plant secondary metabolites in terrestrial slug species need to be strengthened.

## 5. Conclusions

*A. adenophora* is an invasive plant with wide distribution and strong fecundity. the water extract of *A. adenophora* contains flavonoids, saponins and tannins, which show *L. maximus* activity at all stages of the life cycle of yellow slug. it has a certain effect on the reaction mechanism in its body. The extract inhibited egg hatching and induced egg morphological changes. In addition, compared with larvae, adults are more sensitive to the toxicity of extracts. In addition to the massive loss of body water, behavioral changes such as drowsiness and decreased vitality were also observed in adult slugs, suggesting that the extract caused an imbalance in the balance of the *L. maximus*. Therefore, the aqueous extract of *A. adenophora* has the active ingredient to kill slugs. Because of its solubility in water, its extraction process may be environmentally friendly. In addition, it is a promising plant for effective control of biological invasion of *A. adenophora* and slug species aimed at controlling other agricultural importance.

## Declarations

**Author Contributions:** Conceptualization, H.L. and W.C.; methodology, H.L. and H.T.; formal analysis, R.Z. and Y.P.; investigation, H.L. and Y.P.; writing—original draft preparation, H.L.; validation, R.Z.; writing—review and editing, R.Z. and W.C. All authors have read and agreed to the published version of the manuscript.

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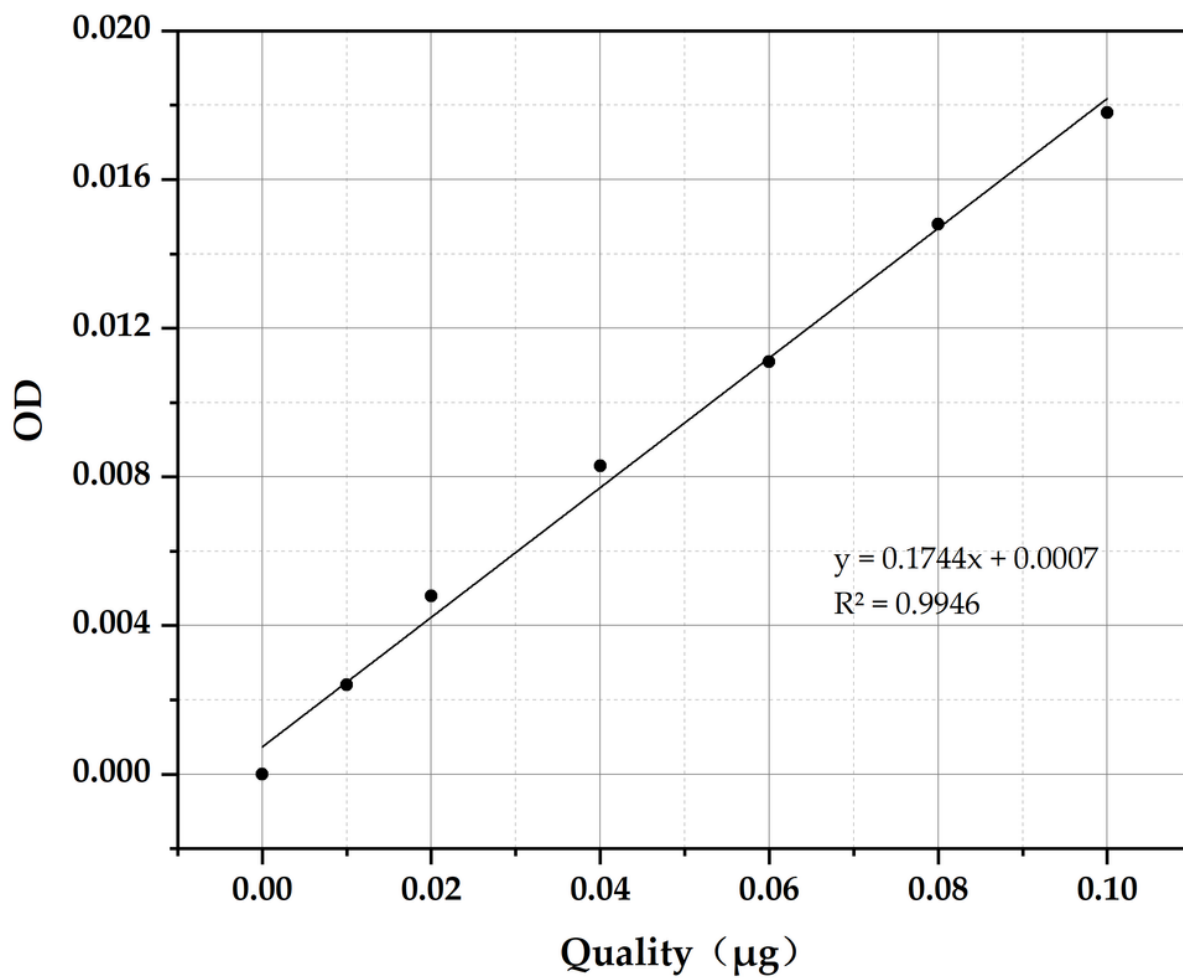
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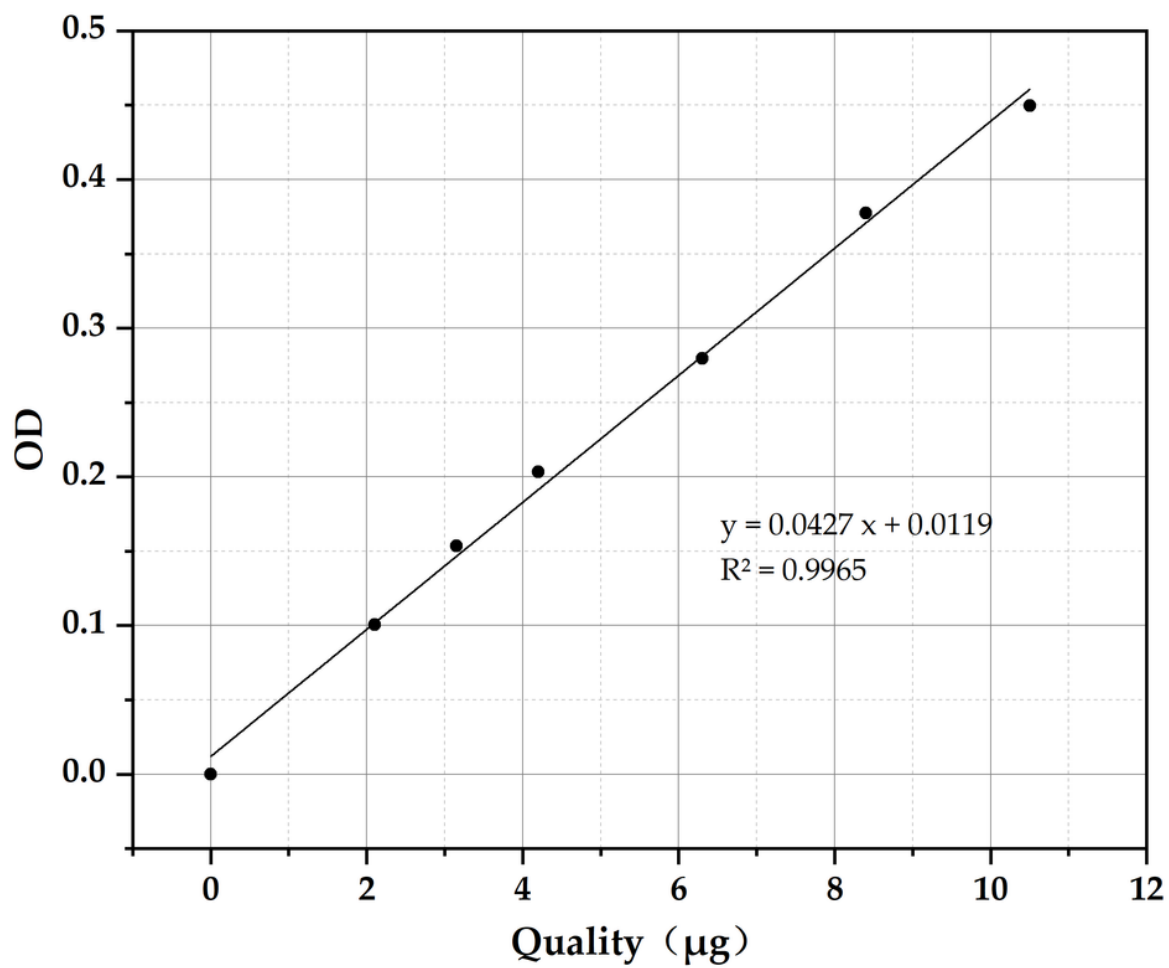
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## Figures



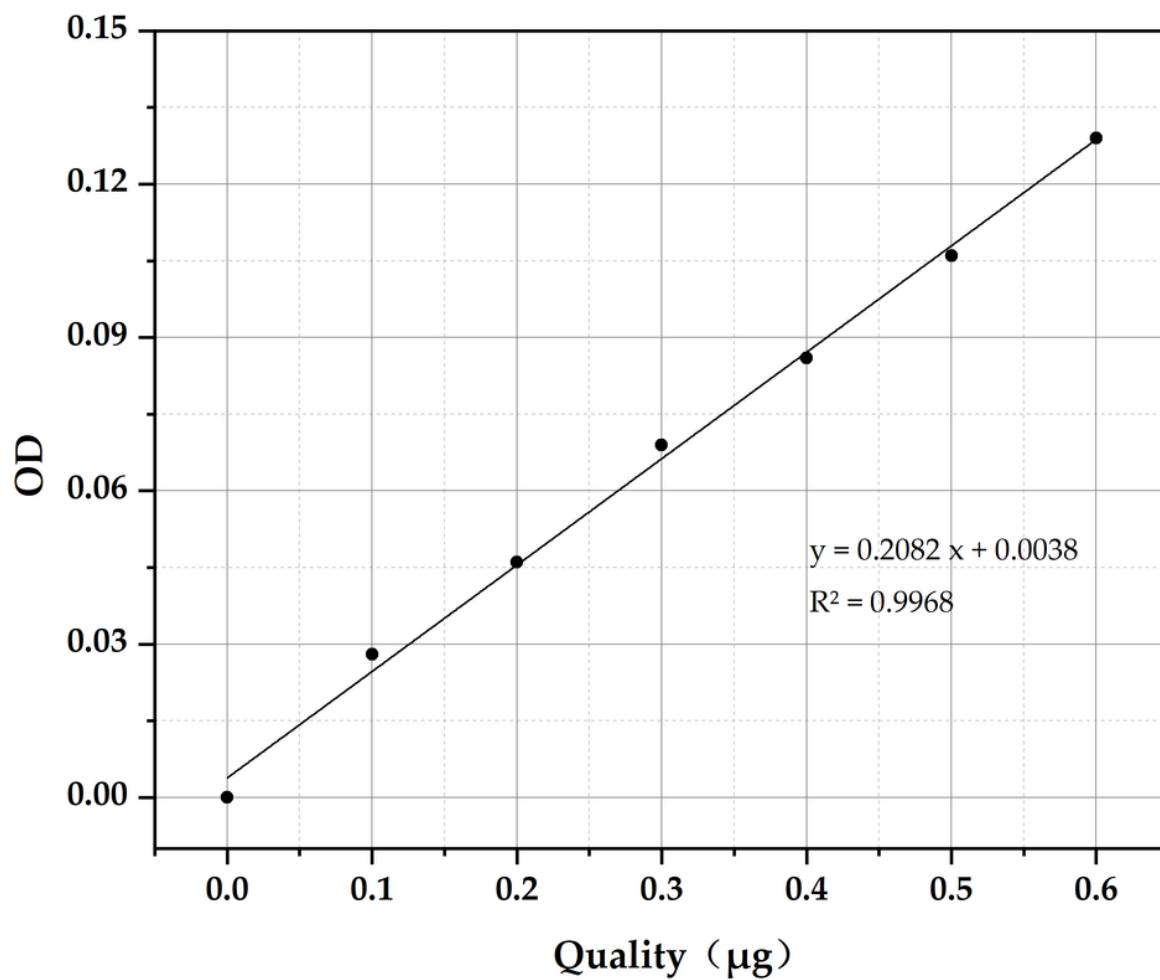
**Figure 1**

Rutin standard curve.



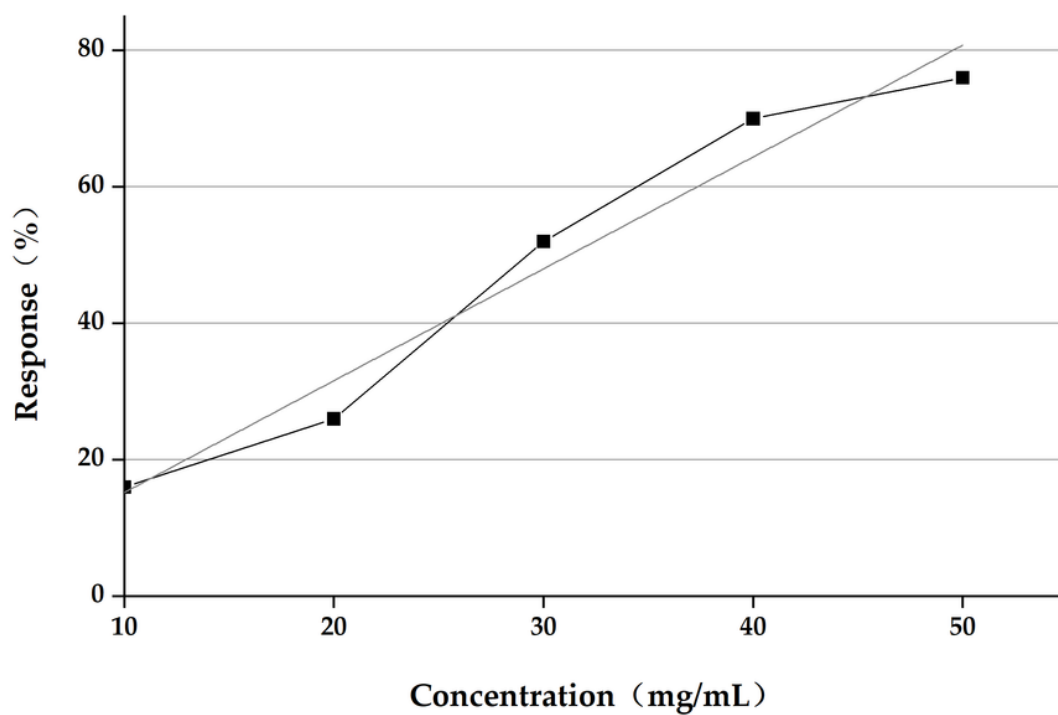
**Figure 2**

Ginsenoside Re standard curve.



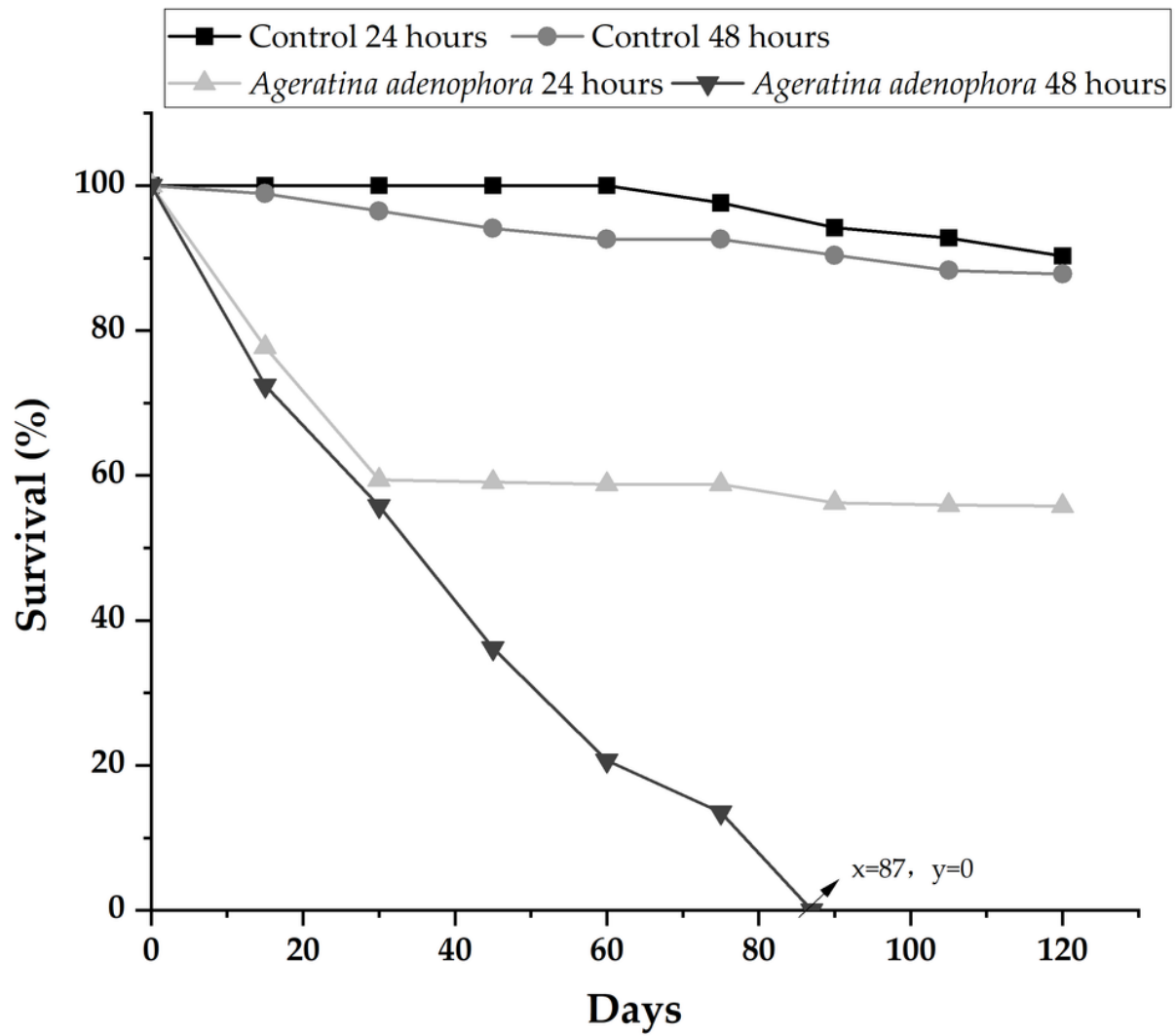
**Figure 3**

Gallic acid standard curve.



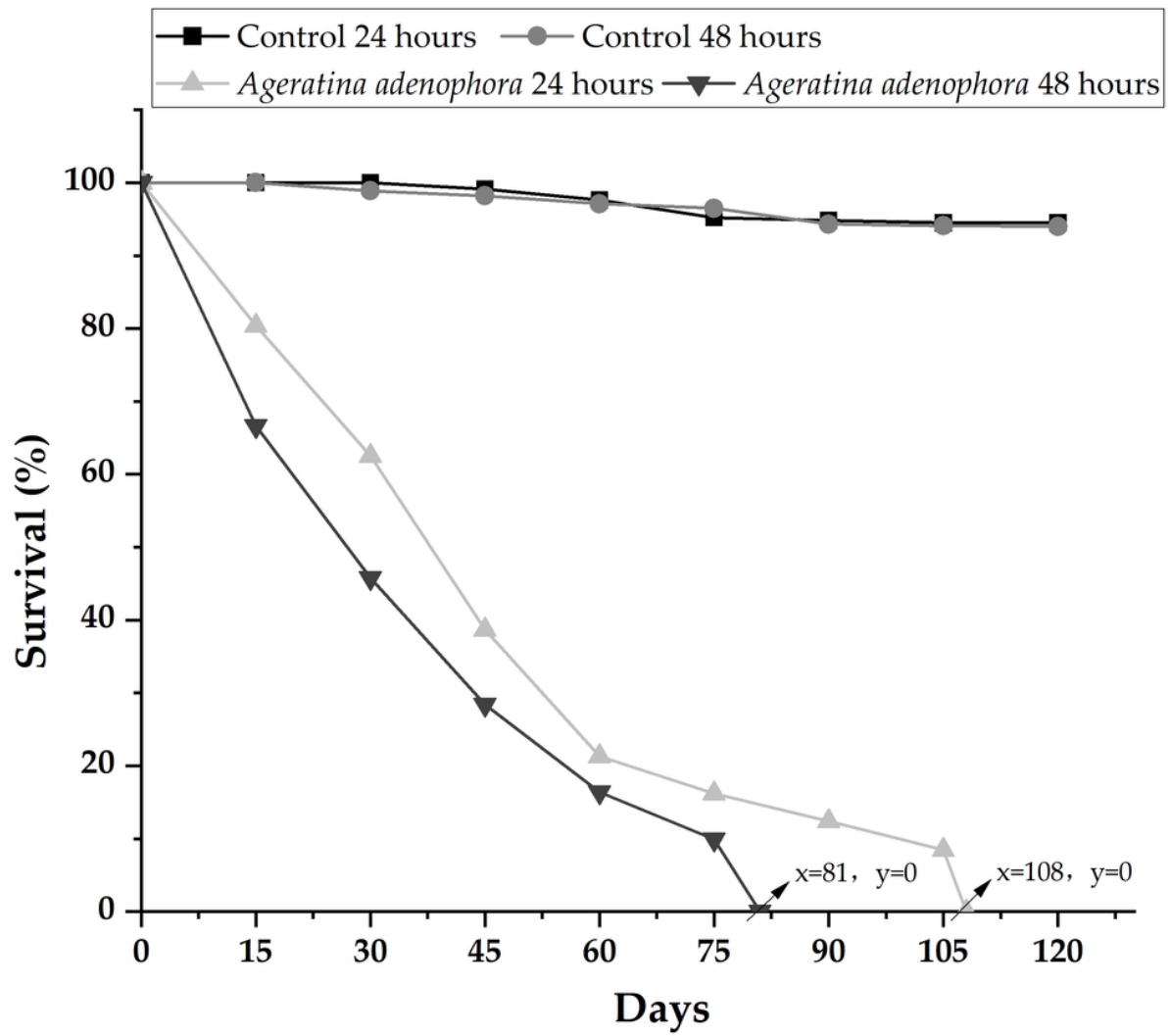
**Figure 4**

Concentration ratio response of *Limax maximus* to *Ageratina adenophora* extract. Assessment response = fatality rate, expressed as a percentage of dead *Limax maximus*.



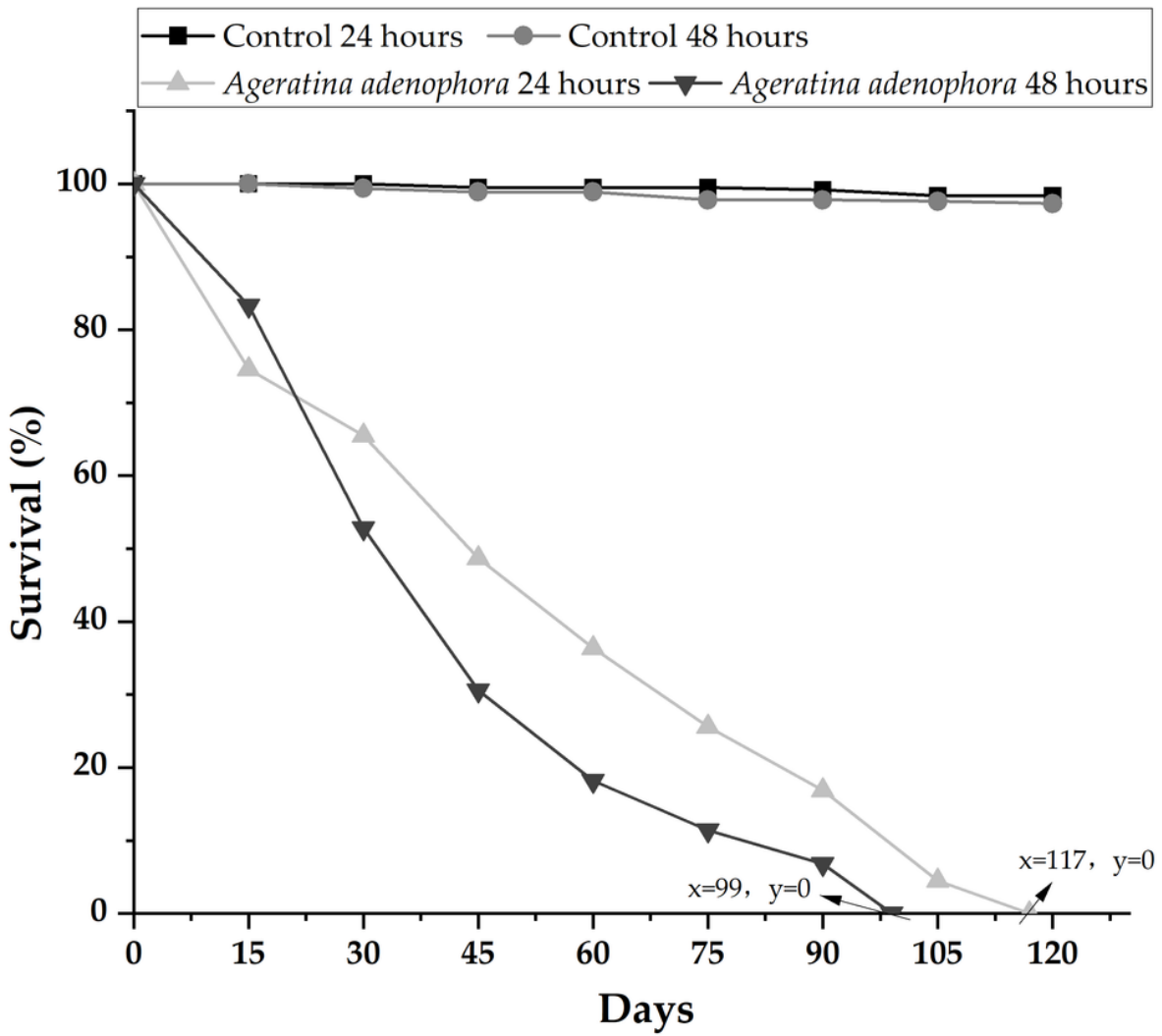
**Figure 5**

The survival rate of newly hatched offspring of eggs exposed to *Ageratina adenophora* extract for 24 hours and 48 hours for 120 days.



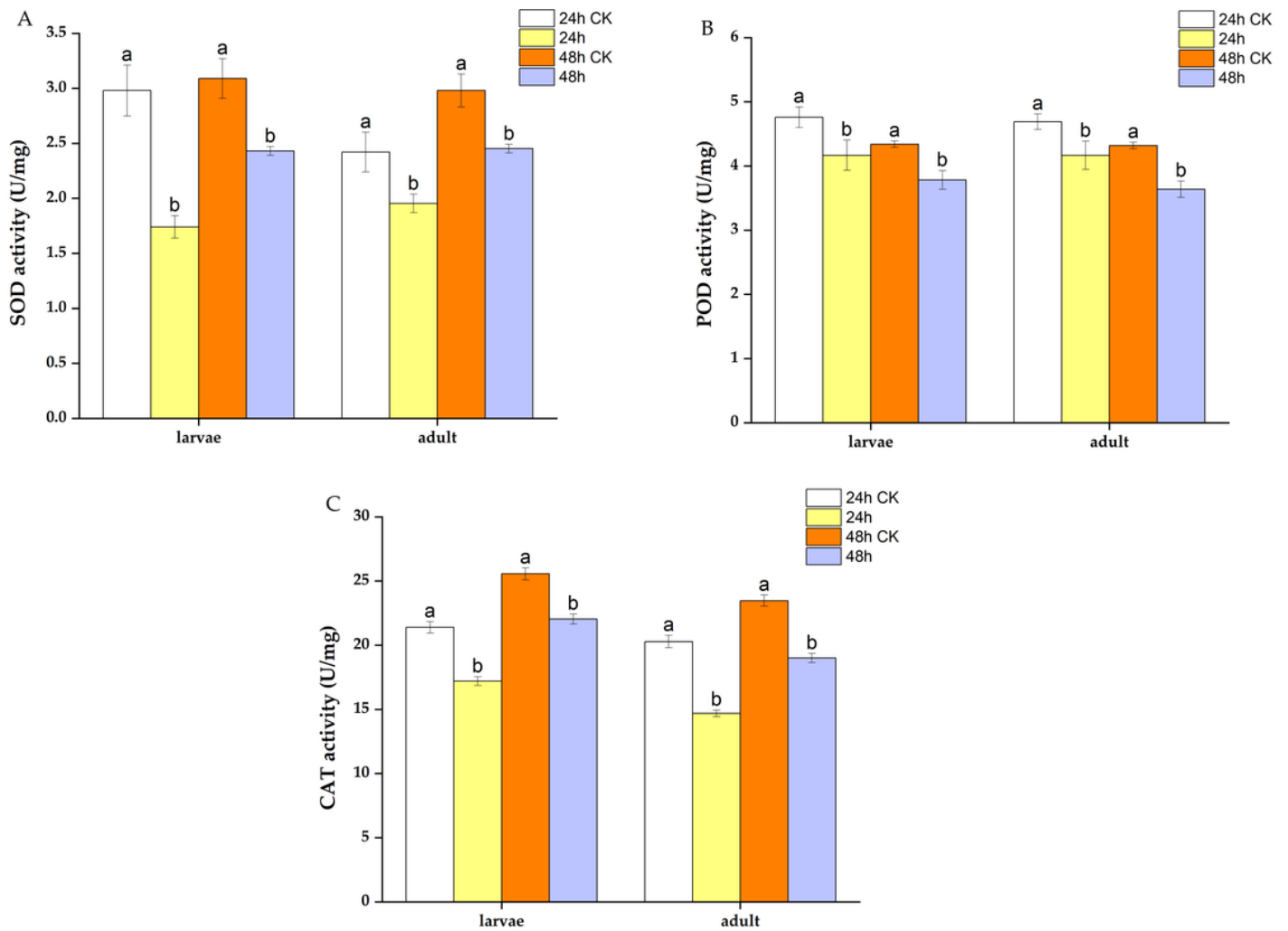
**Figure 6**

Newly hatched slug larvae exposed to *Ageratina adenophora* extract for 24 hours and 48 hours were observed for 120 days.



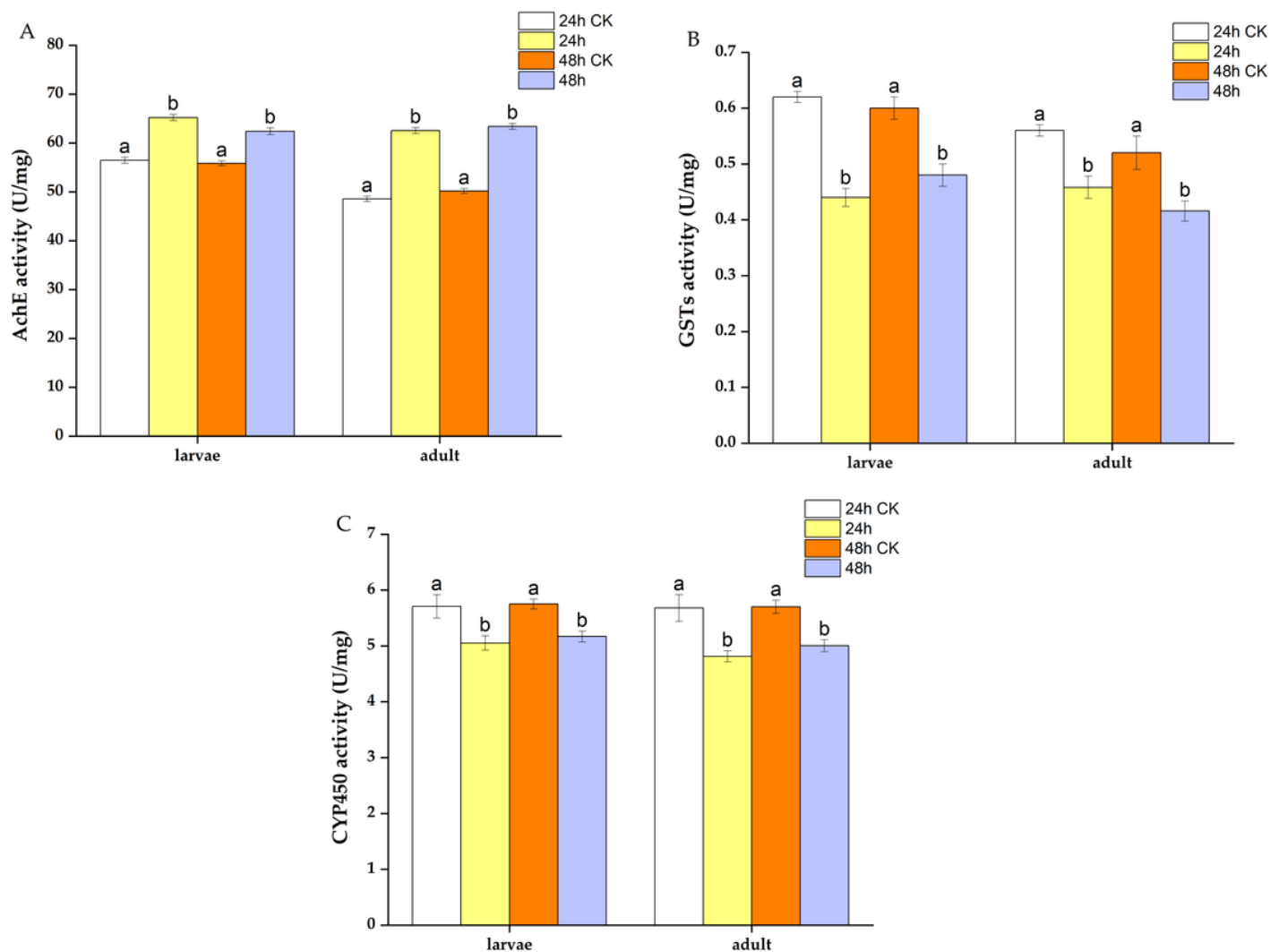
**Figure 7**

The survival rate of 40-day-old *Limax maximus* exposed to *Ageratina adenophora* extract for 24 hours and 48 hours observed for 120 days.



**Figure 8**

The protective enzyme activities of slug larvae and adults treated with  $LC_{50}$  for 24 h and 48 h respectively. (The letters in the above columns represent the results of multiple comparative experiments conducted by Tukey on the enzyme activities of slug larvae and adults after  $LC_{50}$  treatment. Different lowercase letters represent statistically significant differences).



**Figure 9**

The detoxifying enzyme activities of slug larvae and adults treated with  $LC_{50}$  for 24 hours and 48 hours respectively. (The letters in the above columns represent the results of multiple comparative experiments conducted by Tukey on the enzyme activities of slug larvae and adults after  $LC_{50}$  treatment. Different lowercase letters represent statistically significant differences).