Extraction and assessment of Rudbeckia hirta extracts' larvicidal activity on lackey moth (Malacosoma neustria Testacea): toxicity, nutritional effects and enzyme activities

Yajun WANG
Northeast Forestry University
HUNG HAI PHAM
Northeast Forestry University
Weihu Ma
Northeast Forestry University
Kejiao Li
Northeast Forestry University
Guocai Zhang
zhang640308@126.com
Northeast Forestry University
Jie Zhang
Northeast Forestry University

Research Article

Keywords: Rudbeckia hirta, Malacosoma neustria Testacea, Extraction, Nutritional effect, Enzyme activity

Posted Date: March 19th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4099067/v1

Additional Declarations: No competing interests reported.

Abstract

The insecticidal activity of Rudbeckia hirta has been confirmed, however, it has not been adequately studied. In the present study, we optimized extraction conditions and analyzed chemical constituents of ethanol extracts of R. hirta (RHE), the insecticidal activities of RHE on Malacosoma neustria Testacea larvae were investigated, and the safety of RHE for non-target aquatic organisms were evaluated. The results indicated that the optimal extraction conditions of RHE were liquid-solid ratio: 41.4:1 (mL:g), extraction temperature: 41.4°C, and extraction time: 51.99 min, under this condition, the actual extraction rate was 30.27%. RHE contains 22 compounds, flavone and flavonoid derivatives accounting for 75.47% of the total compound contents. More importantly, RHE had a significant biotoxic effect on instar larvae of M. neustria Testacea, the value of LC50 was 6.437 mg/mL at 72 h post-treatment. RHE also significantly affected larval feeding, digestion, and nutrient uptake, six nutritional indices (LWG, RGR, ECI, RCR, AD, and AR) of RHE-treated larvae were suppressed. Under the stress of RHE, the detoxification enzyme activities (ACP, AKP, and GST) were remarkably inhibited, as well as the detoxification enzyme activities (CAT, POD, and SOD) were significantly suppressed in vivo of larvae within 72 h after treatment. Safety experiments showed that RHE was practically nontoxic to Danio rerio. Taken together, RHE has significant insecticidal and antifeeding activities on M. neustria Testacea larvae and can be developed as an environmentally friendly botanical insecticide for M. neustria Testacea control.

1. Introduction

In nature, the interactions between phytophagous insects and their host plants result in a long and continuous co-evolution (Zhou et al., 2023), many plant species resist insect infestation by producing toxic substances, including toxicity, repellent action, antifeedant, and growth regulation properties (Zou et al., 2017). It is the plant's secondary metabolites that play an important role in the plant's defense system (Bouwmeester et al., 2019; Fraenkel 1959; Rothwell et al., 2020). There are many kinds of plants of Asteraceae, and their extracts have been used in food, medicine, and insecticides worldwide (Kachura et al., 2022), the most famous of these is pyrethrum (*Tanacetum cinerariifolium*) and its synthetic analogs, pyrethroids, are key active ingredients in a variety of commercial insecticidal and insect-repelling products (Liu et al., 2021).

Rudbeckia hirta commonly known as the black-eyed susan, has been classified as annual, biennial, or perennial, it is a wildflower native to North America, and that is widespread throughout the world, including Europe, Asia (Kenny et al., 2014). In addition to its ornamental value, the species has long been used in traditional medicine in several regions of North America (B et al., 2021). This plant has been used to treat fevers, colds, and headaches, and was believed to have anti-inflammatory and analgesic properties (Michael et al., 2014). *R. hirta* was also thought to have antimicrobial properties (Burlec et al., 2023; Setzer 2018). The ethanol extracts of *R. hirta* were shown to exert repellent effects against the *Popillia japonica* (Metzger et al., 1932). Another study provided evidence of the light-activated insecticidal properties of the polyacetylenes derivatives in *R. hirta* against *Aedes atropalpus* larvae (Guillet et al., 1997). The use of *R. hirta* extracts to prevent infestations of Lepidopteran insects has not been reported.

The common lackey moth *Malacosoma neustrium* (Lepidoptera: Lasiocampidae) is a univoltine moth that widely distributes throughout the Palaearctic region (Gencer 2023). The subspecies *neustrium* was found in Europe, North Africa, central Russia, and Asia Minor, whereas a distribution of subspecies *testaceum* (*Malacosoma neustria testacea*) in Middle Asia to East Asia (Dubatolov et al., 1992). When infestation of *M. neustrium testacea* is serious, the tree may be defoliated, even resulting in death (Chen et al., 2021). For a long time, chemical agents have been mainly used to control this species of pest, However, the problems of “3R” (Resistance, Residue, and Resurgence) that come with it cannot be ignored (Savary et al., 2019). Consequently, It’s exceeding significance to exploit environmentally friendly botanical insecticides for efficient control of *M. neustrium testacea*.

For the assessment of insecticidal activities of ethanol extracts of *R. hirta* (RHE) and its toxic effects on *M. neustria testacea* larvae, we conducted the following experiments: 1) RHE’s extraction, optimization of extraction conditions and analysis of chemical constituents; 2) Measurement of the toxicity and nutritional effects of RHE on the *M. neustria testacea* larvae; 3) Measurement of detoxification and antioxidant defense system levels in RHE-treated larvae; 4) Evaluation of the safety of RHE for non-target aquatic organisms. Our investigation will demonstrate the possibility for *R. hirta* to become a botanical pesticide, and provide a new way for the non-environmental damage control of *M. neustria testacea*.

2. Materials and methods

2.1 Plant Materials and Extraction

R. hirta plants were harvested from the Experimental Forest Farm of the Northeast Forestry University. The collection time was the above-ground plant part during the peak flowering period. Dried in an electric heating constant temperature blast oven at 60°C (Tianjin Taiste Instrument Co. Ltd. China, Model: DGF-2A). Crushed into powder and screened through an 80 mesh sieve. The active substances of *R. hirta* were extracted by Ultrasonic Wave Extraction Method (Saien et al., 2018). Briefly, the weighed powders were mixed into a fixed volume of anhydrous ethanol, ultrasonic treatment of mixtures with an Ultrasonic Homogenizer (Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China, Model: JY96-IIN), adjusting the extraction time and temperature according to the required experimental conditions. The mixtures were filtered with filter paper, the filtrates were concentrated into paste-like extracts with a constant weight by Rotary Evaporator (Shanghai Shensheng Co., Ltd., Shanghai, China, Model: R-205), extraction rate was calculated by the following formula: extraction rates = weight of paste-like extracts / weight of plant powder × 100.

2.2 Design of single factor and Response surface experiments

We selected three key factors: liquid-solid ratio (10:1, 20:1, 30:1, 40:1 and 50:1, mL:g), extraction temperature (20, 30, 40, 50, 60°C) and extraction time (20, 30, 40, 50, 60 min), respectively. The initial conditions were as follows: extraction temperature was 45°C, extraction time was 35 min and the liquid-

solid ratio was 40:1. Each factor was individually tested to determine the appropriate range of conditions for ultrasonic extraction. According to the design principle of the Box-Behnken experiment (Lai et al., 2013), with the extraction rate as a response value, the extraction conditions of RHE were optimized on analysis of three-levels and three-factors response surface, three-levels and three-factors were shown in Table 1. Under optimal extraction conditions, verifying tests were carried out, actual extraction rate and the model's predicted extraction rate were compared. The RHE prepared under optimal extraction conditions was used in subsequent experiments. Each treatment was repeated independently in triplicate.

Table 1
The design of the Box-Behnken experiment.

Level	Factor		
	Solid-liquid ratio (A) (mL:g)	Temperature (B) (°C)	Extraction time (C) (min)
-1	30:1	30	40
0	40:1	40	50
1	50:1	50	60

2.3 Insect Feeding and Treatments

M. neustria testacea larvae were collected from the Experimental Forest Farm of the Northeast Forestry University (45°48’12.44” N, 126°37’29.10” E, Harbin, China). The larvae were reared in the laboratory at 25°C, 70% relative humidity, and a 14 h-light/10 h-dark cycle. *Salix matsudana* fresh leaves were used to feed the larvae, and leaves were replaced daily. Healthy and newly emerged 3rd instar larvae were selected randomly for each experiment.

2.4 Bioassays and Nutritional Index

Healthy 3rd instar *M. neustrium testacea* larvae were used for bioassay. Briefly, RHE was prepared with distilled water into test reagents at five concentrations: 40, 20, 10, 5, and 2.5 mg/mL. The fresh leaves of *S. matsudana* were soaked in the aqueous solution of extracts for 30 s and then taken out and dried in the shade. The 3rd instar larvae (30 per treatment) starved for 12 h were fed on soaked leaves, and three replicates were set up. The control groups were fed on leaves soaked in distilled water. The number of dead larvae was counted at 12, 24, 48, and 72 h after treatment. The larvae were touched twice with a brush, who did not respond were considered dead. Each treatment was repeated independently in triplicate. The corrected mortalities, toxicity regression equation, median lethal concentration (LC_{50}), and sublethal concentration (LC_{20}) were calculated separately.

The larval nutritional indices were determined using the method of Mohamed (Mohamed et al., 2003), with some modifications. Fresh leaves of *S. matsudana* were treated with sublethal concentration (LC_{20}) of RHE and dried in shade. Briefly, the average weight of the 3rd instar larvae (20 per treatment) was weighed after starvation for 12 h. Larvae were fed with sufficient, weighed fresh RHE-treated leaves for 72 h. Subsequently, each group of larvae, fresh residual leaves, and feces were weighed accurately, baked at

50°C for 24 h, and dried at 100°C to a constant weight, respectively. The fresh and dry weights of the larvae (20 per group) and leaves (10 to 15 pieces) were recorded to calculate the dry/fresh ratio, respectively. The control groups were fed on leaves soaked in distilled water, and each treatment was repeated independently in triplicate. The larval weight gain (LWG), relative consumption rate (RCR), relative growth rate (RGR), efficiency of the conversion of ingested food to body substance (ECI), efficiency of the conversion of digested food into growth (ECD), approximate digestibility (AD), assimilation rate (AR) were calculated, respectively.

2.5 Detoxification enzyme and Antioxidant enzyme activity assays

The larval treatments were the same as nutritional index determination. The healthy larvae after the treatment of RHE for 12, 24, 48, and 72 h were frozen in liquid nitrogen to determine enzyme activities. Briefly, the previously frozen larvae were weighed, and then larvae were homogenized with a pre-cooled extracting solution on ice, according to the manufacturer's kits and respective instructions to extract enzymes and determine activities. These kits included the Acid phosphatase (ACP) assay kit (product ID: G0903F), Alkaline phosphatase (AKP) assay kit (product ID: G0904F), Glutathione S-transferase (GST) assay kit (product ID: G0208F), Carboxylesterase (CarE) assay kit (product ID: G0908F), Catalase (CAT) assay kit (product ID: G0105F), Peroxidase (POD) assay kit (product ID: G0107F), Superoxide dismutase (SOD) assay kit (product ID: G0101F), Polyphenol oxidase (PPO) assay kit (product ID: G0113F), all sourced from Suzhou Grace Biotechnology Co., Ltd, (Suzhou, China).

2.6 Evaluation of the Safety of RHE

Danio rerio (Zebrafish) with a body length of around 2.5 cm, healthy and fast-moving were used for safety testing. The fish were reared in the laboratory at 25°C distilled water, and fish feed was used to feed zebrafish and replaced every third day. During the pre-test, the 0.1g/mL RHE was diluted into four concentrations: 10, 100, 1000, and 10,000 times. *D. rerio* (20 per treatment) were reared in four concentrations of the solution, The control groups were equal volumes of distilled water without the extracts. The poisoning reaction and the number of deaths of zebrafish were recorded 12 h post-treatment. The concentrations of RHE that cause 10% and 90% corrected mortalities were calculated, LC_{10} and LC_{90} were used as the upper limits and lower limits of the concentration interval, respectively. A total of six concentrations (30, 60, 120, 240, 480, and 960 times) were set up. The corrected mortalities, toxicity regression equation, and median lethal concentration (LC_{50}) were calculated separately, and each treatment was repeated independently in triplicate.

2.7 Liquid Chromatography-Mass Spectrometry Analyses

The chemical constituents of RHE were analyzed by HPLC-MS (AB Sciex Pte. Ltd., USA, Model: AB SCIEX Triple TOF 6600). Filtering 0.1mg/mL RHE aqueous solution with a microporous membrane (0.22 μm), filtrates were loaded into the column (Kinetex 2.6 μm C18 100A, 150 mm × 4.6 mm (i.d.) × 2.6 μm; Milford, USA), flow phase A was water (containing 0.1% formic acid), flow phase B was acetonitrile

(containing 0.1% formic acid), the flow rate was 0.6 mL/min, injection volume was 5 μL, and the column temperature was 35°C. The elution gradient of the flow phase was 0 to 1 min, 95% flow phase A and 5% flow phase B; 1 to 22 min, 5% flow phase A and 95% flow phase B; 22 to 25 min, 95% flow phase A and 5% flow phase B. The samples' mass spectrometry signal acquisition was in positive and negative ion scanning mode, and the mass scanning range (m/z) was 50-1000 Da. The positive ion voltage of the ion spray voltage was 5000 V, and the negative ion voltage was − 4000 V; atomization gas pressure: nitrogen was 50 psi and auxiliary heating gas was 50 psi; ion source heating temperature was 550°C; cyclic collision energy was 20 to 40 to 60 V; MSI resolution was 70000; MS2 resolution was 17500.

2.8 Statistical analysis

The data were analyzed using SPSS 26.0 (IBM SPSS Statistics, Chicago, IL, USA). Results were presented as mean ± standard deviation (n = 3). Response Surface analysis and optimization were performed by the Design-Expert 13.0. The analysis of single factor, corrected mortalities, toxicity regression equation, the sublethal concentration of RHE, and safety testing were estimated through one-way analysis of variance (ANOVA, Turkey's test, $p < 0.05$). The larval nutritional index, detoxification, and antioxidant enzyme activities were evaluated using Student's t-test ($p < 0.05$).

3. Results

3.1 Single factor and Response Surface model

The results of single factor experiments show that liquid-solid ratio, extraction temperature, and extraction time significantly affect extraction rates (Fig. 1). The liquid-solid ratio hit 40:1 (mL:g), the extraction rate peaked at 27.83%, although extraction rates were not significant between the two conditions (40:1 and 50:1) ($p > 0.05$). The extraction rates had increased significantly between 30°C and 50°C, and the highest extraction rate was 28.33% at 40°C of extraction temperature. As for the extraction time, the extraction rates showed a trend of first increasing and then decreasing with the extended time, when extraction time reached 50 min, the extraction rate peaked at 30%. After single factor experimental analysis, it can be determined that the optimal conditions for the ultrasonic wave extraction method were: liquid-solid ratio 40:1 (mL:g), extraction temperature 40°C, and extraction time 50 minutes.

The response surface model was used to optimize extraction conditions under the combined action of the three factors (liquid-solid ratio, extraction temperature, and extraction time). The scheme and results of the Box-Behnken design were shown in Table 2. Design-Expert 13.0 software was used for a polynomial regression analysis and the quadratic regression equation model fitting all variables was: $Y = 29.34 + 0.9937A + 1.03B + 1.33C + 1.22AB + 0.6375AC + 0.925BC - 4.62A^2 - 4.82B^2 - 3.88C^2$.

Figure 1. The effects of different factors on extraction rates. A, the independent variable was liquid-solid ratio, the other extraction conditions were fixed (temperature 45°C and time 35 min); B, the independent variable was extraction temperature, the other extraction conditions were fixed (liquid-solid ratio 40:1 and time 35 min); C independent variable was extraction time, the other extraction conditions were fixed

(liquid-solid ratio 40:1 and temperature 45°C). Error bars represented the standard error and data were presented as mean ± S.E. Different letters indicated significant differences between groups.

Table 2
The results of the response surface for RHE.

Test number	Factor			Extraction rate (%)
	A (Liquid-solid ratio) (mL:g)	B (Temperature) (°C)	C (Time) (min)	
1	0	1	-1	19.50
2	1	-1	0	19.35
3	1	0	-1	19.65
4	1	0	1	23.20
5	-1	0	-1	19.75
6	0	0	0	29.35
7	-1	1	0	18.00
8	-1	0	1	20.75
9	-1	-1	0	19.00
10	0	-1	-1	18.75
11	0	-1	1	19.85
12	0	0	0	30.00
13	0	0	0	28.75
14	1	1	0	23.25
15	0	0	0	29.95
16	0	0	0	28.65
17	0	1	1	24.45

The variance analysis of the regression equation showed that the established quadratic regression model was extremely significant ($p < 0.01$). The model correlation coefficient $R^2=0.9878$, indicating that 98.78% of the change in extraction rates was attributed to three selected variables. The linear terms A, B, C, A^2, B^2, and C^2 of the model in the established equation had high significance ($p < 0.01$), and non-significant lack of fit term ($p > 0.05$) indicated that the model had a good fitting degree and accurately predicted the extraction conditions of RHE. Besides, The interaction between liquid-solid ratio and temperature,

temperature, and time both significantly affected extraction rates (AB and BC were both $p < 0.05$), whereas the interaction between liquid-solid ratio and time wasn't (AC was $p > 0.05$) (Table 3). The subsequent response surface figure for the interaction of different factors also demonstrated that steep AB and BC response surface curves, meaning AB and BC have a highly significant impact on extraction rates, while the AC response surface curve was relatively gentle with a non-significant impact on the extraction rates (Fig. 2).

Table 3
Variance analysis of regression model.

Source	Sum of Squares	Degrees of freedom	Mean Square	F-value	p-value	Significance
Model	322.15	9	35.79	62.89	< 0.0001	**
A	7.90	1	7.90	13.88	0.0074	**
B	8.51	1	8.51	14.95	0.0062	**
C	14.05	1	14.05	24.68	0.0016	**
AB	6	1	6.00	10.55	0.0141	*
AC	1.63	1	1.63	2.86	0.1349	ns
BC	3.71	1	3.71	6.51	0.0380	*
A^2	89.87	1	89.87	157.91	< 0.0001	**
B^2	97.82	1	97.82	171.88	< 0.0001	**
C^2	63.47	1	63.47	111.52	< 0.0001	**
Residual	3.98	7	0.5691			
Lack of Fit	2.35	3	0.784	1.92	0.2677	ns
Pure Error	1.63	4	0.408			
Cor Total	326.14	16				

"*" was meaning $p < 0.05$, "**" was meaning $p < 0.01$ and "ns" was meaning no significance.

By solving the regression equation, A, B, and C terms were calculated, and the optimal extraction conditions for the RHE were liquid-solid ratio: 41.4:1 (mL:g), extraction temperature: 41.4°C and extraction time: 51.99 min, under this condition, the extraction rate predicted by the model was 30.00%. The confirmatory experimental results indicated that the actual extraction rate was 30.27% simultaneously.

The relative error of results and predicted value of the model was merely 0.9%, which was within an acceptable range.

3.2 RHE ethanol extracts caused larvae lethal

To evaluate the insecticidal capacity of RHE, larval mortality was determined across varying RHE concentrations, and the lethality was positively correlated with dose ($r = 0.977$; $p < 0.01$). At a concentration of 40 mg/mL, the larval mortality rate reached 96.67% at 72 h after treatment, it was 5 times of the low-concentration group (2.5 mg/mL) and 10 times of the control group in the same period. According to the toxicity regression equation, the values of LC_{50} and LC_{20} of RHE for *M. neustria testacea* larvae were 6.437 mg/mL and 2.628 mg/mL, respectively (Fig. 3, Table 4).

Table 4
The sublethal concentrations of RHE for *M. neustria testacea* larvae.

Treatment	LC_{20} (mg/mL)		LC_{50} (mg/mL)		R^2	Regression equation
	Value	95% confidence interval	Value	95% confidence interval		
RHE	2.628	2.207–3.023	6.437	4.241–9.709	0.955	y = 2.164x + 3.25

3.3 Effect of RHE on larval nutritional effects

To investigate the effects of RHE on the nutritional effects of larvae, seven nutritional indices were determined, which were related to food intake, digestion, and nutrient absorption. After treatment with a sublethal concentration (LC_{20}) of RHE, the results showed that six nutritional indices of RHE-treated insects were suppressed, larval LWG, RGR, ECI, RCR, AD, and AR all showed different degrees of reduction, which were significantly lower than the control group, and the LWG decreased by 54.94%, RCR decreased by 61.17%, RGR decreased by 22.23%, ECI decreased by 16.26%, AD decreased by 58.85%, and AR decreased by 82.12%. Whereas, the ECD of the treatment group was significantly higher than the control group, which was twice of the control group (Fig. 4).

3.4 Effect of RHE on larval detoxification metabolic system

To further reveal the insecticidal mechanism of RHE, we assessed the response of the detoxification metabolic system of *M. neustria testacea* larvae to RHE stress. Sublethal concentration (LC_{20}) of RHE affected the activities of larval four detoxifying enzymes, the activity of ACP was notably reduced compared to the control group ($p < 0.05$) at 12, 24, 48, and 72 h after treatment and reached the lowest at 12 h after treatment, which was 0.53 times of the control group. The activity of GST was significantly lower than the control group at 48 and 72 h after treatment ($p < 0.05$), and the activity of AKP was 0.56 times of the control group at 72 h after treatment. The activity of GST was significantly lower than the

control group at 12, 24, and 48 h after treatment ($p < 0.05$), and the activity of GST was only 0.51 times of the control group at 12 h after treatment. Different from the previous three enzymes, the activity of CarE was significantly stimulated at 12 h and 24 h after treatment ($p < 0.05$), and reached the highest point at12 h after treatment, which was 3.29 times of the control group (Fig. 5).

3.5 Effect of RHE on larval antioxidant defense system

Simultaneously, the antioxidant defense system of *M. neustria testacea* larvae was examined after treatment with sublethal concentration (LC_{20}) of RHE. The results show activities of CAT, POD, and SOD were significantly lower than the control groups ($p < 0.05$) at 12, 24, 48 and 72 h after treatment, the difference was that the activity of CAT reached the lowest value at 48 h after treatment, which was 0.47 times of the control group, while the activities of POD and SOD reached the lowest value at 12 h after treatment, which were only 0.11 and 0.24 times of the control group, respectively. On the contrary, the activity of PPO was significantly activated after treatment ($p < 0.05$) and reached the highest at 72 h after treatment, which was 4.40 times of the control group (Fig. 6).

3.6 Evaluation of the Safety of RHE

Due to the excellent insecticidal activity of RHE against the *M. neustria testacea*, it provides the possibility for RHE to become a botanical insecticide. The toxicity of pesticides to aquatic organisms is one of the important indicators for safety risk evaluation, we estimated the safety of RHE through acute toxicity tests on *D. rerio*. The mortality rates of low-concentration group (960 times) and control were equivalent at 12 h after treatment (Fig. 7). According to the toxicity regression equation, the LC_{50} of RHE for *D. rerio* was 0.229 mg/mL at 12 h post-treatment. The results were compared with the Acute Toxicity Rating Scale (Aquatic organism) provided by the U.S. Fish and Wildlife Service (USFWS), the RHE was classified as practically nontoxic (Tables 5 and Tables S1).

Table 5
The sublethal concentrations of RHE for *M. neustria testacea* larvae.

Treatment	LC_{50} (mg/mL)		R^2	Regression equation
	Value	95% confidence interval		
RHE	0.229	0.189–0.473	0.887	y = 4.15x + 6.817

3.7 Chemical Constituents Analysis of RHE

To clarify the main insecticidal active constituents of RHE, they were isolated and analyzed using HPLC-MS. Total ions flow chromatograms and each compound was shown in Fig. 8 and Fig. S1-23, a total of 22 compounds were detected, ten of them were flavone or flavonoid derivatives, accounting for 75.47% of the total compound contents in the RHE. Flavone was present at the highest content (74%), whereas

Quercetin was present at the lowest concentration (0.009%) among the detected compounds. Besides that, Terpenes, Vitamins, Phenylpropanoids, and other functional ingredients were also found in RHE (Table 6).

Table 6
Mass spectrometry data of 22 compounds.

Peak number	Molecular formula	Molecular weight	Peak area	Retention time (min)	Chemical compound
1	$C_{15}H_{10}O_3$	239.07	1.10E + 03	0.12	Flavonol
2	$C_{15}H_{10}O_2$	223.075	9.37E + 05	0.14	Flavone
3	$C_{16}H_{18}O_9$	355.102	6.54E + 04	0.14	Chlorogenic acid
4	$C_{10}H_{18}O$	155.143	2.83E + 03	0.24	Linalool
5	$C_{15}H_{22}O_3$	251.164	1.12E + 04	0.28	2-Ethylhexyl salicylate
6	$C_4H_{10}N_4O_2$	147.088	1.41E + 03	0.29	Succinic dihydrazide
7	$C_{10}H_{16}O$	153.127	3.21E + 02	0.35	Celery ketone
8	$C_{15}H_{10}O_7$	303.05	1.09E + 02	0.39	Quercetin
9	$C_8H_{12}ClNO_3$	206.058	3.63E + 02	0.46	Pyridoxine hydrochloride
10	$C_{10}H_{16}$	137.132	2.04E + 03	0.49	Cinene
11	C_8H16_O	129.127	2.12E + 02	0.55	Octanal
12	$C_{20}H_{30}O$	287.237	1.31E + 05	0.83	retinol
13	$C_{16}H_{12}O_6$	301.071	2.01E + 03	1.28	Diosmetin
14	$C_{16}H_{12}O_5$	285.076	1.64E + 03	1.28	Acacetin
15	$C_{10}H_{14}O$	151.112	2.53E + 04	1.89	thymol
16	$C_5H_{14}ON$	105.115	1.17E + 02	2.19	Choline
17	$C_5H_5N_5$	136.062	2.13E + 02	3.33	Adenine

Peak number	Molecular formula	Molecular weight	Peak area	Retention time (min)	Chemical compound
18	$C_{15}H_{10}O_6$	287.055	1.83E + 03	10.76	luteolin
19	$C_7H_{14}NO_2$	145.11	7.08E + 04	11.56	stachydrine
20	$C_{15}H_{10}O_5$	271.06	2.61E + 02	12.61	Apigenin
21	$C_9H_{10}O$	135.08	1.09E + 04	13.54	4-Methylacetophenone
22	$C_{24}H_{22}O_{14}$	535.108	4.54E + 02	13.72	Kaempferol-3-O-(6-Malonyl-Glucoside)

Analysis of mass spectrometry data of ethanol extracts of *R. hirta* using HPLC–MS. The underline indicates that the substance belongs to flavonoids or their derivatives.

4. Discussion

During the long time interaction and co-evolution of plants and insect herbivores, both host plants and insects have obtained diverse complicated mechanisms to adapt to each other (Barbero et al., 2023; Luo et al., 2023; Wang et al., 2023). On the one hand, when suffering from insect damage, plants interfere with and weaken insects ingestion, even poison insects by producing a variety of secondary metabolites (Aboshi et al., 2021; Divekar et al., 2022; Fürstenberg-Hägg et al., 2013), on the other hand, insects resist toxic substances in plants through own detoxification metabolic system and antioxidant defense system (Chen et al., 2023; Li et al., 2023; Pang et al., 2023). In the present study, we designed and optimized the extraction conditions of *R. Hirta* by response surface to obtain heavier extracts, the relative error of actual results and predicted value of the model was merely 0.9%, meaning the rationality and reliability of the design and optimization. Previous research has shown that extracts of *R. Hirta* have insecticidal activity against some species of insect (Metzger et al., 1932; Guillet et al., 1997), our research found that RHE exerted insecticidal activity against the 3rd instar larvae of *M. neustria Testacea*, the value of LC_{50} was 6.437 mg/mL. Interference with the feeding and digestion of herbivorous insects is also one of the defense mechanisms for various plants with insecticidal activity (Chabaane et al., 2022; Wari et al., 2022; Zhao et al., 2018), similarly, RHE significantly affected larval feeding, digestion, and nutrient uptake, six nutritional indices (LWG, RGR, ECI, RCR, AD, and AR) of RHE-treated larvae were suppressed, only the ECD was activated, this may be due to the extracts affected the larval excretory system, resulting in abnormal fecal morphology and weight. The interference of RHE with the normal growth and development of larvae was one of the important reasons for larval mortality.

The detoxification metabolic system stimulates the degradation and reduction of external toxins in insects, an important component of their physiological and biochemical defense systems (Koirala et al., 2022). The detoxifying enzymes include ACP, AKP, GST, and CarE (Fan et al., 2023; Sun et al., 2020). Our

findings demonstrate a significant inhibition effect on activities of ACP, AKP and GST following RHE treatment, the activities of ACP, AKP, GST showed varying degrees of decline within 72 h after treatment, the activity of GST was only 0.51-fold of the control group at 12 h after treatment notably, their activities were inhibited and could not perform their biochemical functions normally, this is likely a key reason to the pronounced elevated mortality among *M. neustria testacea* larvae. Interestingly, the activity of CarE was significantly facilitated after treatment, as an early pivotal detoxifying enzyme, CarE hydrolyzed diverse exogenous toxins, its activity increased in the early stages of treatment, and was used to resist the toxicity of RHE.

Meanwhile, antioxidant defense reactions are also crucial in the physiological and biochemical defense system of insects, which resist external biological factors or environmental factors stresses. Various antioxidant enzymes, including CAT, POD, SOD, and PPO participate in the insects' antioxidant defense reactions (Zhang et al., 2023). We found that the activities of CAT, POD, and SOD were significantly suppressed within 72 h after treatment, the activities of POD and SOD were only 0.11 and 0.24-fold of the control group at 12 h after treatment, respectively. The insects' antioxidant defense system is one of the targets of numerous insecticides. Under the stress of RHE, *M. neustria testacea* larvae produce oxidative stress response, resulting in the production of a large number of superoxide anion (O), reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH) and other detrimental in vivo, yet larval antioxidant defense mechanism was weakened, unable to reduce the damage caused by oxidative stress, this is likely another crucial contributor to larval death. Diametrically, the activity of PPO was significantly expanded after treatment, indicating its important role in maintaining the balance of the redox system.

Using insecticides to control agricultural pests may lead to important risks for non-target aquatic organisms (Ba et al., 2018). Previous studies have shown that the methanolic extract of *Annickia chlorantha* had mosquitocidal activity, and a very low toxic effect on on the non-target organism *D. Rerio* (Selim et al., 2022). Our research aligns with these findings, the RHE was practically nontoxic to *D. rerio* compared to the toxicity of *M. neustria testacea* larvae reported here.

Through analysis of the chemical constituents of RHE, we found that RHE contains 22 compounds, ten of which were flavone or flavonoid derivatives, accounting for 75.47% of the total compound contents. The extracts of *R. hirta* have revealed the presence of more than 250 compounds, including sesquiterpene lactones, polyphenolic acids, flavonoids, amino acids, and fatty acids (Burlec et al., 2023). We believe that the insecticidal activity of RHE was mainly attributed to the flavone and flavonoid derivatives. Related studies have shown that flavone or flavonoid derivatives suppressed normal growth, development, feeding, and digestion of insects by inhibiting the expression of genes related to ecdysone synthesis, as well as the activity of detoxifying and digestive enzymes (Felton et al., 1991; Oberdörster et al., 2001; Singh et al., 2021; Wang et al., 2014). Future investigations would elucidate the impact of flavonoids on the expression of genes, which are related to the detoxification metabolic system and antioxidant defense system through transcriptome analysis.

5. Conclusion

In conclusion, we designed and optimized the extraction conditions of RHE, and found that RHE had toxic and anti-feeding activities against the 3rd instar larvae of *M. neustria testacea*. RHE's insecticidal activity was mainly attributed to the flavone and flavonoid derivatives. RHE interfered with the normal growth and development of larvae by weakening larval feeding, digestion, and nutrient uptake. Meantime, RHE inhibited the activities of multiple enzymes of the detoxification metabolic system and antioxidant defense system in vivo of larvae, which further enhanced the insecticidal activity of RHE. Subsequent determination of toxicity to non-target aquatic organisms highlighted the safety of RHE as a potentially exploitative botanical insecticide.

Declarations

Funding

This research was supported by the Project of Forestry Generalized and Demonstrative S & T of Central Finance (2021TG08), Science and Technology Department of Heilongjiang Province (2023TG21) and Joint Guidance of Heilongjiang Province (JJ2023LH0857).

Author contributions

Guocai Zhang and Jie Zhang conceived and designed the research. Yajun Wang and PHAM HUNG HAI conducted the experiments. Weihu Ma and Kejiao Li analyzed the data. Yajun Wang and PHAM HUNG HAI wrote the manuscript. All authors have read and approved the manuscript.

Ethical approval

This article does not contain any studies with human participants performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no conflict of interest with the contents of this article.

References

1. W. Zhou, X. Xia, A. Mendoza-Mendoza, W. Wakil, K.S. Akutse, X. Bing, 2023. Editorial: Insect physiological changes during insect-plant interaction. Frontiers in physiology. 14, 1175813.
2. C. Zou, C. Lv, Y. Wang, C. Cao, G. Zhang, 2017. Larvicidal activity and insecticidal mechanism of *Chelidonium majus* on *Lymantria dispar*. Pesticide biochemistry and physiology. 142, 123–132.
3. H. Bouwmeester, R.C. Schuurink, P.M. Bleeker, F. Schiestl, 2019. The role of volatiles in plant communication. The Plant journal: for cell and molecular biology. 100, 892–907.

4. G.S. Fraenkel, 1959. The raison d'ĕtre of secondary plant substances; these odd chemicals arose as a means of protecting plants from insects and now guide insects to food. Science (New York, N.Y.). 129, 1466–1470.
5. E.M. Rothwell, L.M. Holeski, 2020. Phytochemical defences and performance of specialist and generalist herbivores: a meta-analysis. Ecological Entomology. 45, 396–405.
6. A. Kachura, C.S. Harris, 2022. An ethnobotanical meta-analysis of North American medicinal Asteraceae. Botany. 100, 207–217.
7. F. Liu, Q. Wang, P. Xu, F. Andreazza, W.R. Valbon, E. Bandason, M. Chen, R. Yan, B. Feng, L.B. Smith, J.G. Scott, G. Takamatsu, M. Ihara, K. Matsuda, J. Klimavicz, J. Coats, E.E. Oliveira, Y. Du, K. Dong, 2021. A dual-target molecular mechanism of pyrethrum repellency against mosquitoes. Nature communications. 12, 2553.
8. O. Kenny, T.J. Smyth, D. Walsh, C.T. Kelleher, C.M. Hewage, N.P. Brunton, 2014. Investigating the potential of under-utilised plants from the Asteraceae family as a source of natural antimicrobial and antioxidant extracts. Food chemistry. 161, 79–86.
9. E.V.A. B, J.T.L. C, C.L.Q.C. D, 2021. The genus *Rudbeckia*: A critical review of its traditional medicinal uses, phytochemistry, and pharmacology. Journal of Herbal Medicine.
10. B.R. Michael, S.R. Gedara, M.M. Amer, L. Stevenson, A.F. Ahmed, 2014. Evidence-based medicinal value of *Rudbeckia hirta* L. flowers. Natural Product Research. 28, 909–913.
11. A.F. Burlec, Ł. Pecio, C. Mircea, C. Tuchiluș, A. Corciovă, C. Danciu, O. Cioancă, I.C. Caba, S. Pecio, W. Oleszek, M. Hăncianu, 2023. Preliminary Phytochemical and Biological Evaluation of *Rudbeckia hirta* Flowers. Plants (Basel, Switzerland). 12,
12. W.N. Setzer, 2018. The Phytochemistry of Cherokee Aromatic Medicinal Plants. Medicines (Basel, Switzerland). 5,
13. Metzger, F.W. Grant, H.J.T.B. D., 1932. Repellency to the Japanese Beetle of Extracts Made From Plants Immune to Attack. Technical Bulletins.
14. G. Guillet, B.J.R. Philogène, J. O'Meara, T. Durst, J.T.J.P. Arnason, 1997. Multiple modes of insecticidal action of three classes of polyacetylene derivatives from *Rudbeckia hirta*. Phytochemistry. 46, 495–498.
15. D. Gencer, 2023. Isolation and characterization of a high-efficacy *Beauveria bassiana* strain from the European tent caterpillar, *Malacosoma neustria* Linnaeus (Lepidoptera: Lasiocampidae). Folia microbiologica. 68, 579–586.
16. V.V. Dubatolov, V.V.J.F.n.r. Zolotuhin, 1992. A list of the Lasiocampidae from the territory of the former USSR (Insecta, Lepidoptera). Atalanta (Wurzburg).
17. Y. Chen, J. Yang, G. Zhang, B. Zhang, J. Zeng, H. Zou, T. Li, 2021. Larvicidal activity and microencapsulation of tobacco (*Nicotiana tabacum*) extract on *Malacosoma neustria testacea* larvae. Journal of Forestry Research. 32, 1763–1773.
18. S. Savary, L. Willocquet, S.J. Pethybridge, P. Esker, N. McRoberts, A. Nelson, 2019. The global burden of pathogens and pests on major food crops. Nature ecology & evolution. 3, 430–439.

19. J. Saien, S. Daneshamoz, 2018. Experimental studies on the effect of ultrasonic waves on single drop liquid-liquid extraction. Ultrasonics sonochemistry. 40, 11–16.
20. J. Lai, C. Xin, Y. Zhao, B. Feng, C. He, Y. Dong, Y. Fang, S. Wei, 2013. Optimization of ultrasonic assisted extraction of antioxidants from black soybean (*Glycine max* var) sprouts using response surface methodology. Molecules (Basel, Switzerland). 18, 1101–1110.
21. H.A. Mohamed, K.S. Ghoneim, A.S.J.P.J.o.B.S. Bream, 2003. Neemazal Effects on the Consumption and Utilization of Food in Some Early Larval Instars of the Cotton Leafworm, *Spodoptera littoralis* Boisd. (Noctuidae:Lepidoptera). Pak. J. Biol. Sci.
22. F. Barbero, M.E. Maffei, 2023. Recent Advances in Plant-Insect Interactions. International journal of molecular sciences. 24,
23. M. Luo, B. Li, G. Jander, S. Zhou, 2023. Non-volatile metabolites mediate plant interactions with insect herbivores. The Plant journal: for cell and molecular biology. 114, 1164–1177.
24. H. Wang, S. Shi, W. Hua, 2023. Advances of herbivore-secreted elicitors and effectors in plant-insect interactions. Frontiers in plant science. 14, 1176048.
25. T. Aboshi, C. Iitsuka, I. Galis, M. Teraishi, M. Kamo, A. Nishimura, A. Ishihara, N. Mori, T. Murayama, 2021. Isopentylamine is a novel defence compound induced by insect feeding in rice. Plant, cell & environment. 44, 247–256.
26. P.A. Divekar, S. Narayana, B.A. Divekar, R. Kumar, B.G. Gadratagi, A. Ray, A.K. Singh, V. Rani, V. Singh, A.K. Singh, A. Kumar, R.P. Singh, R.S. Meena, T.K. Behera, 2022. Plant Secondary Metabolites as Defense Tools against Herbivores for Sustainable Crop Protection. International journal of molecular sciences. 23,
27. J. Fürstenberg-Hägg, M. Zagrobelny, S. Bak, 2013. Plant defense against insect herbivores. International journal of molecular sciences. 14, 10242–10297.
28. L. Chen, J. Song, J. Wang, M. Ye, Q. Deng, X. Wu, X. Wu, B. Ren, 2023. Effects of Methyl Jasmonate Fumigation on the Growth and Detoxification Ability of *Spodoptera litura* to Xanthotoxin. Insects. 14,
29. T. Li, L. Yuan, Y. Huang, A. Zhang, D. Jiang, S. Yan, 2023. Assessment of cytisine as an insecticide candidate for *Hyphantria cunea* management: Toxicological, biochemical, and control potential insights. Pesticide biochemistry and physiology. 196, 105638.
30. J. Pang, Y. Peng, T. Di, G. Du, B. Chen, 2023. Virulence of Metarhizium rileyi Is Determined by Its Growth and Antioxidant Stress and the Protective and Detoxifying Enzymes of *Spodoptera frugiperda*. Insects. 14,
31. Y. Chabaane, C. Marques Arce, G. Glauser, B. Benrey, 2022. Altered capsaicin levels in domesticated chili pepper varieties affect the interaction between a generalist herbivore and its ectoparasitoid. Journal of pest science. 95, 735–747.
32. D. Wari, T. Aboshi, T. Shinya, I. Galis, 2022. Integrated view of plant metabolic defense with particular focus on chewing herbivores. Journal of integrative plant biology. 64, 449–475.
33. A. Zhao, Y. Li, C. Leng, P. Wang, Y. Li, 2018. Inhibitory Effect of Protease Inhibitors on Larval Midgut Protease Activities and the Performance of *Plutella xylostella* (Lepidoptera: Plutellidae). Frontiers in

physiology. 9, 1963.

34. B.K.S. Koirala, T. Moural, F. Zhu, 2022. Functional and Structural Diversity of Insect Glutathione S-transferases in Xenobiotic Adaptation. International journal of biological sciences. 18, 5713–5723.
35. R. Fan, Z. Fan, Z. Sun, Y. Chen, F. Gui, 2023. Insecticide Susceptibility and Detoxification Enzyme Activity of *Frankliniella occidentalis* under Three Habitat Conditions. Insects. 14,
36. L. Sun, J. Yin, H. Du, P. Liu, C. Cao, 2020. Characterisation of GST genes from the *Hyphantria cunea* and their response to the oxidative stress caused by the infection of *Hyphantria cunea* nucleopolyhedrovirus (HcNPV). Pesticide biochemistry and physiology. 163, 254–262.
37. K. Zhang, S. Nakamura, S. Furukawa, 2023. Cloak Scavenges the Reactive Oxygen Species around the Larvae of *Drino inconspicuoides* (Diptera: Tachinidae). Insects. 14,
38. M.N. Ba, J.E. Huesing, M. Tamò, T.J.V. Higgins, B.R. Pittendrigh, L.L. Murdock, 2018. An assessment of the risk of Bt-cowpea to non-target organisms in West Africa. Journal of pest science. 91, 1165–1179.
39. T.A. Selim, I.E. Abd-El Rahman, H.A. Mahran, H.A.M. Adam, V. Imieje, A.A. Zaki, M.A.E. Bashar, H. Hwihy, A. Hamed, A.A. Elhenawy, E.S. Abou-Amra, S.E. El-Didamony, A.I. Hasaballah, 2022. Mosquitocidal Activity of the Methanolic Extract of Annickiachlorantha and Its Isolated Compounds against *Culex pipiens*, and Their Impact on the Non-Target Organism Zebrafish, *Danio rerio*. Insects. 13,
40. G.W. Felton, S.S. Duffey, 1991. Protective action of midgut catalase in lepidopteran larvae against oxidative plant defenses. Journal of chemical ecology. 17, 1715–1732.
41. E. Oberdörster, M.A. Clay, D.M. Cottam, F.A. Wilmot, J.A. McLachlan, M.J. Milner, 2001. Common phytochemicals are ecdysteroid agonists and antagonists: a possible evolutionary link between vertebrate and invertebrate steroid hormones. The Journal of steroid biochemistry and molecular biology. 77, 229–238.
42. S. Singh, I. Kaur, R. Kariyat, 2021. The Multifunctional Roles of Polyphenols in Plant-Herbivore Interactions. International journal of molecular sciences. 22,
43. Z. Wang, Z. Zhao, M.M. Abou-Zaid, J.T. Arnason, R. Liu, B. Walshe-Roussel, A. Waye, S. Liu, A. Saleem, L.A. Cáceres, Q. Wei, I.M. Scott, 2014. Inhibition of insect glutathione S-transferase (GST) by conifer extracts. Archives of insect biochemistry and physiology. 87, 234–249.

Figures

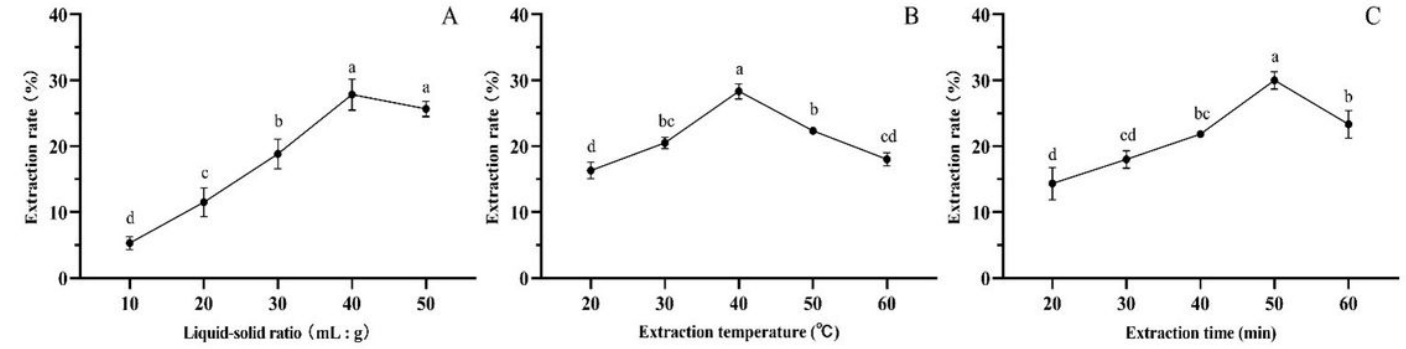

Figure 1

The effects of different factors on extraction rates. A, the independent variable was liquid-solid ratio, the other extraction conditions were fixed (temperature 45 °C and time 35 min); B, the independent variable was extraction temperature, the other extraction conditions were fixed (liquid-solid ratio 40:1 and time 35 min); C independent variable was extraction time, the other extraction conditions were fixed (liquid-solid ratio 40:1 and temperature 45 °C). Error bars represented the standard error and data were presented as mean ± S.E. Different letters indicated significant differences between groups.

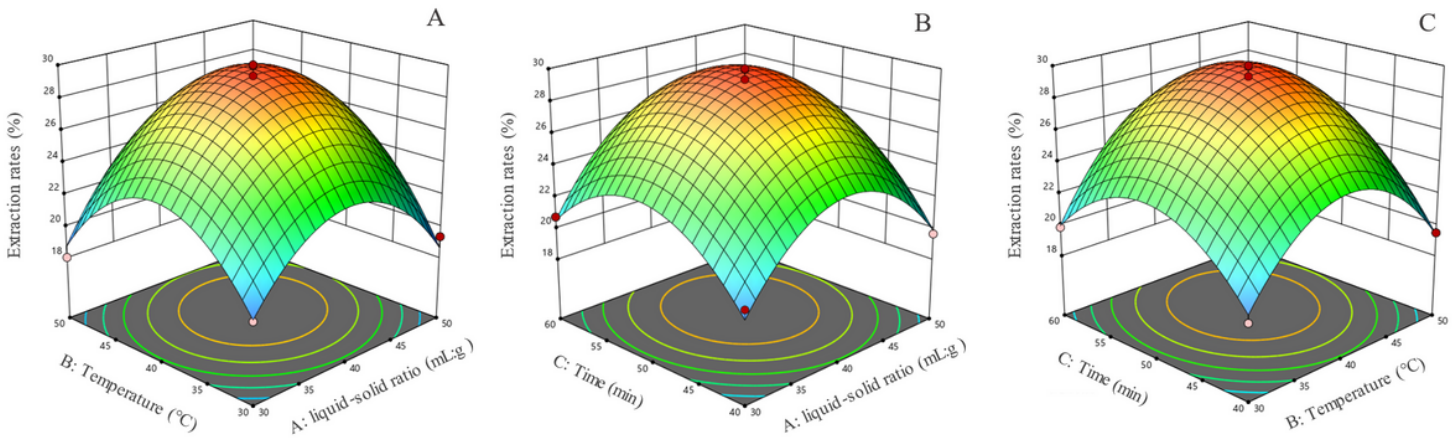

Figure 2

Response surface for the interaction of different factors. Response surface can reflect the degree of influence of experimental factors on the response value. The response surface has a steep slope, and experimental factors have a significant impact on the response value. A, Interaction between liquid-solid ratio and temperature. B, Interaction between temperature and time.C, Interaction between liquid-solid ratio and time. All data represented the average results of each group, and each group was independently repeated in triplicate.

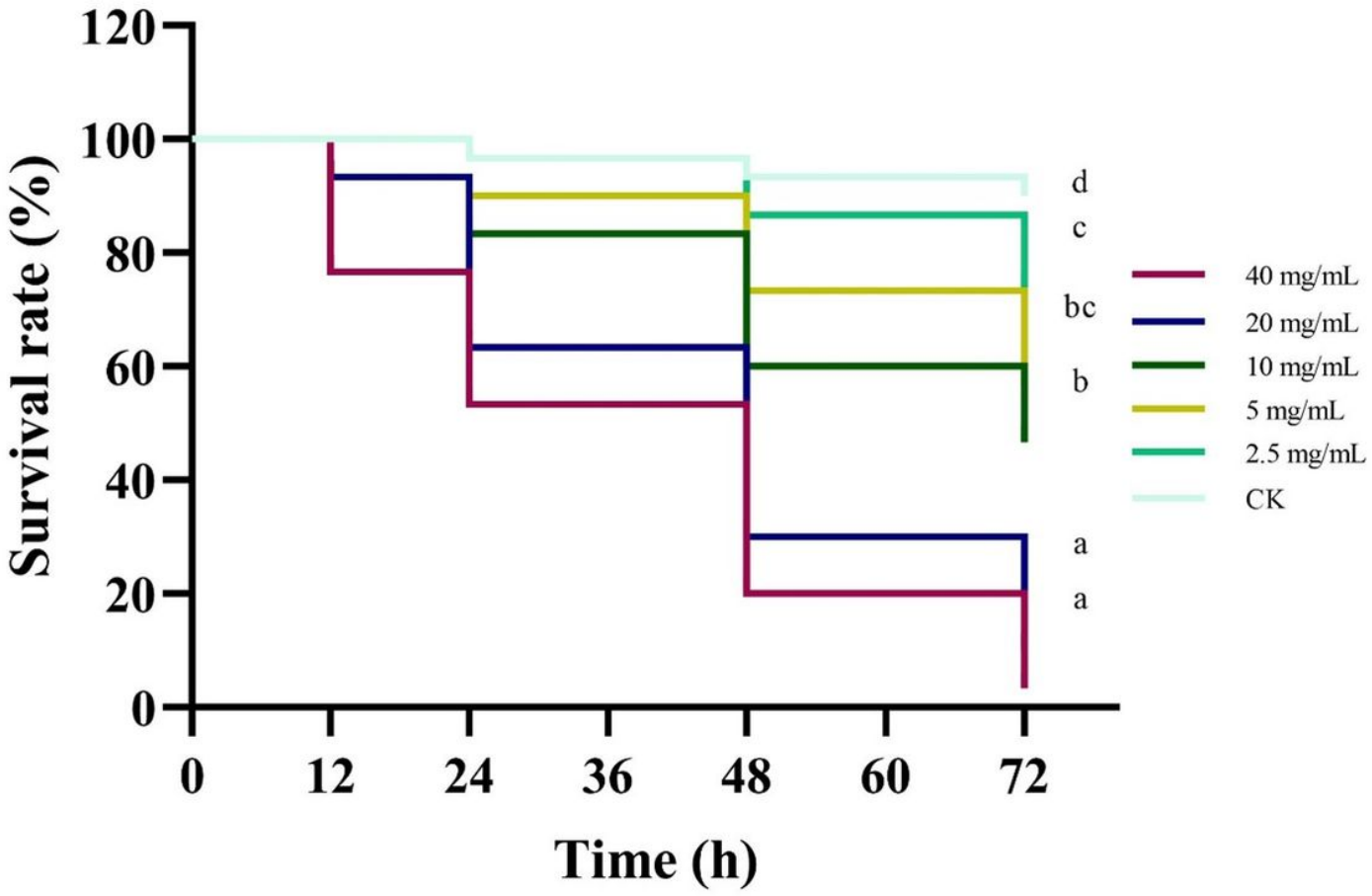

Figure 3

Survival curve analysis of *M. neustria testacea* larvae treated with different concentrations of RHE. Values were mean ± S.E. Different letters indicated significant differences between groups.

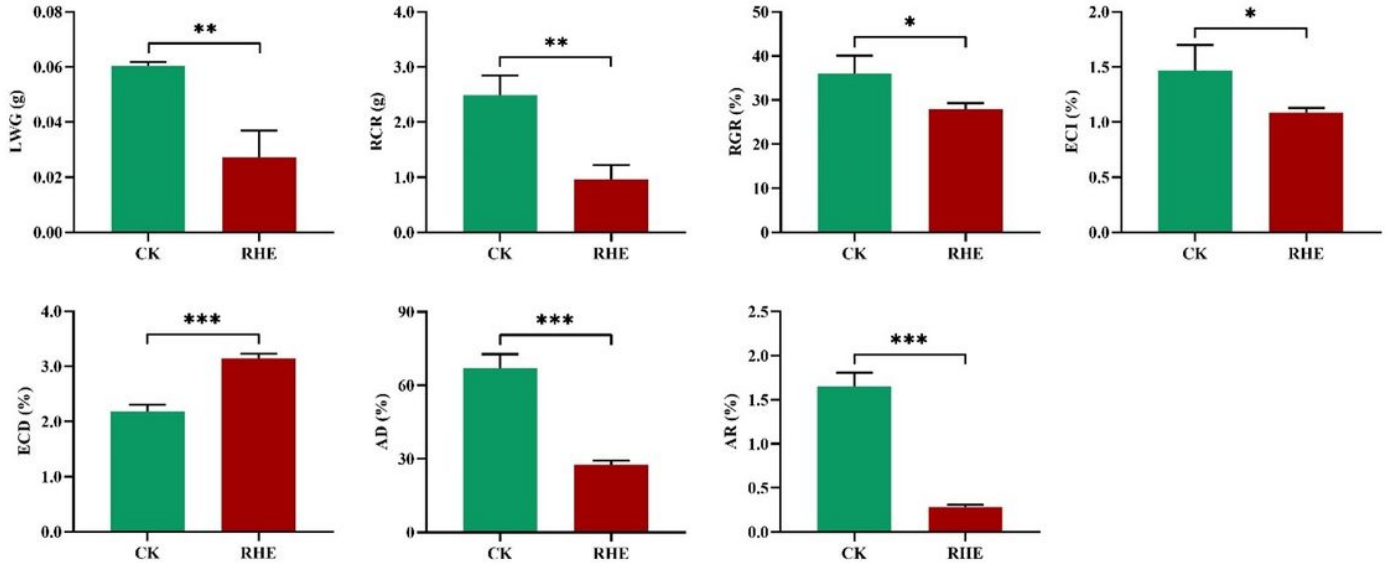

Figure 4

Determination of the nutritional index of *M. neustria testacea*larvae.B, The relative consumption rate (RCR) was calculated as weight of food ingested / (average larval weight during trial × number of days). C, The relative growth rate (RGR) was calculated as larval weight gain (LWG) / (average weight during trial × number of days). D, The efficiency of the conversion of ingested food to body substance (ECI) was measured by dividing the weight gain by the weight of ingested food and multiplying by 100. E, The efficiency of the conversion of digested food into growth (ECD) was evaluated as weight gain / (weight of ingested food − weight of feces) × 100. F, The conversion of ingested food, indicated as approximate digestibility (AD), was determined as 100 × (weight of ingested food − weight of excreta) / weight of ingested food. G, The assimilation rate (AR) of food was estimated by multiplying the RCR by the AD. Error bars represent the standard error, and data were presented as the mean ± S.E. “*” was meaning $p < 0.05$, “**” was meaning $p < 0.01$ and “***” was meaning $p < 0.001$.

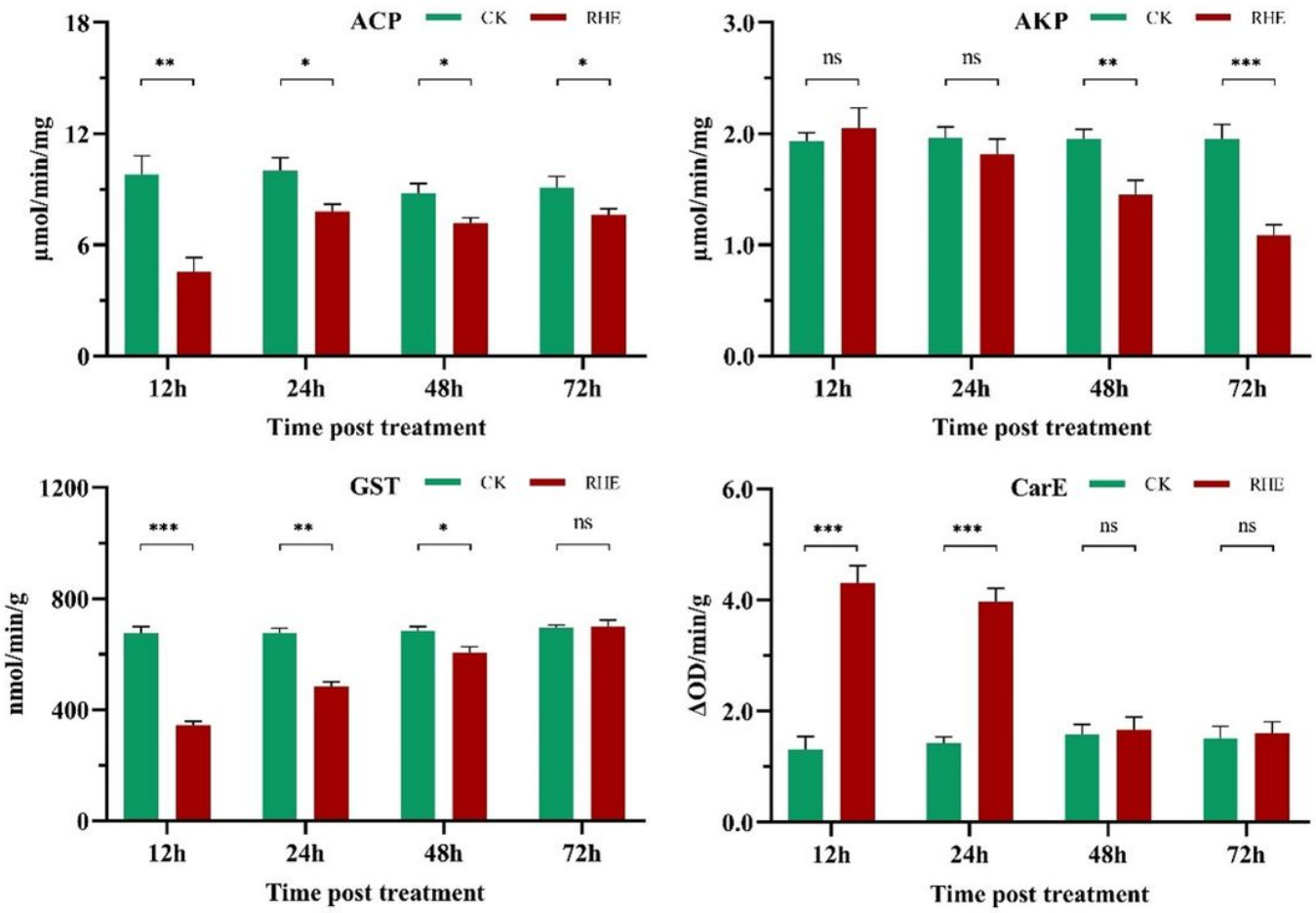

Figure 5

Effect of RHE on larval four detoxifying enzymes activity. The 3rd instar *M. neustria testacea* larvae were treated with RHE (a sublethal concentration of 2.629 mg/mL). The activities of ACP, AKP, GST, and CarE were measured at 12, 24, 48, and 72 h after treatment. Each treatment was independently repeated in triplicate. Error bars represented the standard error, and data were presented as the mean ± S.E. "*" was meaning $p < 0.05$, "**" was meaning $p < 0.01$, "***" was meaning $p < 0.001$ and "ns" was meaning no significance.

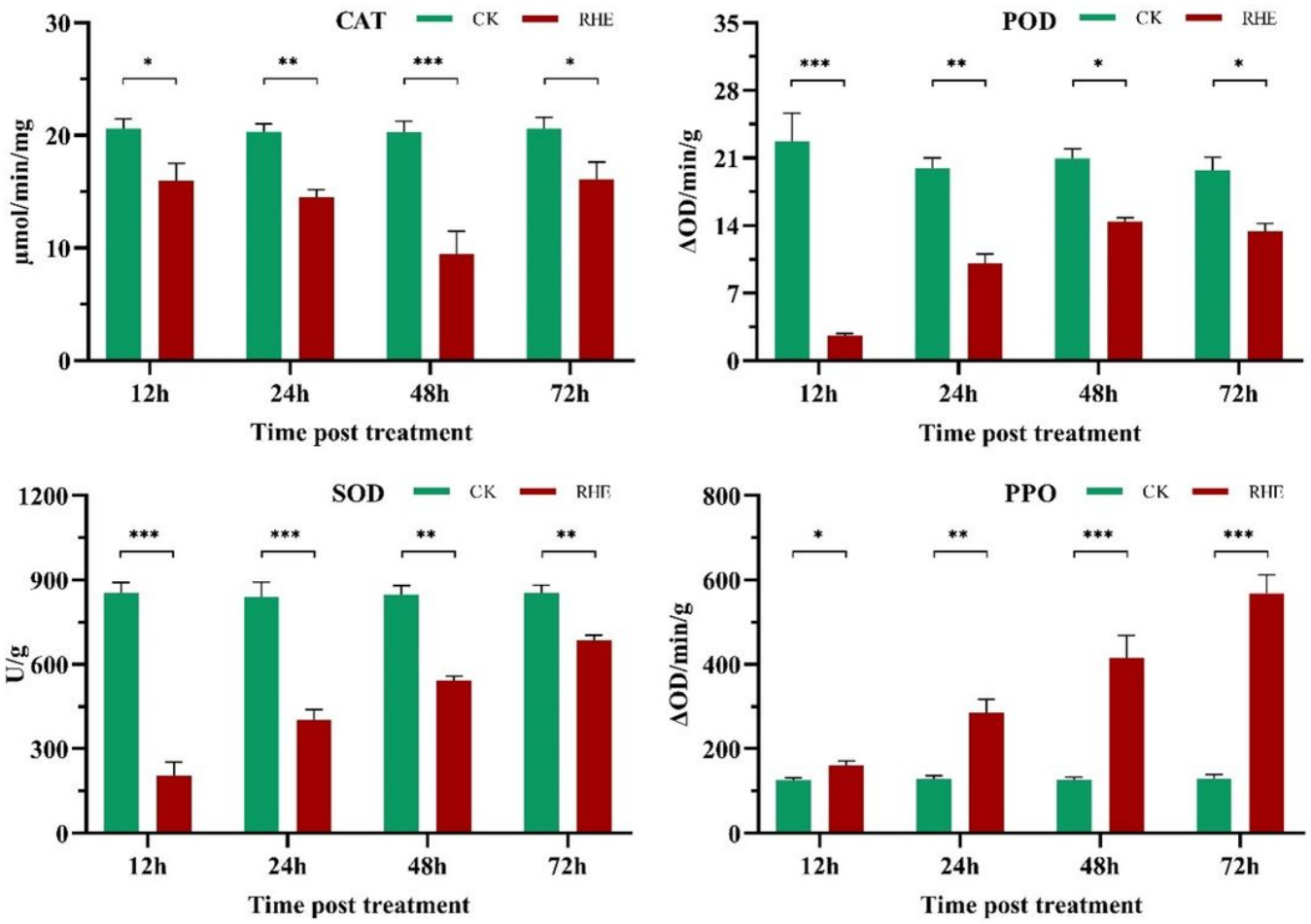

Figure 6

Effect of RHE on larval four antioxidant enzymes activity. The 3rd instar *M. neustria testacea* larvae were treated with RHE (a sublethal concentration of 2.629 mg/mL). The activities of CAT, POD, SOD, and PPO were measured at 12, 24, 48, and 72 h after treatment. Each treatment was independently repeated in triplicate. Error bars represented the standard error, and data were presented as the mean ± S.E. “*” was meaning $p < 0.05$, “**” was meaning $p < 0.01$, “***” was meaning $p < 0.001$ and “ns” was meaning no significance.

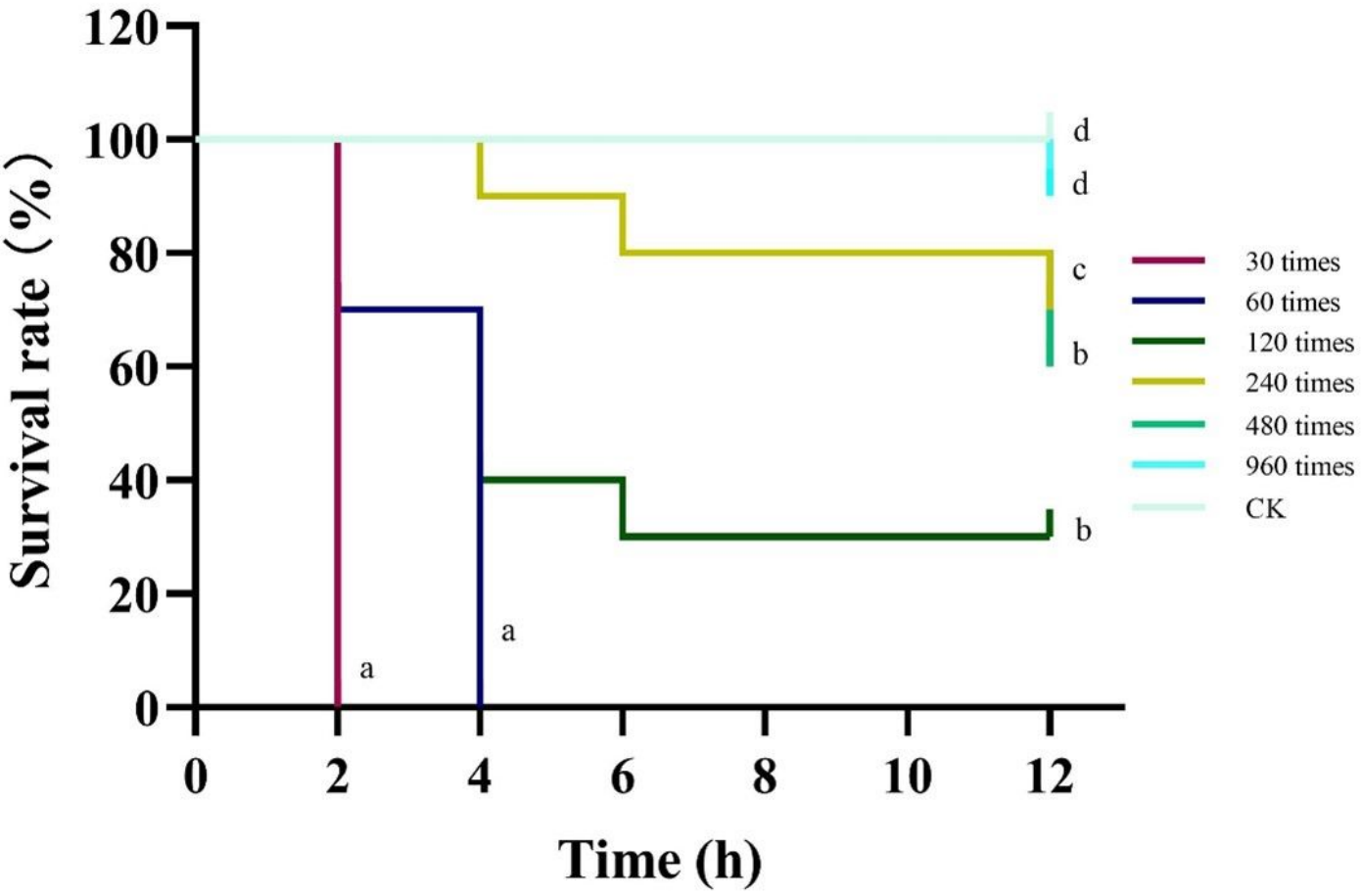

Figure 7

Survival curve analysis of *D. rerio*treated with different concentrations of RHE. Values were mean ± S.E. Different letters indicated significant differences between groups.

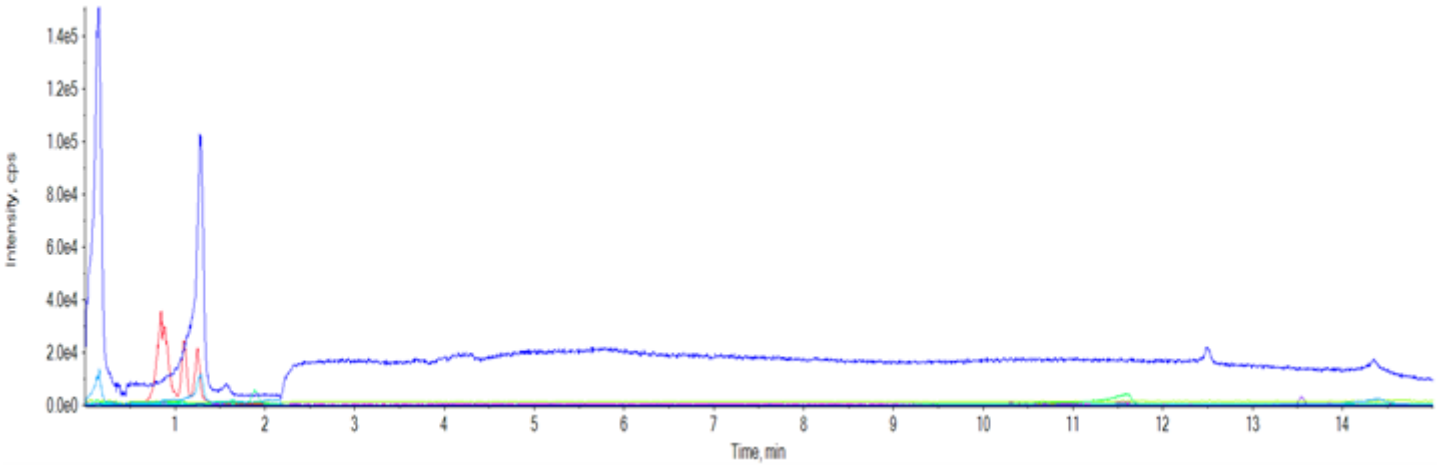

Figure 8

Total ions flow chromatograms of ethanol extracts of *R. hirta*.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GA.jpg
- SupplementaryInformation.docx