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Study on extraction technology and antioxidant activity of "Sibai" extracts based on orthogonal design and *Caenorhabditis elegans*

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ABSTRACT

Objective: This study examined the antioxidant, whitening, anti-aging, and safety properties of "Sibai" extract obtained from *Bombyx Batryticatus*, *Ampelopsis japonica*, *Radix Paeoniae Alba*, and *Atractylodes macrocephala*.
Methods: The "Sibai" extract was optimized by orthogonal design and evaluated for in vitro antioxidant activity through hydroxyl radical scavenging, DPPH scavenging, and total reducing capacity measurements. Tyrosinase inhibition and hemolysis assays assessed its whitening potential and biosafety. Anti-aging effects were examined using *Caenorhabditis elegans (C. elegans)* lifespan and fecundity assays, while antioxidant capacity under H_2O_2, juglone, and heat stress was evaluated. Motor function was analyzed via head thrashing, body bending, and spontaneous locomotion tests. Finally, aging-related changes in lipofuscin, reactive oxygen species (ROS), malondialdehyde (MDA), and catalase (CAT) levels were measured.
Results: The optimal ratio of *Bombyx Batryticatus*, *Ampelopsis japonica*, *Radix Paeoniae Alba*, and *Atractylodes macrocephala* rhizome was determined to be 3:3:1:1. At specific concentrations, the extract demonstrated significant biological activity: the scavenging rate for hydroxyl radicals was 61.70% (at 48 mg/mL), the scavenging rate for DPPH radicals was 93.53% (at 4 mg/mL), and its total reducing power at 48 mg/mL was comparable to that of Vitamin C (Vc). The tyrosinase inhibition rate was 74.90% (at 24 mg/mL), and the hemolysis rate remained below 15%, indicating good in vitro safety. In lifespan experiments using *C. elegans* as a model, treatment with 48 mg/mL extract reduced fecundity and extended the normal lifespan by 38.5%. Under oxidative stress induced by H_2O_2 and juglone, as well as under heat stress, the survival time of *C. elegans* in the 48 mg/mL treatment group increased by 37.9%, 69.5%, and 49.2% respectively. In terms of motor ability, the 48 mg/mL treatment group showed increases in head thrashing and body bending frequencies by 61.4% and 220.7% respectively, along with excellent autonomous movement capacity. At the cellular level, levels of lipofuscin, ROS, and MDA were reduced by 65.8%, 41.5%, and 51.3% respectively, while CAT activity increased by 329.1%.
Conclusion: This study employed an orthogonal experimental design to determine the optimal formulation. Subsequent validation confirmed that the optimized extract possessed remarkable antioxidant and whitening properties. Furthermore, it was found to significantly enhance stress resistance in *C. elegans* and improve age-related phenotypes. These results indicated that this formulation may effectively address issues related to aging and oxidative stress.

1. Introduction

As individuals age, they frequently develop a range of diseases. These diseases include type 2 diabetes and cancer.[1] One mechanism that links aging to age-related diseases is the accumulation of reactive oxygen species (ROS). Under environmental stress, organisms produce and accumulate ROS, leading to oxidative stress that damages cells and tissues.[2] The body experiences an imbalance between antioxidants and

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pro-oxidants, leading to a condition known as oxidative stress.[3] Antioxidants can be categorized as either enzymatic or non-enzymatic, with examples such as catalase (CAT) and glutathione (GSH). Exogenous antioxidants can effectively scavenge free radicals, enhance antioxidant defenses, and mitigate ROS damage.[4]

The *Bombyx Batryticatus*, *Ampelopsis japonica*, *Radix Paeoniae Alba*, and *Atractylodes macrocephala* have been confirmed to contain various chemical substances, such as polysaccharides, volatile oils, polyphenols, and anthraquinones, which are known to possess antioxidant activity.[5] However, the optimal ratios of these substances and their interactions with anti-aging activity remain unclear. This study aims to further investigate the composite ratios of these four substances through orthogonal experiments to enhance the efficacy of the extracts. Additionally, we will assess their potential to delay the aging process. Furthermore, our research aims to address the gap in knowledge regarding the effects of "Sibai" extract on the lifespan, oxidative stress resistance, heat stress tolerance, and motor abilities of *C. elegans*. This will provide a scientific foundation for developing novel methods to delay aging.

2. Materials and methods

Bombyx Batryticatus, *Radix Paeoniae Alba*, *Atractylodes macrocephala*, and *Ampelopsis japonica* were purchased from Zhongshan Xian Yitang Chinese Herbal Medicine Co., Ltd.; *E. coli* OP50 was obtained from Shandong Zhongke Jiayi Biological Engineering Co., Ltd.; *C. elegans* was provided by the Xiamen University Laboratory; NGM medium was from Shandong Top Engineering Co., Ltd.; and the ROS, CAT, and MDA assay kits were purchased from Solarbio Technology Co., Ltd.

2.1. Preparation of "Sibai" extract samples

The four medicinal materials are crushed and sieved through a 60-mesh filter. They are subsequently mixed with 80% ethanol at a material-to-liquid ratio of 1:12 and soaked for 24 h. The extraction procedure is repeated three times, utilizing ultrasound for 30 min each time.[6] The mixture is then filtered under reduced pressure, and the filtrate is concentrated to eliminate the ethanol. Four volumes of anhydrous ethanol are added, and the mixture is allowed to stand overnight prior to centrifugation. The supernatant is subsequently concentrated until devoid of ethanol and diluted to a final concentration of 0.5 g/mL with ultra-pure water, rendering it ready for use.

2.2. Ortho experimental design

The optimization experiment of the compound ratio was conducted using the L9(3^4) orthogonal array.[7,8] The design included nine groups, four factors, and two levels. In this study, the four factors were defined as *Bombyx Batryticatus* (A), *Ampelopsis japonica* (B), *Radix Paeoniae Alba* (C), and *Atractylodes macrocephala* (D), with a total amount of 10 g. Two indicators were used, namely hydroxyl radical scavenging capacity and tyrosinase inhibition ability, the ratio of the two is 50% each.

2.3. Antioxidant activity test

The antioxidant capacity of the extract was evaluated by measuring its hydroxyl radical scavenging activity, DPPH radical scavenging activity, and total reducing power. For hydroxyl radical scavenging, samples (3–48 mg/mL) were reacted with a mixture of salicylic acid, $FeSO_4$, and H_2O_2 at 37 °C for 15 min, and absorbance was measured at 510 nm. For DPPH scavenging, samples (1–10 mg/mL) were mixed with 0.1 mmol/L DPPH ethanol solution, reacted in the dark for 30 min, and absorbance was measured at 517 nm. For total reducing power, samples (3–48 mg/mL) were incubated with potassium ferricyanide at 50 °C for 20 min, followed by the addition of trichloroacetic acid and ferric chloride, and absorbance was measured at 700 nm. Vc was used as a positive control in all assays. The aforementioned methods were performed with reference to literature.[9–12]

2.4. Tyrosinase inhibition rate test

As outlined in section 2.3, samples of varying concentrations were prepared (3–48 mg/mL). A volume of 1 mL was aliquoted, followed by the sequential addition of 0.5 mL of tyrosinase (100 U/mL). The mixture was incubated at 37 °C for 10 min, after which 2 mL of a 1 mg/mL L-DOPA solution was introduced. The mixture was allowed to react for 5 min. Absorbance was measured at 475 nm at the conclusion of the reaction.[13]

2.5. Erythrocyte haemolysis assay

Wash the rabbit blood with phosphate-buffered saline (PBS) at pH 7.4 to eliminate impurities. Centrifuge the sample at 10,000 rpm for 2 min. Discard the supernatant and repeat this procedure three times. The washed erythrocytes were mixed with PBS at a 1:9 ratio. Prepare samples of varying concentrations as described in Section 2.3, utilizing PBS as a blank control and 1% SDS as a positive control. Measure the absorbance according to the established reference method.[14]

This study was approved by the Animal Ethics Committee of the Laboratory Animal Center, Guangdong Pharmaceutical University (Approval No: GDPULAC2023315). All animal procedures were performed in accordance with the committee's guidelines.

2.6. Evaluating the impact of "Sibai" extract on aging in C. elegans

2.6.1. Cultivation of C. elegans

All *C. elegans* were cultivated on Nematode Growth Medium (NGM) plates with an *E. coli* OP50 lawn serving as the primary food source. The *E. coli* OP50 strain was cultured for 12 h at 37 °C. The eggs were harvested using a bleaching solution and subsequently washed with M9 buffer. Following 48 h of synchronization, the *C. elegans* were administered "Sibai" extract solutions of varying concentrations, with Vc serving as the positive control.[15]

2.6.2. Lifespan assay

Transfer L4 stage *C. elegans* to drug-containing Nematode Growth Medium (NGM) plates, ensuring 30 *C. elegans* per plate, and establish three groups of replicates. Change the culture medium every 24 h for the first 12 d, and then every 48 h thereafter, while observing the *C. elegans* daily. *C. elegans* were considered dead and removed from the dish if they did not respond to repeated gentle mechanical prodding. Record the numbers of dead and alive *C. elegans*. Finally, calculate the survival rate and plot the survival curve.[16]

2.6.3. Egg-laying capacity assay

Transfer one L4-stage *C. elegans* to a culture dish coated with "Sibai" extract and designate this as day 0. Transfer the *C. elegans* to a new culture dish every 24 h, while recording the number of eggs produced each day until egg production ceases.[17]

2.6.4. Stress resistance assessment

Heat Stress Experiment: After feeding the *C. elegans* with the "Sibai" extract solution for 3 d, transfer them to an incubator set at 37 °C for cultivation. Record the number of dead *C. elegans* every 2 h until all are deceased. Oxidative Stress Experiment: Using the same *C. elegans* as in the previous experiment, transfer them to NGM plates containing a 3% H_2O_2 solution and juglone. Record the number of dead *C. elegans* every hour until all are deceased. Each experiment includes three parallel groups.[18–20]

2.6.5. Movement and head movement rate assay

Head Movement Experiment: Place *C. elegans* that have been treated

with medication for 3 d onto a microscope slide containing a drop of M9 buffer solution. Record the number of head swings to the left and right, along with the number of body bends, within 1 min. In the spontaneous movement experiment, *C. elegans* were observed moving on blank NGM plates on days 3, 7, and 11 of the feeding period and classified into three distinct groups: those that can move autonomously without external stimulation (Active Locomotion); those that require external stimulation, such as touch, to produce movement (Stimulus Response); and those that only exhibit slight movement after stimulation (Minimal Movement).[21] The proportion of *C. elegans* in each movement class was calculated and expressed as a percentage. The calculation is defined by the following general formula:

$$\text{Proportion}\ (\%) = \left(\frac{N_x}{N_{\text{total}}}\right) \times 100$$

Where N_x represents the number of *C. elegans* in a specific movement class, and N_{total} represents the total number of *C. elegans* assessed per group.

2.6.6. Quantification of lipofuscin accumulation

The accumulation of lipofuscin granules was quantified by measuring the fluorescence intensity of lipofuscin relative to a background measurement. After a seven-day treatment period, the *C. elegans* were immobilized using 20 μM NaN_3 on 1% agarose pads. Lipofuscin fluorescence was quantified using a fluorescence microscope (Olympus S-BX43), with excitation at 340–380 nm and emission at 430 nm. Fluorescence intensity was measured using ImageJ software[22]

2.6.7. Measuring the activity of enzymes that scavenge free radicals and determining the levels of reactive oxygen species (ROS)

More than 3000 synchronized L4 *C. elegans* were fed with different concentrations of "Sibai" extract. On day 5, all *C. elegans* were collected and subsequently washed three times with M9 buffer. To evaluate antioxidant enzyme activity, the *C. elegans* were homogenized, and the resulting supernatant was collected through centrifugation. These supernatants were subsequently utilized to measure CAT activity, MDA content, and ROS levels following the instructions provided in the kit.[23,24]

2.7. Statistical analysis

All data were analyzed using IBM SPSS Statistics version 24, and the results were expressed as the mean ± standard deviation. The mean comparisons among all groups were performed using one-way analysis of variance (ANOVA) with the Duncan test, and differences were deemed significant at $P < 0.05$.

3. Results

3.1. Optimization of extraction process

Range analysis was conducted to determine the mean (K) and range of influence (R) of the four herbs on hydroxyl radical scavenging and tyrosinase inhibition. A higher value of R indicates a greater influence of variations in that factor's level on the experimental results. The order of influence of the four herbs was presented in Table 1 as follows: A > B > C > D. The Fisher F-test was employed in ANOVA to assess whether the factors significantly influenced the experimental indicators. A higher F-value indicates a stronger influence of the factor on the experimental indicators. As shown in Table 2, all four factors exhibited extremely significant statistical differences ($P < 0.001$). Based on the F-values, the order of significance among the four factors was A > B > C > D, which aligned with the results of the range analysis. In conclusion, the optimized formulation was A:B:C:D = 3:3:1:1.

Table 1
Factors and levels of the orthogonal design L9 (3^4) and experimental results for two stability indicators.

Test group	Factors				Experimental results (IC_{50})
	A	B	C	D	
1	1	1	1	1	45.3465 ± 0.5212
2	1	2	2	2	48.6100 ± 1.2439
3	1	3	3	3	46.0035 ± 0.8307
4	2	1	2	3	46.0060 ± 0.4571
5	2	2	3	1	31.0025 ± 0.5499
6	2	3	1	2	29.2800 ± 0.8466
7	3	1	3	2	39.1160 ± 1.3111
8	3	2	1	3	36.4025 ± 0.6336
9	3	3	2	1	30.4695 ± 0.5781
K1	46.6533	43.4895	37.0097	35.6062	
K2	35.4295	38.6717	41.6952	39.0020	
K3	35.3293	35.2510	38.7073	42.8040	
R	11.3240	8.2385	4.6855	7.1978	

Table 2
Orthogonal experimental variance analysis.

Factors	Sum of Squares, SS	Degrees of Freedom, d_f	Mean Square, MS	F-value	Statistical significance (*P*)
A	741.829	2	370.914	2160.00	***
B	320.829	2	160.415	932.698	***
C	108.988	2	54.494	316.846	***
D	233.539	2	116.770	678.933	***

Note: Comparison with the Control group: ***$P < 0.001$.

3.2. Antioxidant activities and the tyrosinase inhibition ability of "Sibai" extract

When the concentration of the "Sibai" extract extraction solution reached 48 mg/mL, the hydroxyl radical scavenging rate was 61.70%. Meanwhile, at a concentration of 4 mg/mL, the DPPH radical scavenging rate was 93.53%, indicating a significant dose-response relationship in the scavenging of hydroxyl and DPPH radicals by the "Sibai" extract (Fig. 1A and B).

Furthermore, we used the potassium ferricyanide method to determine the total reducing capacity of the "Sibai" extract. The test results showed that when the concentration of the "Sibai" extract reached 24 mg/mL, its total reducing capacity significantly increased. When the concentration reached 48 mg/mL, the total reducing capacity of the "Sibai" extract was essentially equivalent to that of Vc (Fig. 1C), indicating that at this concentration, the "Sibai" extract already possessed a reducing capacity similar to that of Vc.

Within the specified sample concentration range, the "Sibai" extract exhibited effective inhibition of tyrosinase activity. When the sample concentration reached 24 mg/mL, the concentration-inhibition rate curve for tyrosinase activity stabilized at an inhibition rate of 74.90% (Fig. 1 D). This indicates that the "Sibai" extract composite extract is effective for skin whitening.

3.3. The results of the hemolysis test

The hemolysis rate of red blood cells within a concentration range of 3 mg/mL to 96 mg/mL of the "Sibai" extract was below 15% (Fig. 2). This indicated that the "Sibai" extract exhibited a low hemolytic effect on red blood cells. A hemolysis rate below 15% was generally considered safe, indicating that the extract has low toxicity to red blood cells within the experimental concentration range, as well as low irritancy and good safety.

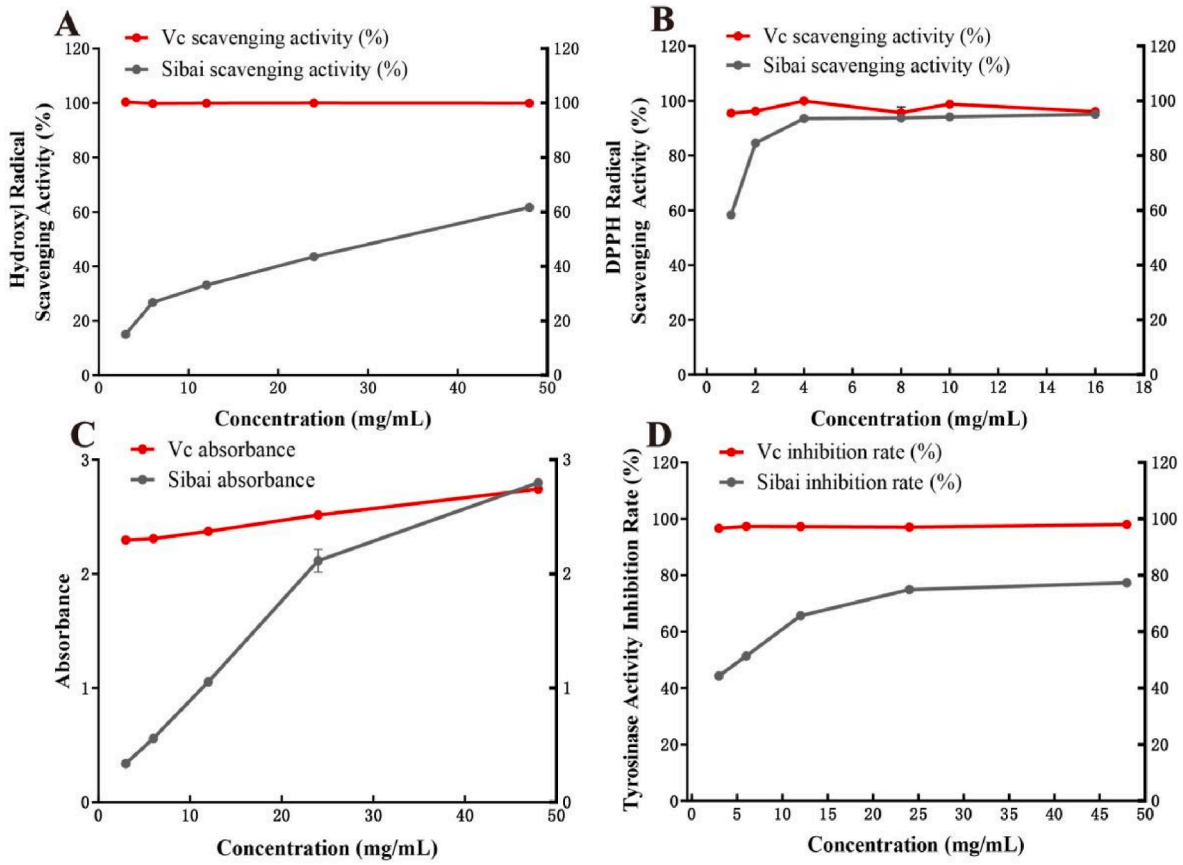

Fig. 1. Effect of "Sibai" extract on hydroxyl radical-scavenging activity (**A**), DPPH radical-scavenging activity (**B**), total reducing power (**C**), and the tyrosinase inhibition rate of the extract (**D**).

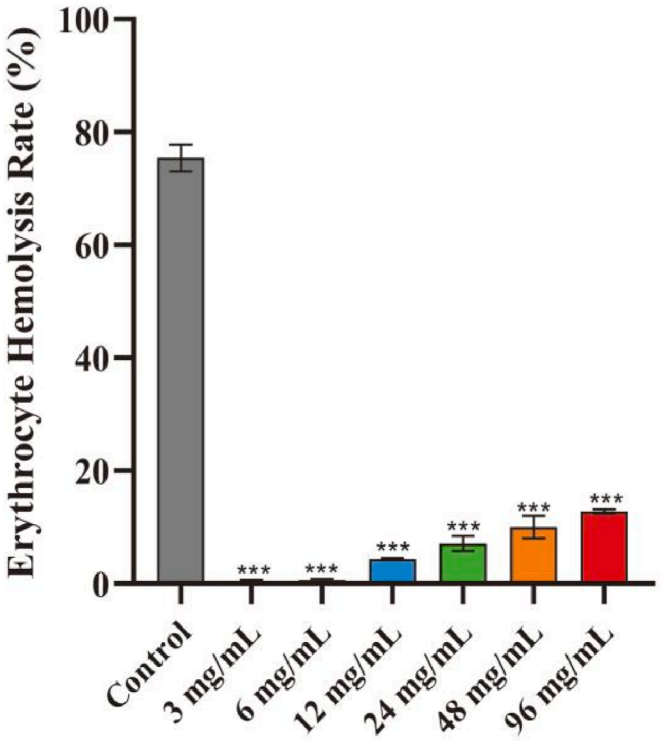

Fig. 2. The erythrocyte hemolysis rate of the "Sibai" extract.
Note: Comparison with the Control group: ***$P < 0.001$.

3.4. *In vivo anti-aging effects of "Sibai" extract*

3.4.1. *Prolonging the lifespan of C. elegans through the treatment of "Sibai" extract*

An extended lifespan is a definitive marker of anti-aging effects. The mean and maximum lifespans of *C. elegans* increased in all "Sibai" extract treatment groups. The average lifespan of *C. elegans* exposed to 48 mg/mL of "Sibai" extract was 20 d, compared to the control group, which had an average lifespan of only 14 d. Compared to the control group, the mean lifespans of *C. elegans* treated with 3 mg/mL and 48 mg/mL of "Sibai" extract were extended by 17.6% and 38.5%, respectively (Fig. 3 A). We propose that the effect of "Sibai" extract on *C. elegans* may be dose-dependent. At lower concentrations, the effect may be less pronounced, leading to a relatively diminished capacity to increase lifespan. However, as concentrations increase, optimal biological activity may be achieved, resulting in an extended lifespan.

The egg-laying capacity of *C. elegans* was closely associated with their lifespan. On day 4, egg production in the treated groups was significantly lower than that in the control group ($P < 0.001$). In the 48 mg/mL group, total egg production significantly decreased ($P < 0.001$) by 33.7%, with no notable difference compared to the Vc group. The results of the lifespan experiments align with the optimal anti-aging efficacy of the "Sibai" extract, suggesting that it can extend the lifespan of *C. elegans* by reducing both lipofuscin accumulation and egg-laying capacity (Table 3).

3.4.2. *Enhancing the stress resistance of C. elegans through the treatment of "Sibai" extract*

Stress tolerance is often correlated with anti-aging capacity and can serve as an indicator of aging. In our experiments, we exposed *C. elegans* to H_2O_2 and carnosine quinone to establish an aging model. The results

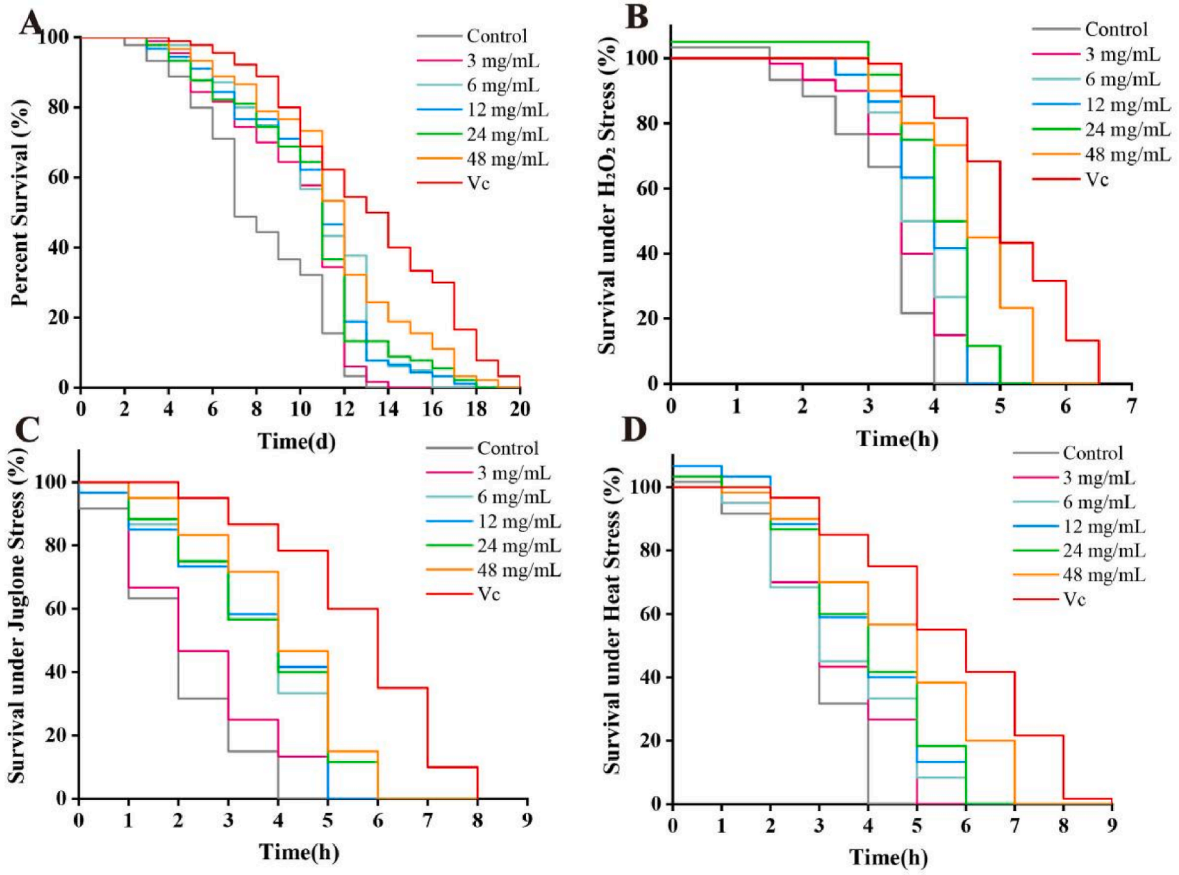

Fig. 3. Effects of "Sibai" extract on *C. elegans* lifespan and stress resistance. Lifespan extension under normal conditions (**A**). Survival curves of *C. elegans* under oxidative stress induced by H_2O_2 (**B**), juglone (**C**), and thermal stress (**D**) at varying concentrations of "Sibai" extract.

Table 3
The egg-laying quantity of *C. elegans*. under different concentrations of "Sibai" extract.

Group	1d	2d	3d	4d	Totality
Control	34.0 ± 11.1	98.7 ± 6.3	41.7 ± 7.3	34.0 ± 5.5	208.3 ± 26.7
3 mg/mL	18.7 ± 7.5	101.0 ± 4.6$^{\#}$	41.7 ± 6.0	12.0±0.6***	173.3±8.7$^{\#\#}$
6 mg/mL	25.7 ± 7.8	101.3 ± 6.9$^{\#}$	43.0 ± 1.7	3.0±1.5***	173.0±7.5$^{\#\#}$
12 mg/mL	11.0 ± 1.0	91.7 ± 1.2	38.3 ± 4.2	2.0±0.6***	143.0±4.6**
24 mg/mL	10.7 ± 1.9	91.7 ± 9.9	37.3 ± 2.7	6.0±2.0***	145.7±3.9**
48 mg/mL	11.0 ± 0.6	89.3 ± 7.9	34.7 ± 1.2	3.0±0.6***	138.0±8.4***
Vc	10.0 ± 2.1	78.7 ± 6.1	29.3 ± 2.3	7.0±1.5***	125.0±6.6***

Note: Comparison with the Control group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Comparison with the Vc group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

demonstrated that stress resistance significantly increased in all treatment groups compared to the control group. After the introduction of H_2O_2 solution and juglone, the lifespan of all *C. elegans* in this study decreased (Fig. 3 B, C, D). However, when compared to the control group, the average lifespan of *C. elegans* treated with 48 mg/mL "Sibai" extract showed increases of 37.9% and 69.5%, respectively ($P < 0.05$).

After an 8 h exposure to heat stress at 35 °C, the *C. elegans* in the control group exhibited a survival time of only 4 h. In contrast, the *C. elegans* in the group treated with 48 mg/mL exhibited a significantly higher survival rate of 49.2% ($P < 0.05$). Therefore, the "Sibai" extract enhanced the overall health of the *C. elegans* by improving their motility and stress resistance.

3.4.3. Enhancing the locomotion ability of C. elegans through the treatment of "Sibai" extract

An extended lifespan in *C. elegans* is associated with enhanced overall health. Motility, which is linked to the quality of life in *C. elegans*, serves as an indicator of muscle integrity. On day 3, the average number of head swings in the control group was 110 times; after treatment with the "Sibai" extract, this value increased to 140–170 times; the number of body bends also rose from 20 times in the control group to 30–50 times. Both parameters peaked at a concentration of 48 mg/mL, showing significant differences compared to the control group ($P < 0.001$), but no significant difference compared to the Vc treatment group ($P > 0.05$), indicating a significant enhancement in motor ability (Fig. 4A and B).

Meanwhile, in the autonomous movement experiment, we evaluated the movement patterns of *C. elegans* on days 3, 7, and 11 after treatment. On days 3 and 7, the *C. elegans* generally exhibited the "active locomotion" type, with the 48 mg/mL treatment group and the Vc treatment group showing significantly better autonomous movement performance than the control group ($P < 0.001$). By day 11, although overall movement activity showed a significant decline, the 48 mg/mL treatment group still maintained a higher proportion of "active locomotion", and the proportion of "minimal movement" was significantly lower than that of the control group ($P < 0.001$) (Fig. 4C). These results revealed that the "Sibai" extract effectively enhanced the overall health of *C. elegans* by

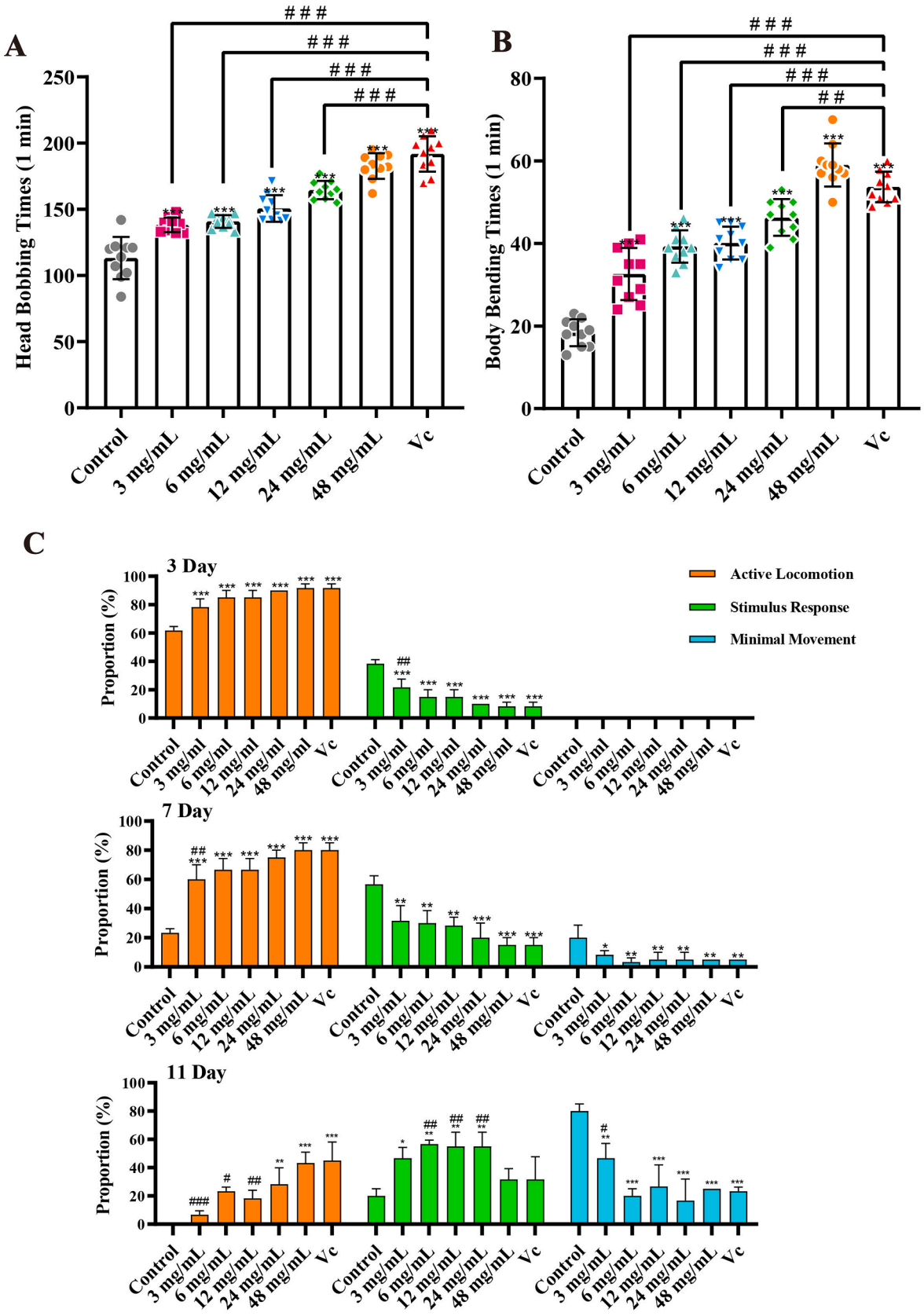

Fig. 4. The effect of different concentrations of "Sibai" extract on head bobbing frequency (**A**), body bending frequency (**B**) and voluntary movement on days 3, 7, and 11 (**C**) in *C. elegans*.
Note: Comparison with the Control group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Comparison with the Vc group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

improving their movement capacity and stress resistance.

3.4.4. Improvement in the antioxidant capacity of C. elegans by the treatment of "Sibai" extract

As lipofuscin accumulates in aging *C. elegans*, a reduction in its accumulation may indicate the anti-aging effects of the drugs. Lipofuscin accumulation in *C. elegans* decreased across all dose groups, especially in those treated with the "Sibai" extract solution at a concentration of 48 mg/mL, which significantly reduced ($P < 0.05$) the relative fluorescence intensity of lipofuscin (Fig. 5 A).

According to the oxidative stress theory, the accumulation of oxidative damage contributes to aging. It is well established that the overproduction of ROS induces oxidative damage in the body. The relative fluorescence intensity of ROS showed moderate reductions across all dose groups compared to the control group. The ROS fluorescence intensity significantly decreased in the 48 mg/mL treatment group (Fig. 5 B). Additionally, we evaluated the activity of various antioxidant enzymes. Compared to the control group, CAT levels increased significantly, and the activities of antioxidant enzymes varied across all dose groups. Among all dose groups, CAT activity levels were highest in the 48 mg/mL group, showing a significant difference from the control group (Fig. 5 D). Additionally, the 48 mg/mL group exhibited a significant reduction in malondialdehyde (MDA) content compared to the control group ($P < 0.05$) (Fig. 5C). These results indicated that "Sibai" extract reduced ROS production and safeguards the antioxidant enzyme system in *C. elegans* from oxidative damage.

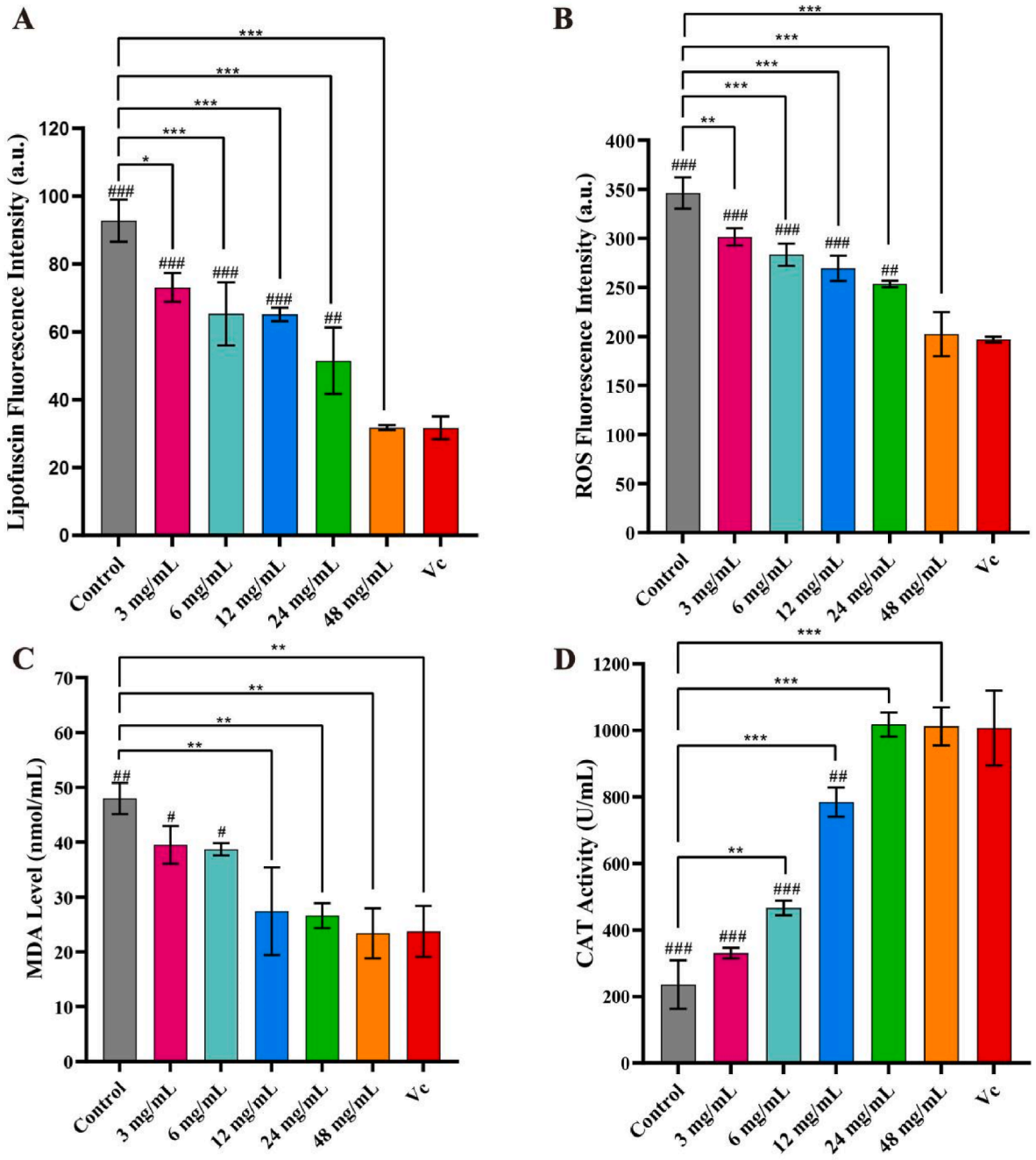

Fig. 5. The effects of varying concentrations of "Sibai" extract on the reduction of elevated lipofuscin (**A**), ROS fluorescence levels (**B**), MDA content (**C**), and CAT activity (**D**) in *C. elegans*.
Note: Comparison with the Control group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Comparison with the Vc group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

4. Discussion

This study initially focuses on the extraction process, followed by the application of chemical methods for authentication, and ultimately incorporates in vivo validation using a *C. elegans* model. This approach reflects a systematic research strategy that transitions from fundamental to applied research and from in vitro to in vivo studies.

Currently, anti-aging research has developed a multi-faceted evaluation system that encompasses various indicators from the cellular to the organ level. At the cellular level, shortened telomere length,[25] decreased mitochondrial function,[26] and ROS levels increased. At the metabolic level, reduced insulin sensitivity, abnormal lipid metabolism, and elevated glucocorticoid levels are closely associated with premature aging.[27–29] The aging of the immune system is reflected in changes in immune cell populations and impaired function.[30] Additionally, aging also involves aspects such as DNA methylation.[31]

These multidimensional evaluation strategies are not only widely applied in human and mammalian models but have also established a systematic assessment framework in the model organism *C. elegans*. As an ideal model for anti-aging research, *C. elegans* offers multiple advantages: its transparency allows for direct observation, its short lifecycle enables rapid acquisition of experimental results, and it is easily cultivated and preserved at low cost in the laboratory. Furthermore, this model organism can yield synchronized samples with consistent genetic backgrounds in bulk, thereby reducing experimental errors. Its hermaphroditic nature avoids the interference of inbreeding, and at the genetic level, *C. elegans* shares a high degree of similarity with human genes, carrying multiple conserved signaling pathways.[32] In this experiment, we developed a comprehensive *C. elegans* aging assessment system that spans from macro phenotypes (lifespan, locomotion, stress response) to intracellular damage markers (lipofuscin, MDA, ROS) and cellular defense capabilities (antioxidant enzymes). This system encompasses the external manifestations of aging, the intrinsic cellular mechanisms, and the defense functions, representing a commonly used multi-dimensional evaluation combination in anti-aging research utilizing *C. elegans* models.

This study found that 48 mg/mL of the "Sibai" extract extended the normal lifespan of *C. elegans* by 38.5%, and increased their survival under oxidative stress induced by H_2O_2 and juglone, as well as under heat stress, by 37.9%, 69.5%, and 49.2%, respectively. Additionally, locomotor ability was significantly enhanced, and levels of ROS, lipofuscin, and MDA decreased markedly, while CAT activity increased substantially. These results are comparable to, or even superior to, the effects reported for various plant extracts in *C. elegans* anti-aging models in recent years, for example, bitter melon saponins (0.1–0.2 mg/mL) extended the average lifespan of *C. elegans* by approximately 10.6%, while deer antler polysaccharides (0.8 mg/mL) extended lifespan by 25.46%,[33,34] both effects were lower than that of "Sibai" extract. In terms of stress resistance, L-theanine (10 μmol/L) extended the maximum lifespan of *C. elegans* under oxidative stress from 17.0 min to 31.0 min, representing an 82% increase compared to the control group,[21] which was superior to "Sibai" extract. Additionally, recent studies showed that ginkgo biloba flavonoid extracts (0.2–0.8 mg/mL) exhibited a concentration-dependent effect in extending lifespan and reducing ROS and lipofuscin,[35] consistent with the dose-response trend of "Sibai" extract. In summary, "Sibai" extract demonstrated significant activity across multiple anti-aging indicators, with overall effects superior to or comparable to existing plant extracts.

Although this study optimized the extraction ratio of "Sibai" extract, and demonstrated its antioxidant, skin-whitening, and anti-aging effects in *C. elegans*, several limitations should be acknowledged. First, the findings are derived from an invertebrate model, necessitating further validation in mammalian systems (e.g., mice) to evaluate their potential for translation. Second, although the extract demonstrated low hemolytic activity, its long-term toxicity and optimal dosage for therapeutic applications remain to be established. Third, while the study associated "Sibai" extract with reduced ROS and enhanced stress resistance, the precise molecular mechanisms necessitate further investigation.

While this study optimized the composition ratio of the Sibai extract, further investigation is needed on other key process parameters, such as extraction time, temperature, and solvent ratio. Future research should focus on optimizing the extraction process to improve efficiency and yield. Additionally, while the active components of the four individual drugs have been documented, the overall chemical composition of the "Sibai" extract compound extracts and their synergistic mechanisms remain unclear.Thus, we recommend employing a multi-omics integration strategy, including metabolomics, transcriptomics, and network pharmacology, to systematically analyze the active compounds and their regulatory networks in future studies.Concurrently, further preclinical studies using higher animal models and exploring formulation optimization strategies, such as nano-delivery or solubilization, are necessary to enhance bioavailability. Such studies will establish a robust scientific foundation for developing "Sibai" extract as a potential anti-aging intervention.

5. Conclusion

This study investigated the anti-aging activity of "Sibai" extract by optimizing the ratio of *Bombyx Batryticatus*, *Ampelopsis japonica*, *Radix Paeoniae Alba*, and *Atractylodes macrocephala* in the "Sibai" extract through orthogonal design experiments. The optimal ratio was determined to be 3:3:1:1. Under this optimal ratio, antioxidant activity assays demonstrated that "Sibai" extract effectively scavenged hydroxyl and DPPH radicals. Consequently, the extract may possess a high level of antioxidant capacity. Additionally, the extract was found to effectively inhibit tyrosinase, showcasing its whitening effect while also exhibiting good safety.

"Sibai" extract enhanced the longevity of *C. elegans*, improving their locomotion and stress resistance. Moreover, the "Sibai" extract treatment significantly improved heat stress tolerance, elevated CAT levels, and reduced ROS, MDA, and lipofuscin levels in *C. elegans*. Overall, our findings highlighted the significant potential of "Sibai" extract for the development of dietary supplements or pharmaceuticals aimed at aging intervention.

CRediT authorship contribution statement

Zhenghao Chen: Writing – review & editing, Methodology, Investigation, Formal analysis. **Haoxian Chen:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Xiaohua Ye:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Ping Zhao:** Project administration, Funding acquisition. **Yan Wang:** Validation, Supervision.

Ethical approval

This study was approved by the Animal Ethics Committee of the Laboratory Animal Center, Guangdong Pharmaceutical University (Approval No: GDPULAC2023315).

Declaration of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhip.2025.11.006.

References

1. Campisi J, Kapahi P, Lithgow GJ, et al. From discoveries in ageing research to therapeutics for healthy ageing. *Nature*. 2019;571(7764):183–192.
2. Wan QL, Fu XD, Dai WY, et al. Uric acid induces stress resistance and extends the life span through activating the stress response factor DAF-16/FOXO and SKN-1/NRF2. *AG-US*. 2020;12(3):2840–2856.
3. Cho CH, Kim E, Kim J, et al. N-Adamantyl-4-methylthiazol-2-amine suppresses amyloid β-induced neuronal oxidative damage in cortical neurons. *Free Rad Res*. 2016;50(6):678–690.
4. Wang X, Liu YJ, Yu J, et al. Effects of fermentation on the alleviation of oxidative damage in *Caenorhabditis elegans* by broccoli glucosinolates. *Food Sci (N Y)*. 2023;44(6):205–213.
5. Yen HH, Shahidi F. Antioxidant activity of green tea and its catechins in a fish meat model system. *J Agric Food Chem*. 1997;45:4262–4268.
6. Meng JM. *Study on the Preparation Process and Quality Standards of the New Qibai spot-removing Gel*. Wuhan: Hubei University of Chinese Medicine; 2019.
7. Qiu J, Xu X, Guo J, et al. Comparison of extraction processes, characterization and intestinal protection activity of *Bletilla striata* polysaccharides. *Int J Biol Macromol*. 2024;263(1), 130267 - 130267.
8. Li Y, Gu QY, Liu YM, et al. Formulation of whitening ingredients from traditional Chinese medicine and the efficacy of fermented compound Chinese herbal extract. *Acta Microbiol Sin*. 2018;58(8):1483–1491.
9. Zhang YL. Capability of several marine active substances to eliminate hydroxyl radicals. *J Phys: Conf Ser*. 2020;1549(2):22–42.
10. Manickam V, Mani G, Muthuvel R, et al. Green fabrication of silver nanoparticles and it's in vitro anti-bacterial, anti-biofilm, free radical scavenging and mushroom tyrosinase efficacy evaluation. *Inorg Chem Commun*. 2024;162:112–199.
11. Tian JH, Zhang CY, Wei L. Optimization of extraction process and antioxidant activity of total flavonoids from sea buckthorn pomace. *Nat Prod Res Dev*. 2021;33(1):65–72.
12. Qi L, Wang ZY. Study on the antioxidant activity of pine nut polypeptides. *Food Sci Technol*. 2010;35(10):110–113.
13. Zhang HM, Geng J, Zhou QC. Study on the inhibition of tyrosinase activity by flavonoid extracts from *Xanthoceras sorbifolia* husk. *J Chin Cereals Oils Assoc*. 2013;28(10):96–100.
14. Liu XG, Bai WJ, Tian N, et al. Comparative study on the replacement of rabbit eye irritation test with different erythrocyte hemolysis experiments. *Sci Technol Vision*. 2019;32:232–233, 216.
15. Wu DF, Li MQ, Wang Y. Optimization of extraction technology of total flavonoids from *Pouzolzia zeylanica* (L.) Benn and its effect on stress ability of nematode. *J Guangdong Pharm Univ*. 2024;40(1):43–49.
16. Komura T, Ikeda T, Yasui C, et al. Mechanism underlying prolongevity induced by bifidobacteria in *Caenorhabditis elegans*. *Biogerontology*. 2013;14(1):73–87.
17. Hu R, Zhang Y, Qian WY, et al. *Pediococcus acidilactici* promotes the longevity of *C. elegans* by regulating the insulin/IGF-1 and JNK/MAPK signaling, fat accumulation and chloride ion. *Front Nutr*. 2022;9:821685.
18. Wang XH, Chen XD, Wang D, et al. Antioxidant and anti-aging effects of polysaccharide LDP-1 from wild *Lactarius deliciosus* on *Caenorhabditis elegans*. *Food Nutr Res*. 2022;9:66–78.
19. Li ZW. *Study on the Anti-aging Effects of Cervus elaphus Antler Aqueous Extract on Caenorhabditis elegans*. Changchun: Changchun University of Technology; 2022.
20. Li SM, Mao HY, Dong FX, et al. Study on the anti-aging effects and mechanism of *Paeonia lactiflora* polysaccharides on *Caenorhabditis elegans*. *J Food Saf Qual*. 2024;15(4):283–291.
21. Du ZF, Li YF, Jia R, et al. L-theanine delays aging in *Caenorhabditis elegans* under stress conditions. *J Fuyang Norm Univ*. 2023;40(4):27–32.
22. Xiao M, Chen Y, Han L, et al. Effects of flavonoid extract from *Ginkgo biloba* leaves on antioxidation and anti-aging of *Caenorhabditis elegans*. *Food Ferment Ind*. 2024;50(23):148–155.
23. Wang SN, Chen DN, Hu KJ, et al. Analysis of total flavonoids in Eleusine coracana and their anti-aging activity in Caenorhabditis elegans [J/OL]. *Food Sci*. 2024;45(14):94–102.
24. Lin C, Chen Y, Lin Y, et al. Antistress and anti-aging activities of *Caenorhabditis elegans* were enhanced by *Momordica* saponin extract. *Eur J Nutr*. 2021;60(4):1819–1832.
25. Lin J, Epel E. Stress and telomere shortening: insights from cellular mechanisms. *Ageing Res Rev*. 2022;73:101507.
26. Harrington JS, Ryter SW, Plataki M, et al. Mitochondria in health, disease, and aging. *Physiol Rev*. 2023;103(4):2349–2422.
27. Kurauti MA, Soares GM, Marmentini C, et al. Insulin and aging. *Vitam Horm*. 2021;115:185–219.
28. Kuhla A, Blei T, Jaster R, et al. Aging is associated with a shift of fatty metabolism toward lipogenesis. *J Gerontol A Biol Sci Med Sci*. 2011;66(11):1192–1200.
29. Yu JL, Seckl JR. Local amplification of glucocorticoids in the aging brain and impaired spatial memory. *Front Aging Neurosci*. 2012;4:24.
30. Wrona MV, Ghosh R, Coll K, et al. The 3 i's of immunity and aging: immunosenescence, inflammaging, and immune resilience. *Front Aging*. 2024;5:1490302.
31. Horvath S, Raj K. DNA methylation-based biomarkers and the epigenetic clock theory of ageing. *Nat Rev Genet*. 2018;19(6):371–384.
32. Zhang CH. *Study on the Anti-aging Effects of Soy Isoflavone Metabolites on Caenorhabditis elegans and its Mechanism*. Baoding: Hebei Agricultural University; 2014.
33. Bai J, Zhang JF, Zhang PX, et al. Effects of *Momordica charantia* saponins on the lifespan of *Caenorhabditis elegans* and its mechanism. *J Food Sci*. 2022;43(7):165–173.
34. He Y. *Isolation of Pilose Antler Polysaccharide and its Anti-aging Activity*. Changchun: Jilin Agricultural University; 2021.
35. Chen Y. *Study on the Lifespan Extension Effect of Ginkgo biloba Leaf Flavonoids on Caenorhabditis elegans and its Mechanism of Action*. Chongqing: Chongqing Three Gorges University; 2021.