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Blackcurrant Extract Regulates Lipid Metabolism in *Caenorhabditis elegans via* SBP-1 and NHR-49

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ABSTRACT: Blackcurrant extract (BCE), rich in anthocyanins, has demonstrated significant potential in lipid level reduction. In this study, network pharmacology was applied to predict the lipid-lowering metabolic mechanism of BCE. Upon treatment with BCE (100 μg/mL), lipid accumulation was significantly reduced in both the HepG2 cells in *vitro* and the high-glucose-fed *Caenorhabditis elegans* (*C. elegans*) in *vivo* by 46.06% and 56.68%. Additionally, the lifespan of *C. elegans* was significantly prolonged by BCE treatment, its stress tolerance and antioxidant capacity were enhanced, and aging-related markers (e.g., lipofuscin accumulation) were altered. Subsequent qPCR analysis revealed that genes associated with the SBP-1/SREBP pathway (*sbp-1*, *fat-6*, and *fat-7*) were down-regulated by BCE, while genes in the NHR-49/PPARα pathway (*nhr-49*, *acs-2*, *ech-1.1*, and *cpt-2*) were up-regulated. Furthermore, Oil Red O staining and triglyceride (TG) content assays demonstrated that the hypolipidemic effect of BCE was absent in *nhr-49*, *fat-6*, and *fat-7* mutant strains of *C. elegans*. The current findings were validated by molecular docking, indicating that abnormal fat accumulation in *C. elegans* was alleviated by BCE treatment via the SBP-1 and NHR-49 pathways. In summary, these findings indicated that BCE exerted a lipid-lowering effect and provided novel insights and research materials for the development of natural lipid-lowering medications and health products.

Keywords: Blackcurrant extract (BCE), HepG2 cells, *Caenorhabditis elegans*, Lipid metabolism, SBP-1 and NHR-49 signaling pathways

1. Introduction

Obesity is a chronic metabolic disease caused by changes in metabolism or excessive energy intake, resulting in abnormal fat accumulation[1]. It is strongly associated with the rising global incidence of other chronic illnesses, including type 2 diabetes (T2DM), hypertension, and cardiovascular disease[2]. Numerous studies have demonstrated that dietary substances could regulate lipid metabolism by reducing adipose tissue mass. For instance, red bean (*Phaseolus vulgaris*) and its bioactive extracts inhibited adipocyte differentiation

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and attenuated high-fat diet-induced obesity in murine models by downregulating key adipogenic transcription factors[3]. Anthocyanins, phytochemicals abundant in black rice, soybeans, and purple corn, have exerted protective effects against high-fat diet-induced hepatic inflammation by modulating oxidative stress and inflammatory signaling pathways[4]. Additionally, grape-derived anthocyanins have been shown to mitigate obesity-associated inflammatory responses, highlighting their potential to improve metabolic dysfunction[5]. Consequently, there is growing interest in natural functional compounds with minimal side effects, and well lipid-metabolism-regulating activities.

Blackcurrant berries (*Ribes nigrum L.*), rich in anthocyanins, are widely recognized for their bioactive properties[6]. Numerous pieces of evidence have demonstrated that blackcurrants exhibit significant lipid-lowering activity. Supplementation with blackcurrant pomace extract induced beneficial changes in serum lipids, antioxidant status in rabbits fed with a high-fat diet[7]. Studies in C57BL/6 obese mice showed that blackcurrant powder extract reduced body weight[8], decreased levels of inflammatory markers[9], and alleviated intestinal disorders[10]. Microbial analysis revealed that blackcurrant anthocyanins supplementation (BCA) combats obesity and hepatic steatosis by altering gut bacterial structure and composition[11]. In addition, dietary supplementation of 2 g/kg cyanidin-3-O-glucoside (one of the main anthocyanins in blackcurrants) was found to promote the release of adiponectin and leptin from adipocytes in rats, thereby preventing obesity[12]. Notably, while existing studies suggest a link between blackcurrant extract (particularly its anthocyanin-rich fractions) and improved lipid homeostasis, the molecular mechanisms underpinning this effect remain further clarification.

This study aimed to investigate the potential mechanisms of blackcurrant extract (BCE) in regulating lipid metabolism. The possible anti-obesity molecular mechanism of BCE was predicted in this study using network pharmacology, a novel method for examining biological activities in medicinal plants[13]. Additionally, the interaction between medications, targets, and disorders was visualized. Oil red O staining (ORO) and triglyceride (TG) were used as *in vitro* indicators of cell viability to investigate the lipid-lowering effect of BCE on HepG2 cells. Furthermore, this study revealed a potential regulatory mechanism of BCE in *C. elegans*, which enabled a better understanding of the antioxidant and anti-obesity properties of BCE. Lastly, molecular docking was used to examine the binding potential between the essential proteins and the majority of active components of BCE. These analyses provide a fresh concept for the creation of BCE as a medication and natural health product that lowers obesity and cardiovascular diseases.

2. Materials and methods

2.1 Chemicals and reagents

The blackcurrant extract (BCE) was derived from the fresh ripe fruits of *Ribes nigrum L.* (a species belonging to the Grossulariaceae family), prepared via a series of processes including 50% ethanol extraction, concentration, and drying, and purchased from Tianjin Jianfeng Natural Products Research and Development Co., Ltd. (Tianjin, China). Reactive oxygen species (ROS) assay kit was obtained from Beyotime Biotechnology Co., Ltd. (Shanghai, China). The superoxide dismutase (SOD, No. A001-3-2), catalase

(CAT, No. A007-1-1), glutathione peroxidase (GSH-Px, No. A005-1-2), malondialdehyde (MDA, No. A003-1-2), triglyceride (TG, No. A110-1-1) and total cholesterol (TC, No. A111-1-1) assay kits were purchased from Nanjing JianCheng Bioengineering Institute (Nanjing, China). Ultra SYBR Mixture, the Trizol Universal Reagent, and HiFiScript cDNA Synthesis kit were purchased from ComWin (Beijing, China). HepG2 cells were obtained from the Cell Resource Center of Shanghai Institute of Life Sciences, Chinese Academy of Sciences. *E. coli* OP50 and *C. elegans* strains were purchased from the Caenorhabditis Genetics Center (CGC) (Minnesota, USA). In this investigation, all other chemicals were obtained from commercial suppliers and were of reagent grade.

2.2 Analysis of anthocyanin components in BCE

The anthocyanin components of BCE were characterized using the previously reported method[14]. A high-resolution liquid chromatography-mass spectrometry system equipped with a photodiode array (PDA) detector, an electrospray ionization (ESI) source, and an ion trap-time-of-flight (IT-TOF) mass analyzer was employed for analysis. Chromatographic separation was achieved on a Venusil XBP C18 column (2.1 mm × 150 mm, 5 μm), with mobile phases consisting of solvent A (0.1% formic acid in water) and solvent B (acetonitrile). The eluent was delivered at a constant flow rate of 0.2 mL/min under gradient conditions.

2.3 Network pharmacology

The PubChem database was chosen to obtain the secondary structure of cyanidin-3-O-rutinoside (C3R), cyanidin-3-O-glucoside (C3G), delphinidin-3-O-glucoside (D3G), and delphinidin-3-O-rutinoside (D3R), and the target information was predicted in the Swiss Target Prediction database. The human gene targets of obesity were identified in OMIM, GeneCards, and TTD databases. The Venny 2.1.0 platform screened out the common targets of drugs and diseases. Then, the String database and Cytoscape 3.9.0 software were employed to identify the critical targets. Furthermore, the DAVID online database was used to conduct KEGG enrichment analysis and GO functional annotation, and Cytoscape 3.9.0 was used to generate the drug-target-disease network diagram.

2.4 The lipid-lowering effect of BCE in HepG2 cells

2.4.1 Cell culture and viability assay

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 μg/mL), and streptomycin (100 μg/mL) at 37℃ in a humidified incubator with 5% CO_2. The cell model for high lipid accumulation was established by exposing HepG2 cells to oleic acid (OA, 0.25 mmol/L) for 24 h, as described previously[15].

BCE (7.5, 15, 25, 50, 100, 200 μg/mL) and OA with BCE (7.5, 15, 25 μg/mL) were added into the 96-well plate for 24 h, 100μL MTT (5 mg/mL) was applied and incubated for 4 h before adding DMSO (150 μL) to solubilize the crystals and measuring absorbance at 492 nm (Thermo, MA, USA)[16].

2.4.2 Determination of lipid accumulation in cells

HepG2 cells were seeded (1.5×10^5 cells/well) into a 6-well plate and incubated with or without OA (0.25 mmol/L) or BCE (7.5, 15, 25 μg/mL) for 24 h. The cells were washed with PBS and fixed with paraformaldehyde for 40 min, stained with Oil Red O for 30 min, then washed thrice with PBS and photographed with a microscope. Finally, 1 mL isopropanol was added to dissolve for 15 min, and the absorbance was measured at 510 nm using a microplate reader (Thermo, MA, USA).

After treatment as described above, HepG2 cells were collected for the determination of intracellular TG and TC content as described in the previous study[17].

2.5 The Lipid-lowering Effect of BCE on C. elegans

2.5.1 Strains and treatment conditions

Both strains of *C. elegans* and *E. coli* OP50 were obtained from the Caenorhabditis Genetics Center (CGC). Nematode growth medium (NGM) was used to maintain normal growth and reproduction of worms[18]. N2 strains were employed in the investigation for the phenotypic, fat content test, and lifespan experiments. The transgenic strains of RB1716 [*nhr-49* (ok2165) I.], CF1038 [*daf-16* (mu86) I], BX106 [*fat-6* (tm331) IV], and BX153 [*fat-7* (wa36) V] were used for the mutant Oil red O and TG experiment. The strains of LIU1 [*dhs-3p*::dhs-3::GFP + unc-76(+)], BX150 [*fat-5*::GFP + lin15(+)], BX115 [*fat-6*::GFP + lin15(+)], BX113 [*fat-7*::GFP + lin15(+)], WBM170 [*acs-2p*::GFP + rol-6(su1006)] and CE548 [*sbp-1*::GFP::SBP-1 + rol-6(su1006)] were chose to measure the fluorescence intensity. Groups were divided into normal, model (2% D-glucose), and treatment (25, 50, 100 μg/mL BCE + 2% D-glucose). In subsequent tests, 150 μM floxuridine (FUDR) (Beyotime, China) was added to the NGM medium to avoid reproduction effects unless otherwise indicated. For BCE treatment, NGM medium (containing 2% glucose) was prepared by autoclaving (121℃, 20 min), cooling molten NGM to 45℃ in a water bath, then adding BCE (pre-dissolved in sterile distilled water) and mixing well to final concentrations of 25, 50, and 100 μg/mL. The BCE-containing NGM was immediately poured into sterile 60-mm Petri dishes and allowed to solidify at room temperature for 2 h in the dark. During the experiment, worms cultured on BCE-NGM plates were maintained in a dark incubator at 20℃ to minimize photodegradation of anthocyanins.

Synchronized eggs were obtained via the lysis simultaneous operation (5% NaClO, 5 mol/L NaOH), followed by 6 washes with M9 buffer. Synchronized L1 larvae were generated by incubating these eggs in M9 buffer at 20℃ for 12 h. Thereafter, the L1 larvae were transferred to NGM medium and cultured at 20℃ for 48 h to yield L4-stage worms. All experiments consistently used synchronized L4-stage worms as the initial stage to ensure uniformity. All experiments were repeated thrice independently.

2.5.2 E. coli OP50 organism growth assay

The *E. coli* OP50 organism growth assay was carried out using the methodology of a recent study to exclude indirect effects of BCE on worm physiology via altered bacterial viability or growth[19]. Briefly, 10 μL *E. coli* OP50 bacteria (OD_{600} = 0.4) was added to 10 mL LB medium containing 25, 50, 100 μg/mL BCE, respectively. The samples were incubated at 37℃ (170 r/min) with shaking, and the optical density at 600 nm was measured within 24 h using a UV spectrophotometer.

2.5.3 Lifespan and lipofuscin assay

The safe dose of BCE was determined by the poisonousness experiment, which was based on the previous methodology[20]. In brief, different doses of BCE (25, 50, 100, 200, and 500 μg/mL) were randomly inserted into NGM coverings containing at least 100 L4-synchronized *C. elegans*. *C. elegans* survival was observed daily during the trial. The level of auto-fluorescent lipofuscin in the gut was measured on 6 d using an upright fluorescence microscope (Olympus BX53, Japan), and the intensity was quantified by Image J software.

2.5.4 Antioxidant activity assay of BCE treatment

The N2 (0, 25, 50, 100 μg/mL, 3d) were mixed with 50 μmol/L 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA), then incubated for 30 min at 37℃ in the dark. Image J software was used to quantitatively analyze the fluorescence intensity of *C. elegans*. The enzyme activities of SOD, CAT, GSH-Px and the content of MDA in *C. elegans* were measured according to reported studies with minor modifications[21].

2.5.5 Oil Red O staining and TG assay

The L4-stage worms were treated with BCE for 6 days, after which the Oil Red O staining experiment (ORO) was performed. Briefly, 50 *C. elegans* were collected and washed three times with PBST solution (a mixture of 1 × PBS and 0.01% Triton X-100). And 600 μL isopropanol (v/v: 40%) was added to the samples to shake for 3 min, and then centrifuged to discard the supernatant. Then, 600 μL ORO working solution (stock solution: water = 3:2) was added for 2 h staining, and washed with PBST for 30 min to remove residual dye solution. Stained *C. elegans* were taken using an inverted microscope. (Olympus BX53, Japan). Finally, 600 μL isopropanol was added to dissolve for 15 min, and the absorbance was measured at 510 nm using a microplate reader (Thermo, MA, USA).

At least 100 *C. elegans* were grown in BCE-treated NGM medium for 6 d. Then, *C. elegans* was collected in PBS solution, homogenates were collected by grinding on ice, and supernatants were collected for TG studies by centrifugation. The TG content was measured with assay kits according to a previous study[22].

2.5.6 Fluorescence Intensity observation and quantification

L4-stage GFP transgenic *C. elegans*, LIU1, CE548 and WBM170 were anesthetized using 2% sodium azide after incubation with different concentrations of BCE up to the 6^{th} day. Fluorescence imaging of *C. elegans* with 488 nm excitation and 500/525 nm emission filters was conducted. A minimum of 30 *C. elegans* in each group were measured. The worms were isolated and imaged individually, while the remaining population was temporarily stored in the dark. Fluorescence intensity was quantified with Image J software.

2.5.7 Motricity, reproduction, growth rate, and pharyngeal pumping assay

Starting from the L4 stage, at least 100 *C. elegans* were selected to determine the frequency of head swing, body bending, and pharynx pumping on the 2^{nd} and 6^{th} days of BCE treatment. The frequency of head swing was defined as the number of head swings from one side to the other side and back again in 30 s. The

quantity of sinusoids created by worms during locomotion within 30 s was described as body bending frequency[23]. The frequency of the pharynx pump was determined based on the previous study[24]. To determine whether BCE affects nematode reproduction, L4 adults of *C. elegans* were moved to NGM plates with or without 2% D-glucose and BCE (25, 50, or 100 μg/mL), followed by daily transfers to new NGMs until no eggs or larvae were found. Each set had ten parallel groups, with one nematode on each plate.

2.5.8 Quantitative real-time PCR

The methodology was an improvement on previous research conducted[25]. Starting from the L4 stage, *C. elegans* were treated with BCE (25, 50, and 100 μg/mL) for 5 d. The RNA extraction kit was used to extract all of the RNA. The primer reverse transcription master mixing kit's operational instructions were followed in the cDNA synthesis process. Rapid real-time PCR technology and UltraSYBR Mixture were used to perform a quantitative real-time PCR experiment. The $2^{-\Delta\Delta CT}$ method was utilized to quantify the mRNA level of the genes in relation to *act-1*.

2.6 Molecular docking

The molecular structures of D3R (CAS: 58285-26-0), D3G (CAS: 50986-17-9), C3G (CAS: 7084-24-4), C3R (CAS: 28338-59-2), were obtained from the chemical book website. The amino acid sequences of NHR-49 (UniProKB: O45666) and SBP-1 (UniProKB: Q9XX00) were downloaded from the UniProt database. The model was tested with the condition "Seq Identity > 30%" after ligands and protein receptors were created using SWISS-MODEL. CDOCKER and related modules in Discover Studio 3.5 (Accelrys) were then used to conduct a molecular docking investigation.

2.7 Statistical analysis

The graphs were created with Origin 8.6 and GraphPad Prism 8.0.2. Depending on the primary analysis, one or two-way analysis of variance (ANOVA) was employed for *in vivo* endpoints, followed by a Tukey post hoc test to verify differences between groups. The difference was statistically significant ($P < 0.05$ and $P < 0.01$).

3. Results

3.1 Network pharmacology

In order to investigate the potential mechanisms of BCE, KEGG pathway enrichment analysis and GO functional annotation were systematically conducted on the intersection targets between four main anthocyanin constituents of BCE (Fig.S1) and obesity-related targets. Based on the KEGG pathway enrichment analysis, we believed that the core signaling pathways, such as fatty acid degradation, steroid hormone biosynthesis, EGFR tyrosine kinase inhibitor resistance, C-type lectin receptor signaling pathway, and TNF signaling pathway, might be the key pharmacological mechanisms (Fig. 1a).

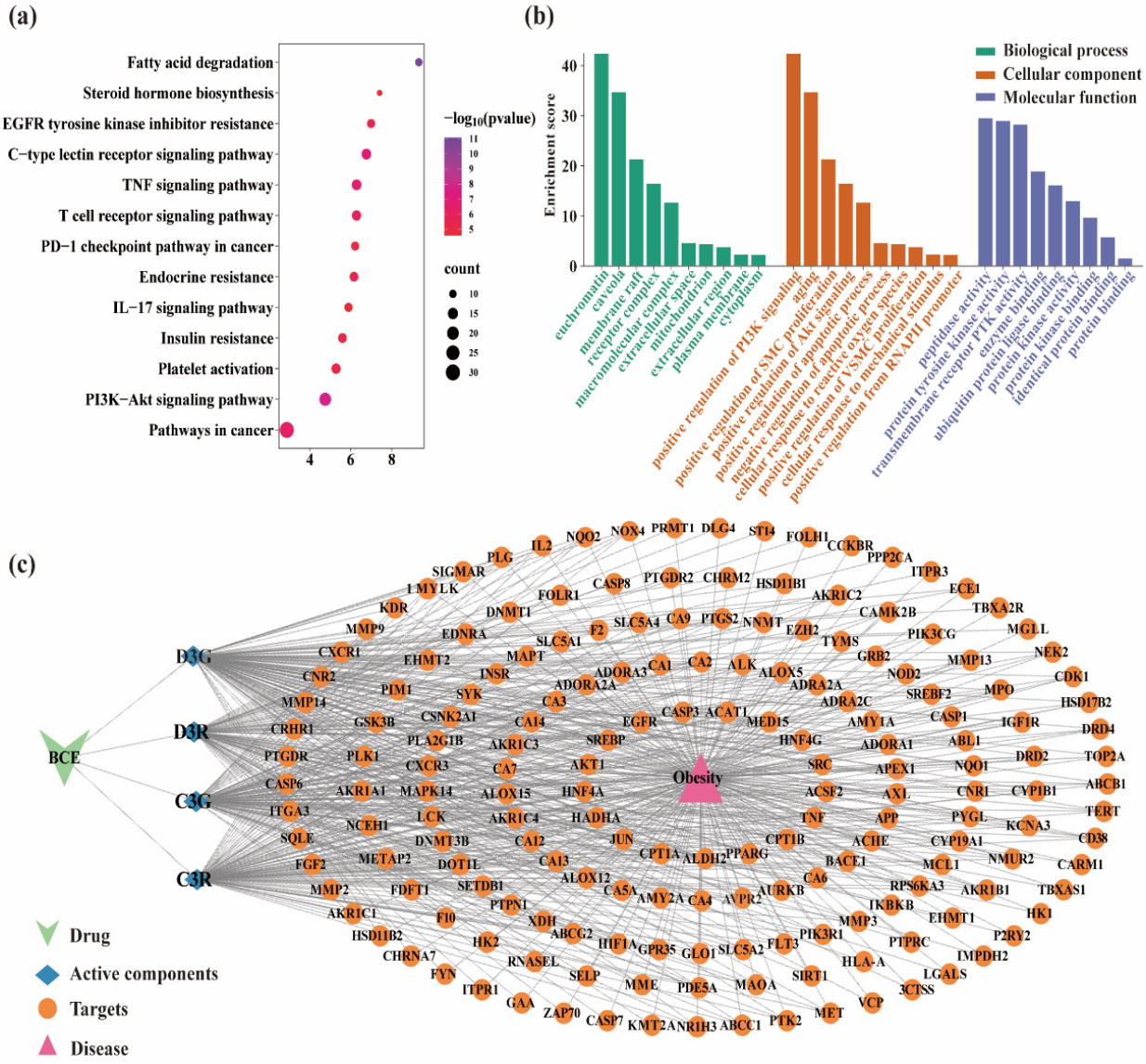

Fig.1 Network pharmacology enrichment analysis predicted key targets and associated pathways of major components in BCE: including D3G, D3R, C3G, C3R. (a) The result of the KEGG pathway enrichment of BCE. (b) GO functional enrichment analysis indicators. (c) The drug-target-pathway-disease network diagram.

The GO analysis was divided into three parts: biological process (BP), cellular component (CC), and molecular function (MF) (Fig.1b). The main BP involved in the anti-obesity function of BCE were euchromatin, caveola, and membrane raft; the main CC were positive regulation of PI3K-AKT signaling, aging, and positive regulation of SMC proliferation; the main MF were peptidase activity, protein tyrosine kinase activity and transmembrane receptor PTK activity. The compound-target-disease network was constructed by Cytoscape (Fig. 1c), BCE shared 182 common targets with obesity, which were identified as the core targets for the anti-obesity activity of BCE.

3.2 The lipid-lowering effect of BCE to HepG2 cells

3.2.1 The effect of BCE on the viability of HepG2 cells

We evaluated whether BCE in the concentration range from 7.5 to 200 μg/mL could affect cell viability. Among the tested groups, the high doses of BCE (50-200 μg/mL) had a significant decrease compared to the control group. (Fig. 2a). Subsequently, we evaluated the effect of BCE (7.5-25 μg/mL) on the viability of

HepG2 cells cultured in DMEM supplemented with or without OA (0.25 mmol/L). BCE did not significantly affect the cell viability under either normal or OA conditions (Fig. 2b). As a result, the concentrations of 7.5, 15, and 25 μg/mL were set as the intervention concentrations.

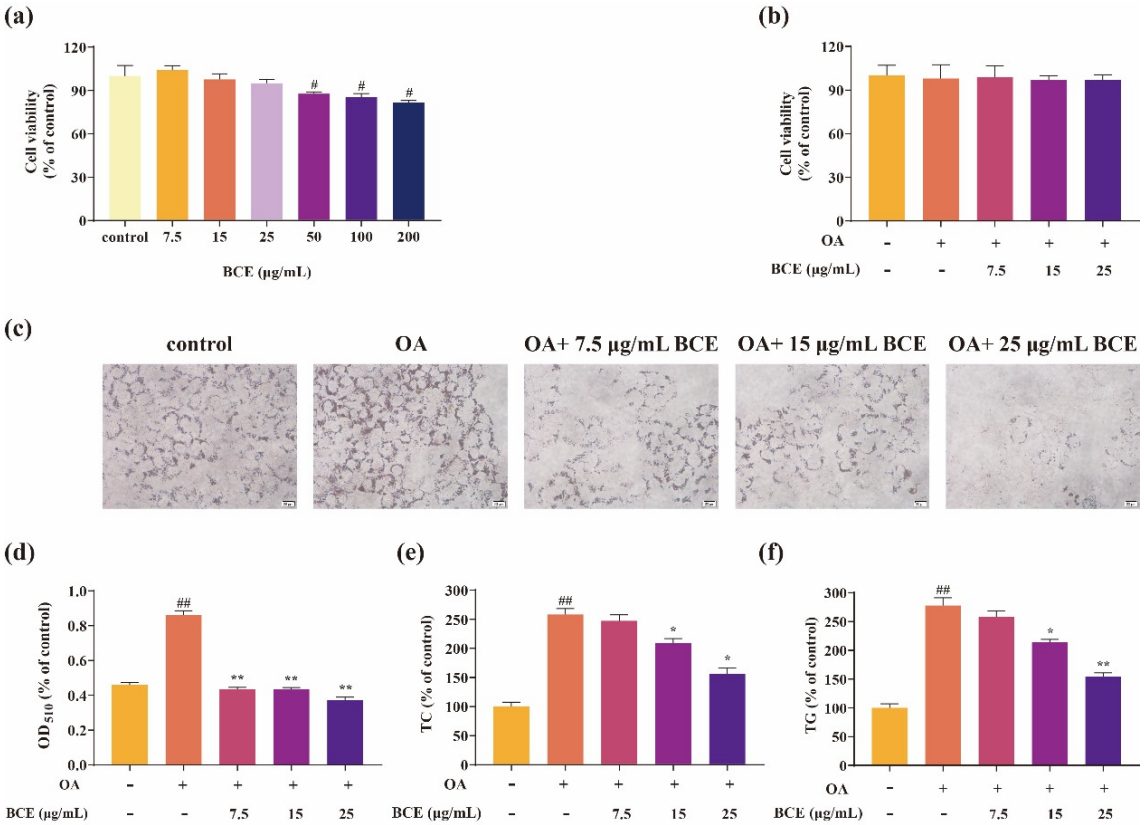

Fig.2 The effect of BCE treatment on HepG2 cells. (a) The impact of BCE treatments (0, 7.5, 15, 25, 50, 100, 200 μg/mL) on HepG2 cells. (b) Effect of BCE on cell viability damaged by OA (1.5 mM). (c) Representative pictures of oil red O staining of captured. (d) oil red O and data analysis of stained areas. (e–f) The change of the contents of TC and TG. *Indicated significant difference ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) between control and model groups. $^{\#}$represented a significant difference ($^{*}P < 0.05$ and $^{**}P < 0.01$) between model and BCE-treated groups.

3.2.2 BCE attenuated lipid accumulation and reduced TG, TC content in HepG2 cells

Oil red O staining results demonstrated that OA treatment could induce abnormal lipid accumulation in HepG2 cells[15], while BCE treatment significantly reversed this trend. Compared to the OA group, cellular lipid droplets were reduced by 49.44% ($P < 0.01$), 49.46% ($P < 0.01$), and 56.68% ($P < 0.01$) following treatment with different concentrations of BCE (Fig. 2c & d). This inhibitory effect was paralleled by a concentration-dependent reduction in lipid parameters: treatment with the highest dose of BCE significantly decreased intracellular TC and TG levels by 39.44% ($P < 0.05$) and 26.63% ($P < 0.05$) compared to the OA group (Fig. 2e-f). Similar to the findings of the current study, the barley polyphenol extract[26] and the ethyl acetate extract of *I. polycarpa* fruits[27] also exhibited superior lipid-lowering activity in OA-induced HepG2 cells, suggesting a potential ability among plant-derived polyphenols in regulating lipid metabolism.

3.3 The effect of BCE on E. coli OP50 and the establishment of the obesity model

The safe dose of BCE that does not affect the growth of *E. coli* was investigated. The results indicated that 25 μg/mL BCE had no significant effect on the growth of *E. coli*, whereas 50–500 μg/mL BCE inhibited growth (Fig. 3a).

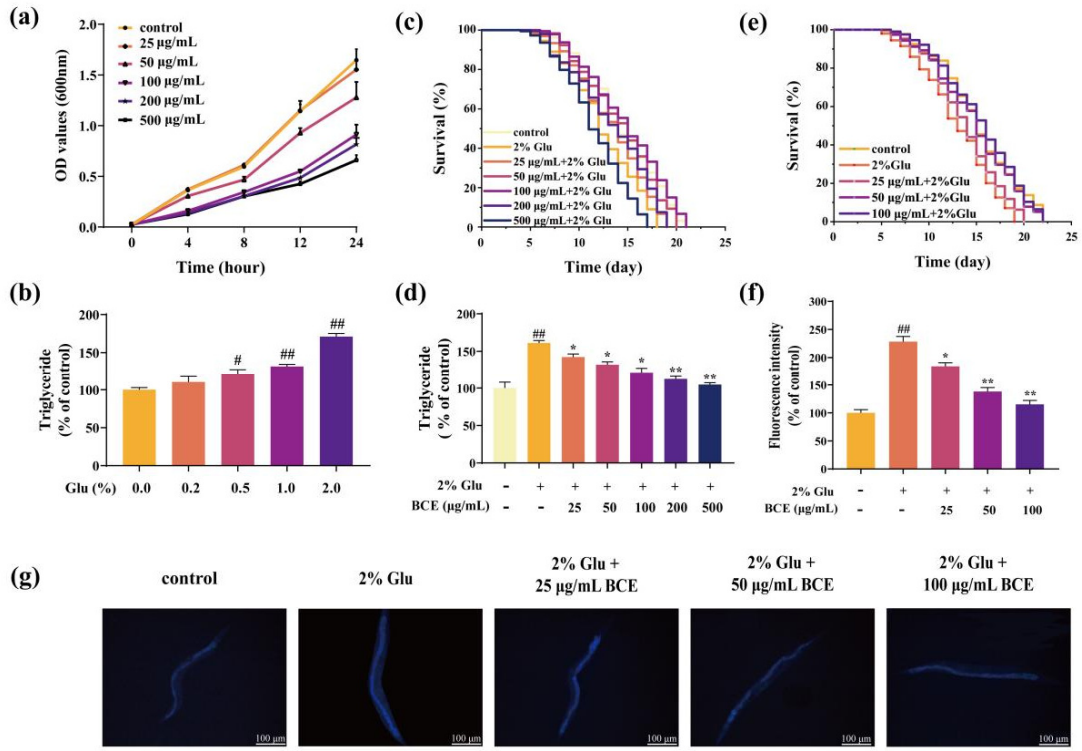

Fig.3 Effect of BCE on the growth of *E. coli* as well as *C. elegans*. (a) Impact of BCE on the absorbance of *E. coli* bacterial broth. (b)Effects of different concentrations of glucose on TG content in *C. elegans*. (c) BCE (200, 500 μg/mL) was poisonous to worms. (d) Effect of BCE on TG content in *C. elegans* under high glucose conditions. (e) Effects of different concentrations of BCE on nematode lifespan. (f-g) *C. elegans* lipofuscin data analysis and sample images. *Indicated significant difference ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) between control and model groups. $^{\#}$represented a significant difference ($^{*}P < 0.05$ and $^{**}P < 0.01$) between model and BCE-treated groups.

For TG content, the 0.2% Glu group showed no significant change compared with the control group, while the 0.5%-2.0% Glu groups exhibited significantly increasing TG values (Fig. 3b). To ensure modeling stability, 2.0% Glu was selected as the obesity modeling condition for subsequent experiments. The significant increase in TG content in the 2.0% Glu group aligned with previous studies linking high glucose intake to lipid accumulation in obesity models[28].

3.4 BCE extended C. elegans longevity and inhibited lipofuscin accumulation

To screen the safe dosing range of BCE, toxicity experiments were conducted using N2 *C. elegans*. At a concentration of 500 μg/mL, BCE was observed to have detrimental effects on *C. elegans* (Fig. 3c-d). Concentrations of 25 μg/mL and 200 μg/mL BCE demonstrated comparable efficacy in extending the lifespan of the nematodes. Notably, 50 μg/mL and 100 μg/mL BCE proved to be the most effective in promoting longevity. In light of these findings, the subsequent experiments in this study utilized blackcurrant extracts at concentrations of 25, 50, and 100 μg/mL.

BCE treatment at 25, 50, and 100 μg/mL significantly prolonged median lifespan by 6.47% ($P < 0.05$), 13.78% ($P < 0.05$), and 18.77% ($P < 0.01$) compared to the model group (Fig. 3e). Lipofuscin, an age-related lysosomal byproduct, could be used to measure the degree of aging in *C. elegans*. Its accumulation correlates with impaired cellular homeostasis and shortened lifespan. BCE treatment markedly reduced lipofuscin accumulation on the 6th day (Fig. 3f-g), with fluorescence intensity decreasing by 23.87% ($P < 0.05$), 39.19%

($P < 0.01$), and 50.95% ($P < 0.01$) (Fig. 3f), indicating its potential to delay aging. This aligns with prior studies demonstrating that natural products, such as *Pterodon emarginatus* dried fruit oil extract[29] and Silkworm Pupa oil[30], could extend nematode lifespan. The mechanism may involve regulating lipid metabolism and reducing oxidative damage, common strategies for promoting longevity.

3.5 The effect of BCE on ROS, MDA, and antioxidant enzymes in C. elegans

BCE treatment effectively decreased ROS concentration relative to the model group (Fig. 4a). The fluorescence intensity following 50 and 100 μg/mL of BCE treatment decreased by 16.12% ($P < 0.05$) and 31.70% ($P < 0.01$), respectively (Fig. 4b). Moreover, treatment with 100 μg/mL BCE led to significant increases in the activities of SOD (87.53%, $P < 0.01$), CAT (52.83%, $P < 0.05$), and GSH-Px (87.79%, $P < 0.05$) compared to the model group (Fig. 4c-e). MDA, a final product of lipid peroxidation, serves as an indicator of lipid oxidation levels. BCE treatment resulted in a decrease in MDA concentration (30.32% for the 100 μg/mL BCE) (Fig. 4f). These results collectively demonstrated that BCE effectively alleviated oxidative stress caused by abnormal lipid accumulation, consistent with previous studies on the antioxidant effect of astaxanthin-anthocyanin nanoparticles in *C. elegans*[31].

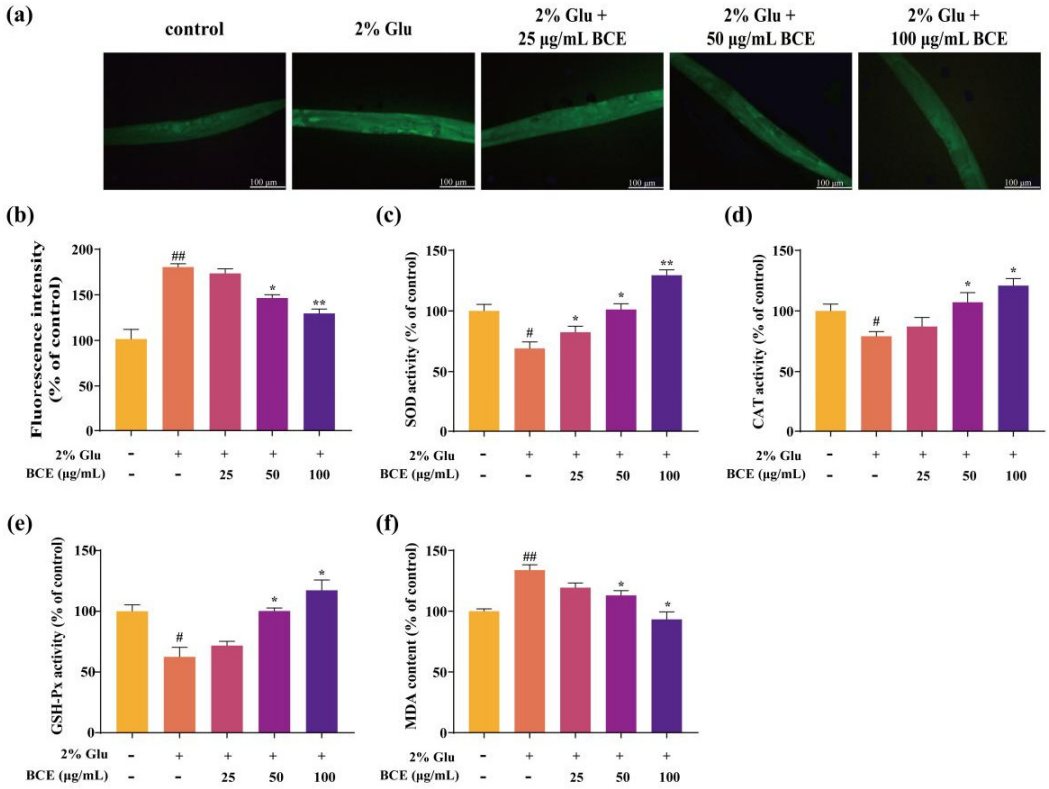

Fig.4 The impacts of BCE on antioxidant-related indexes in *C. elegans*. (a) Typical images showing ROS in *C. elegans*. (b) Using Image J software, the levels of ROS accumulation were calculated as a percentage of control. (c–f) Following BCE treatment, alterations in SOD, CAT, GSH-Px activity, and MDA content. *Indicated significant difference ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) between control and model groups. $^{\#}$ represented a significant difference ($^{*}P < 0.05$ and $^{**}P < 0.01$) between model and BCE-treated groups.

3.6 BCE reduced lipid accumulation and regulated lipid metabolism in C. elegans

Treatment with 25, 50, and 100 μg/mL BCE exhibited dose-dependent and significant reduction in lipid accumulation assessed by ORO (22.04%, 35.56%, 46.06%, $P < 0.05$). This trend was also observed in the fluorescence intensity of LIU1 *C. elegans*.

Following treatment with BCE (25, 50, 100 μg/mL), the fluorescence intensity decreased by 18.35% ($P < 0.05$), 34.75% ($P < 0.05$), and 47.04% ($P < 0.01$), (Fig. 5b). Meanwhile, the TG level was significantly increased by exposure to 2.0% Glu but was decreased after BCE treatment (15.42%, 25 μg/mL; 26.81%, 50 μg/mL; 34.63%, 100 μg/mL) (Fig. 5c).

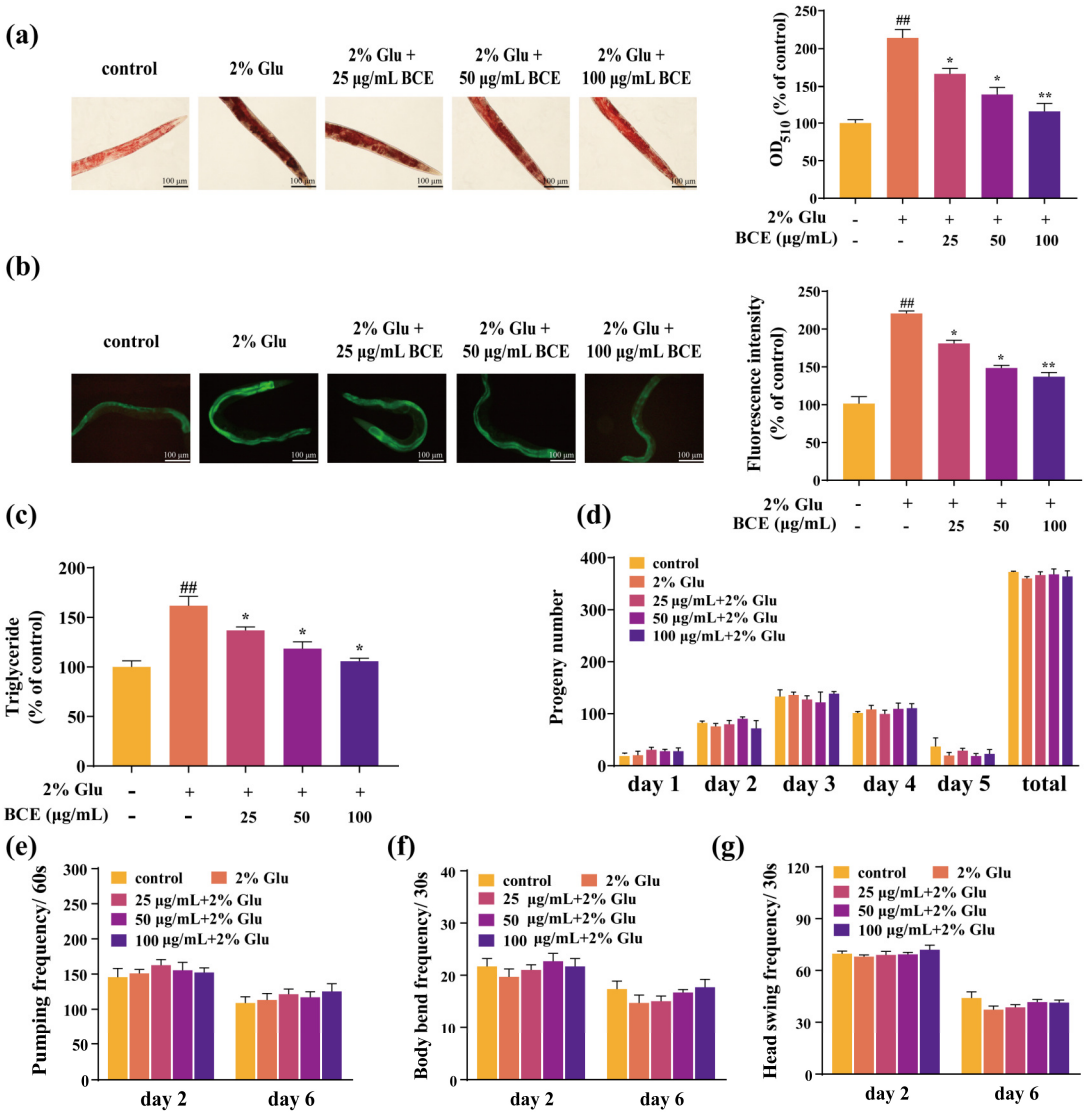

Fig.5 Effects of BCE on lipid accumulation and healthy lifespan of *C. elegans*. (a) Representative images stained with oil red O and data analysis of stained areas. (b) Fluorescence intensity of DHS-3::GFP in *C. elegans* and data analysis. (c) BCE treatment reduced the content of TG. (d-e) The effect of BCE on reproduction rate and pumping frequency. (f-g) BCE did not affect the body bend and head swing frequency of *C. elegans*. *Indicated significant difference ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) between control and model groups. #represented a significant difference ($^{*}P < 0.05$ and $^{**}P < 0.01$) between model and BCE-treated groups.

Functional assessments of pharyngeal pumping rates, reproductive capacity, and locomotor activity confirmed that BCE treatment did not impair the fundamental physiological functions of *C. elegans* (Fig. 5d-g). These findings excluded nonspecific factors (such as reduced food intake or generalized toxicity) as potential contributors to the observed lipid reduction, thereby validating the specificity of BCE's action on lipid metabolic pathways. This aligned with prior investigations that demonstrated bioactive compounds such as epigallocatechin-3-gallate and caffeine mitigated lipid accumulation in *C. elegans* through targeted metabolic regulation rather than systemic disruption[32].

3.7 BCE reduced lipid accumulation in C. elegans through multiple targets of lipid metabolism

The nematode nuclear hormone receptor 49 (NHR-49), a functional homolog of mammalian peroxisome proliferator-activated receptor alpha (PPARα), forms a transcriptional complex with mediator subunit MDT-15 to regulate key genes in fatty acid β-oxidation: acyl-CoA synthetase-2 (*acs-2*) for long-chain fatty acid activation, carnitine palmitoyltransferase-1/carnitine palmitoyltransferase-2 (*cpt-1/cpt-2*) for acyl-CoA mitochondrial transport, and enoyl-CoA hydratase-1.1 (*ech-1.1*) for the β-oxidation step. Consistent with this regulatory axis, the expression levels of *nhr-49* and its downstream genes (*ech-1.1*, *cpt-2*, and *acs-2*) were significantly lower than those of the control group after 2% glucose induction. By contrast, intervention with BCE up-regulated the gene expression of *nhr-49*, *acs-2*, *ech-1.1*, and *cpt-2* (Fig. 6a).

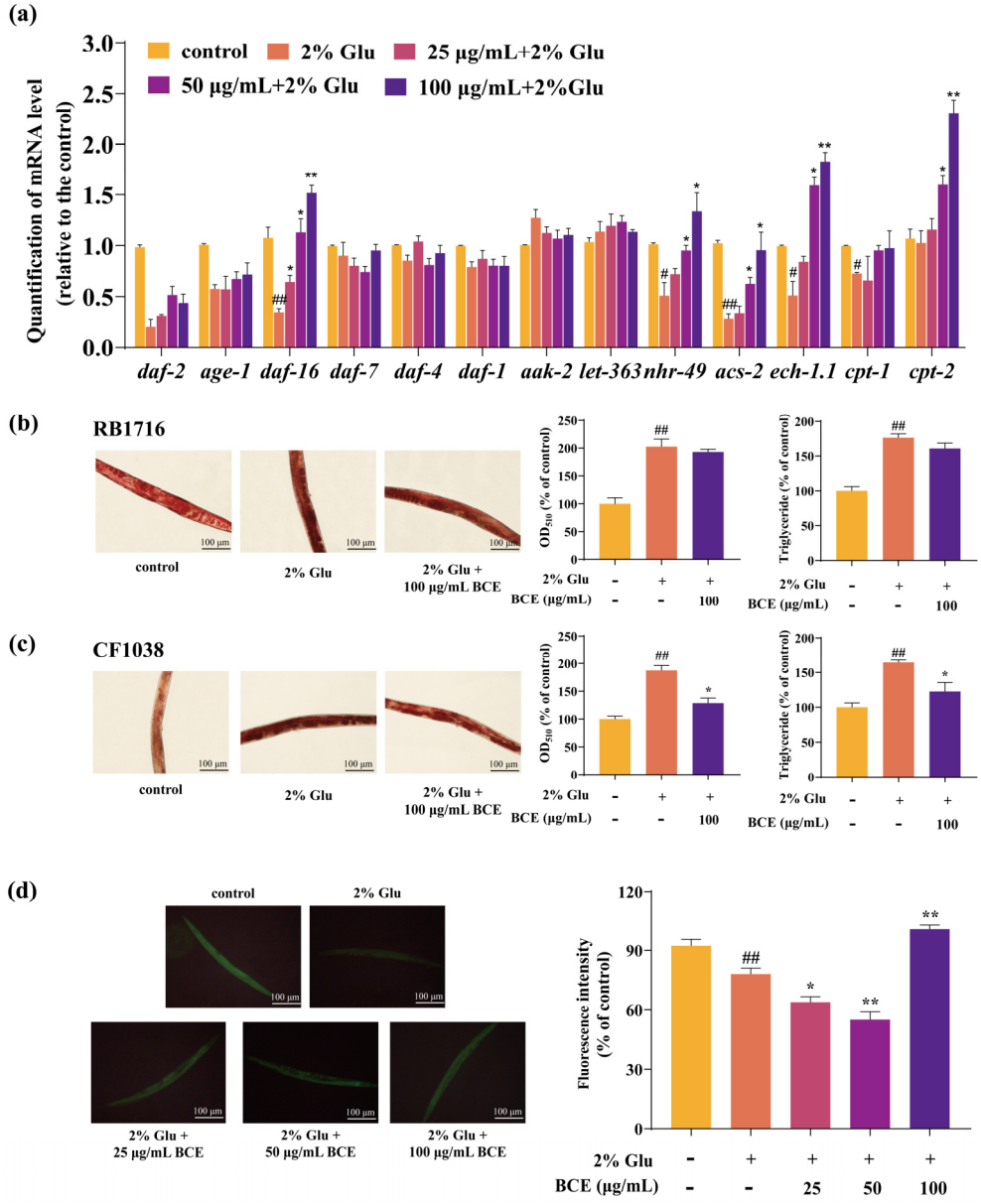

Fig.6 Effects of BCE on genes involved in energy metabolism pathway and NHR-49 pathway and transgenic *C. elegans*. (a) mRNA expression levels of genes regulating lipid metabolism. (b-c) Effects of BCE on changes in lipid accumulation and TG content in *nhr-49* (*ok2165*) and *daf-16* (*mu86*) mutant strains. (d) Representative fluorescence images of WBM170 strains and the fluorescence intensities of ACS-2::GFP was quantitatively analyzed by Image J software. *Indicated significant difference ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) between control and model groups. $^{\#}$represented a significant difference ($^{*}P < 0.05$ and $^{**}P < 0.01$) between model and BCE-treated groups.

The insulin/IGF-1 signaling (IIS) pathway-associated daf-16 gene, a functional ortholog of the mammalian Forkhead box O (FOXO) transcription factor, plays a critical role in regulating lipid metabolism, stress responses, and longevity in *C. elegans*[33]. Notably, treatment with increasing BCE concentrations (25, 50, and 100 μg/mL) significantly up-regulated *daf-16* gene by 27.70% ($P < 0.05$), 73.10% ($P < 0.05$), and 109.18% ($P < 0.01$) compared to the model group (Fig. 6a). This dose-dependent activation of daf-16 suggested that BCE might enhance daf-16 transcriptional activity, thereby mediating its lipid-lowering effects via IIS pathway.

ORO staining and TG content determination (Fig. 6b&c) showed that after ingestion of 2% glucose, the intensity of ORO staining in *nhr-49* and *daf-16* mutant *C. elegans* increased significantly, with lipid contents rising by 120.50% ($P < 0.01$) and 87.36% ($P < 0.01$), respectively, and TG contents increasing by 76.40% and 64.02% ($P < 0.01$), respectively. Following intervention with 100 μg/mL BCE, the lipid content in daf-16 mutants decreased by 31.37%, and the TG content decreased by 25.28% ($P < 0.05$), while no significant changes were observed in the lipid and TG contents of nhr-49 mutants. These results suggested that *nhr-49* was critically involved in the lipid-lowering effect of BCE. Moreover, BCE treatment dramatically increased the fluorescence intensity of *acs-2* (Fig. 6d). These results collectively indicated that BCE modulated pertinent NHR-49 pathway genes, consistent with previous reports demonstrating that isorhamnetin-mediated cholesterol reduction is contingent upon the NHR-49 pathway[34].

In *C. elegans*, sterol regulatory element-binding protein 1 (SBP-1, a homolog of mammalian SREBP) and nuclear hormone receptor 80 (NHR-80, a homolog of mammalian hepatocyte nuclear factor 4) jointly regulate fatty acid synthesis by targeting phosphatidylcholine diacylglycerol choline phosphotransferase 2 (pod-2) and fatty acid synthase 1 (*fasn-1*), which encode enzymes for fatty acid synthesis, as well as the fatty acid desaturase genes *fat-5*, *fat-6*, and *fat-7*, thereby maintaining lipid homeostasis[35]. The model group had higher expressions of *mdt-15*, *sbp-1*, *fat-5*, *fat-6*, *fat-7*, and *nhr-80* genes compared with the control group. Following treatment with a 100 μg/mL concentration of BCE, the expression levels of the genes encoding *sbp-1*, *fat-6*, and *fat-7* dropped by 108.89% ($P < 0.05$), 128.64% ($P < 0.01$), and 137.02% ($P < 0.01$), respectively (Fig. 7a), suggesting that the glucose-induced lipid accumulation effect might be associated with increased fatty acid desaturase activities[36].

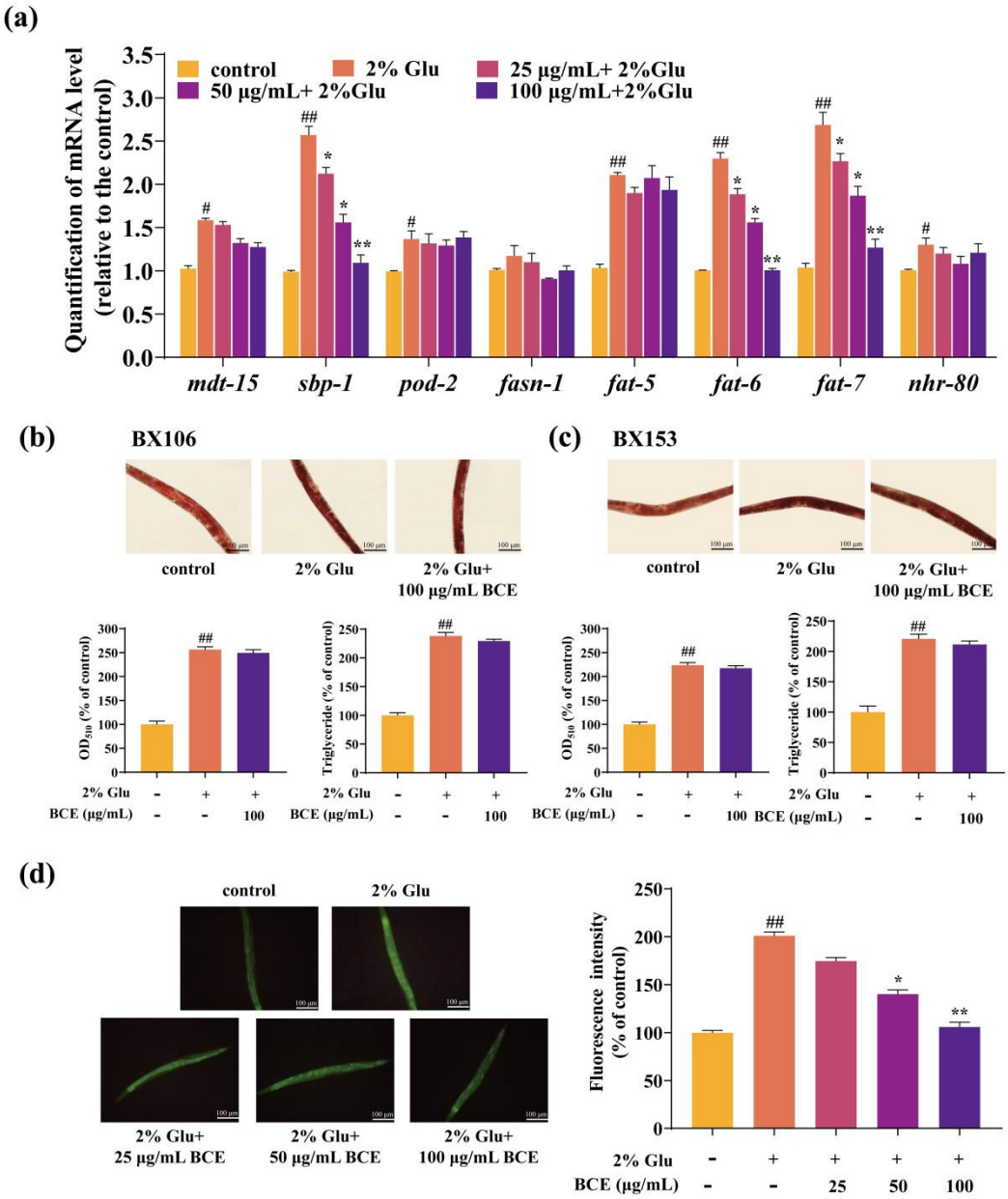

Fig.7 Effects of BCE on genes involved in SBP-1 pathway and transgenic *C. elegans*. (a) mRNA expression levels of genes regulating lipid metabolism. (b-c) Effects of BCE on changes in lipid accumulation and TG content in *fat-6* (*tm331*) and *fat-7* (*wa36*) mutant strains. (d) Representative fluorescence images of CE548 strains and the fluorescence intensity of SBP-1::GFP was quantitatively analyzed by Image J software. *Indicated significant difference ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) between control and model groups. $^{\#}$represented a significant difference ($^{*}P < 0.05$ and $^{**}P < 0.01$) between model and BCE-treated groups.

ORO staining and TG content analysis revealed no significant reduction in lipid accumulation in *fat-6* and *fat-7* mutant obese *C. elegans* following BCE treatment. This indicated that BCE's lipid-lowering effects were compromised in these mutants, underscoring the essential role of fat-6 and fat-7 in mediating BCE's impact on lipid metabolism (Fig. 7b-c). Moreover, BCE treatment significantly attenuated *sbp-1* gene fluorescence intensity (Fig. 7d). Quantitative analysis indicated minimal variation in fat-5 fluorescence intensity (Fig. 8a-b), whereas fat-6 and fat-7 fluorescence were markedly reduced (Fig. 8c-d), corroborating the PCR results. These observations implied that BCE's hypolipidemic effect might be attributed to the down-regulation of *sbp-1* and its downstream target genes, *fat-6* and *fat-7*. This mechanism aligned with

previous studies demonstrating that Fuzhuan brick tea exerted lipid-lowering effects via SBP-1 regulation[37].

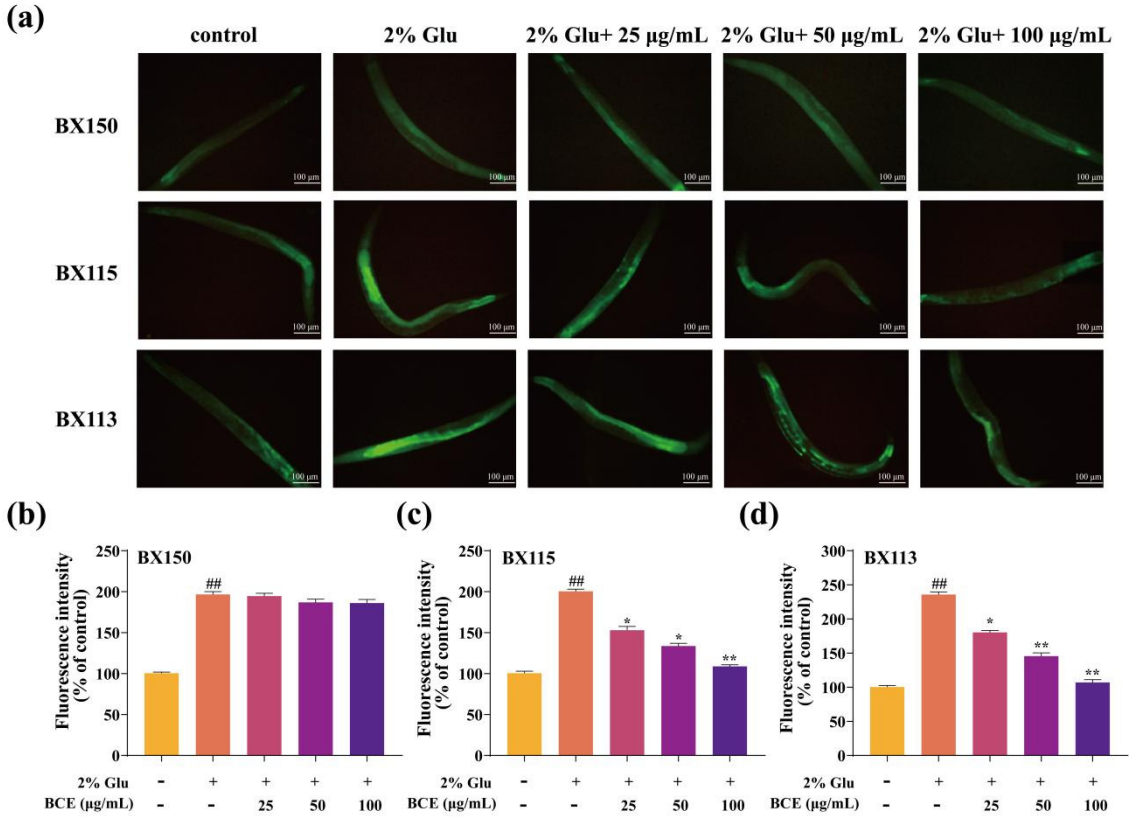

Fig.8 Effect of BCE on the expression of FAT-5::GFP, FAT-6::GFP, and FAT-7::GFP. (a) Representative fluorescence images were obtained using strains BX150, BX115, BX113. The fluorescence intensities of FAT-5::GFP (b), FAT-6::GFP (c) and FAT-7::GFP (d) were quantitatively analyzed. *Indicated significant difference ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) between control and model groups. $^{\#}$represented a significant difference ($^{*}P < 0.05$ and $^{**}P < 0.01$) between model and BCE-treated groups.

3.8 Molecular docking

The major active components of BCE could bind to the same site of SBP-1/MDDT-15 and NHR-49 signaling pathway-related proteins (Fig. 9). D3G, D3R, C3G, and C3R had binding energies of -214.88 kJ/mol, -272.41 kJ/mol, -103.87 kJ/mol, and -188.24 kJ/mol to SBP-1, respectively. The binding energies of D3G, D3R, C3G, and C3R to NHR-49 were -362.06 kJ/mol, -153.87 kJ/mol, -155.82 kJ/mol, and -327.95 kJ/mol, respectively. The results of molecular docking showed that the main active component of BCE could bind to lipid metabolism-related proteins.

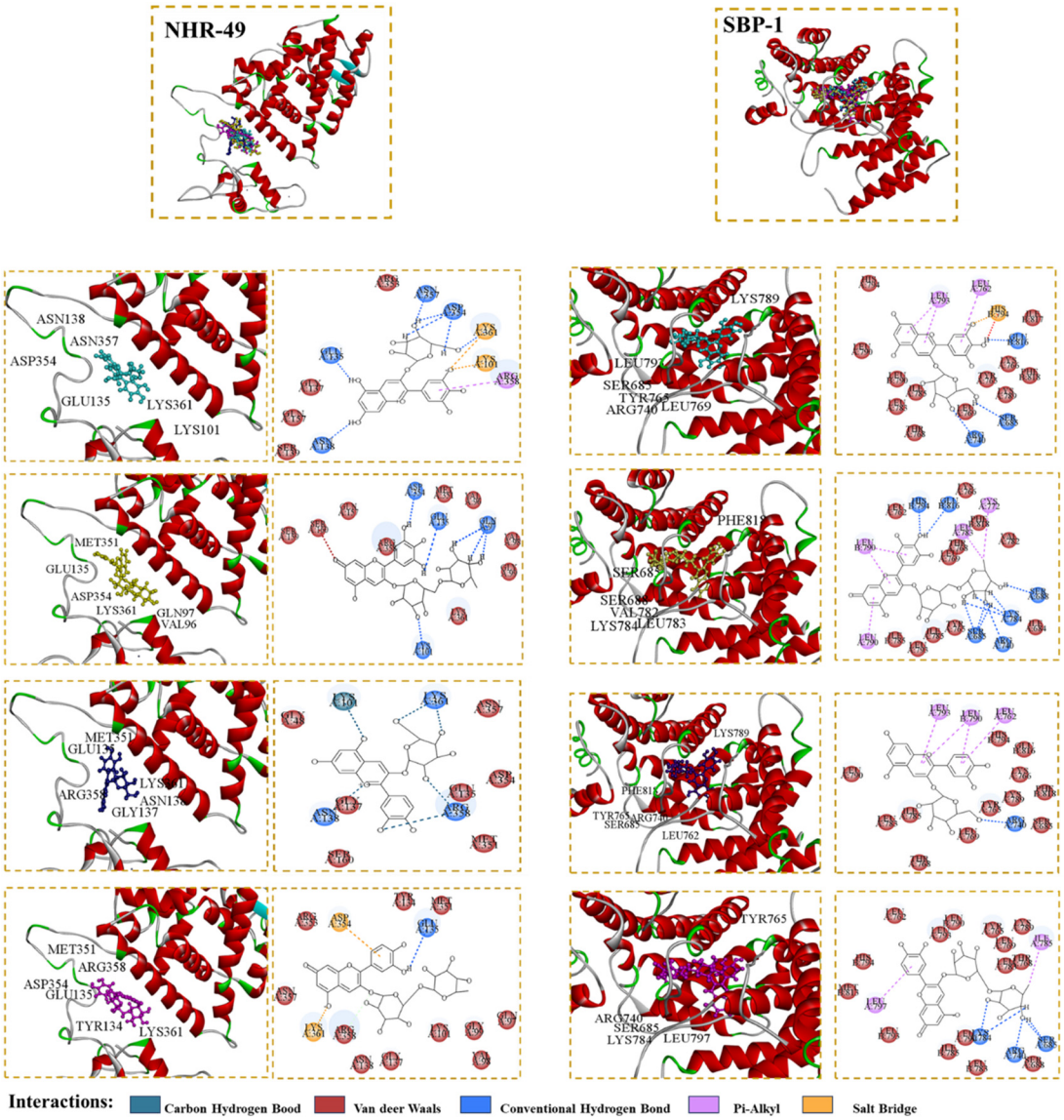

Fig.9 Molecular docking results of the four major anthocyanins (D3G, D3R, C3G, C3R) in BCE with target proteins NHR-49 and SBP-1.

4. Discussion

Obesity prevention and treatment are still in urgent need of new approaches. Natural functional compounds like BCE have shown promise in regulating lipid metabolism, but their specific active components and underlying mechanisms remain unclear. In the present study, network pharmacology was employed to predict the lipid-lowering pathways of major components of BCE (e.g., D3G, D3R, C3G, and C3R). KEGG analysis revealed that the key anti-obesity targets of BCE were linked to fatty acid degradation, similar to the results of quercetin on enhancing fatty acid oxidation[38]. Previous studies have also utilized network pharmacology to validate various lipid-lowering mechanisms of natural products, such as those of lotus leaf in obesity[39], *Polygonum perfoliatum L.* in non-alcoholic fatty liver disease[40], yerba mate in atherosclerosis[41]. Subsequently, *in vitro* experiments demonstrated that BCE could alleviate lipid abnormalities in OA-induced HepG2 cells. We further investigated the mechanisms underlying BCE-mediated regulation of lipid metabolism using the *C. elegans* model.

Notably, obesity often exacerbates inflammation and promotes excessive intracellular ROS accumulation, leading to lipid peroxidation and the generation of MDA, which poses significant threats to organismal health[42]. High glucose exposure significantly shortened the lifespan and increased lipofuscin accumulation in N2 worms, whereas BCE treatment reversed these detrimental effects. This protective activity of BCE was analogous to mulberry anthocyanin extract, which has been shown to ameliorate oxidative damage in HepG2 Cells and prolong the lifespan of *C. elegans*[43]. Mechanistically, such effects might be linked to the ability of BCE to enhance antioxidant enzyme activities for mitigating ROS-mediated damage. For instance, BCE treatment could up-regulate the activities of SOD, CAT and GSH-Px, thereby counteracting obesity-induced oxidative stress and metabolic dysfunction, aligned with the findings of purple wheat extract[44].

In our study, high-glucose exposure was conducted to induce obesity and lipid metabolic dysregulation in *C. elegans*, characterized by increasing TG content and lipid accumulation, which was in lines with reported studies[45]. This phenomenon was subsequently reversed by BCE treatment, indicated that BCE played a critical role in reducing lipid accumulation in obese *C. elegans*. Similarly, chia seed oil reduced fat content and TG levels in *C. elegans*[46]. The short-chain dehydrogenase DHS-3, localized to intestinal lipid droplets in *C. elegans*, serves as a validated lipid droplet marker protein in the organism[47]. Using lipid droplet fluorescence visualization, we observed that BCE significantly reduced intestinal lipid droplet fluorescence intensity in obese *C. elegans* induced by high-glucose exposure. This finding is consistent with previous reports showing that Oenothein B decreased lipid droplet fluorescence intensity in *C. elegans*[48], pointing out a mechanism for BCE in modulating lipid accumulation.

It was well known that growth and reproductive development caused energy consumption[49]. However, BCE treatment did not alter pharyngeal pumping rate, reproductive rate, or locomotor activity in worms, indicating that its lipid-lowering effects are independent of growth and reproduction. This observation aligned with the regulatory characteristics of natural plant-derived components. Such as chia seed oil treatment did not affect pharyngeal pumping rate[46], and supplementation with pu-erh tea could not impair reproductive capacity in *C. elegans*[50].

In *C. elegans*, nhr-49 acts as a pivotal transcription factor governing β-oxidation and the peroxisomal pathway, serving as the regulatory hub of lipid metabolism[51]. In line with our expectations, the results indicated that BCE treatment significantly upregulated the mRNA expression of *nhr-49* and downstream genes *acs-2*, *ech-1.1*, and *cpt-2* in *C. elegans*. This observation was similar with prior evidence regarding that *Baccharis trimera* leaf extract exerted lipid-lowering effects via a mechanism dependent on the transcription factor NHR-49[52]. Mechanistically, Volvariella volvacea polysaccharides have been shown to reduce lipid accumulation in an NHR-49-dependent manner[53].

Sbp-1 was a key transcriptional regulator of fat and sterol synthesis pathways[54]. Knockdown of *sbp-1* could alter the expression of adipogenic genes such as SCDs[55]. In the present study, BCE significantly down-regulated *sbp-1* and some of the target genes, *fat-6* and *fat-7*, but did not affect the expression levels of

mdt-15, *pod-2*, *fasn-1*, and *fat-5*. In line with our results, curcumin[56] and green coffee extract[57] reduced nematode fat accumulation by modulating *sbp-1*. Consistent with the qPCR results, BCE treatment significantly reduced the mean fluorescence intensity of SBP-1::GFP, FAT-6::GFP, and FAT-7::GFP, but did not change the mean fluorescence intensity of FAT-5::GFP. Meanwhile, the lipid-lowering effect of BCE remained in the *fat-5* mutant, but was eliminated in the *fat-6* mutant and *fat-7* mutant, which indicated that BCE could inhibit fatty acid synthesis by down-regulating the expression of *sbp-1* and the target genes *fat-6* and *fat-7*, thereby inhibiting lipid accumulation in *C. elegans*. Taken together, these findings collectively demonstrated that BCE modulated lipid homeostasis by orchestrating both lipid catabolism (via *nhr-49*) and anabolism (via *sbp-1*/fatty acid synthesis axis), providing mechanistic insights into its anti-obesity effects.

Molecular docking technology enables visualization of the interaction between a molecule and the binding site of its target protein, revealing their binding state[58]. The four main anthocyanin active substances in BCE could be successfully bound to NHR-49 and SBP-1 via hydrogen bonding. The active site residues of the primary targets and BCE contained van der Waals forces and carbon-hydrogen bonds, which were crucial for complex formation and stability. Nevertheless, the precise mechanism of such intermolecular interactions and their functional consequences in shaping SBP-1/NHR-49 activity remain incompletely elucidated, leaving several questions open for further investigation: for instance, how specific variations in the number and positional distribution of hydroxyl/glycosyl moieties quantitatively regulate the binding affinity and dynamic properties of anthocyanin-proteins associations; whether these structural disparities confer different functional roles (e.g., whether specific anthocyanin components serve as “scaffolds” to enhance the targeting efficiency of other components toward SBP-1 and NHR-49).

5. Conclusion

Obesity has evolved into a widespread health issue affecting most people. BCE was found to decrease lipid formation *in vitro*. In *vivo*, BCE increased the healthy lifespan and reduced lipid accumulation in *C. elegans*. Finally, BCE might regulate lipid metabolism in *C. elegans* by up-regulating *nhr-49* and downstream target genes to enhance fatty acid *β*-oxidation and down-regulating *sbp-1* and its target genes to inhibit fatty acid synthesis. These findings can serve as a solid foundation for the possible use of BCE as a bioactive ingredient in obesity prevention and management.

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