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Comparative Profiling of Free and Bound Polyphenols from Five Wild *Boletus edulis* Varieties: Antioxidant Activity and Longevity-Regulating Effects in *Caenorhabditis elegans*

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ABSTRACT: *Boletus edulis* is rich in phenolic compounds, but the composition and bioactivity of its free and bound polyphenols remain insufficiently compared. In this study, free and bound polyphenols from five wild edible *Boletus edulis* samples were profiled, and their antioxidant and longevity-regulating effects were evaluated in vitro and in *Caenorhabditis elegans*. A total of 27 phenolic compounds were identified, showing marked variation among samples and fractions. The free fraction of *Leccinum rugosiceps* (LrS-F) and the bound fraction of *Boletus edulis* Bull.: Fr. (BeB-B) showed the best overall antioxidant and lifespan-promoting performance, reducing reactive oxygen species and malondialdehyde levels, enhancing superoxide dismutase and catalase activities, and extending nematode lifespan. Mutant-based, transcriptional, and protein-level analyses indicated that these effects involved *daf-16*-dependent antioxidant regulation and additional JNK-1/SKN-1-related stress responses. These findings highlight *B. edulis* polyphenols as natural antioxidant resources with potential longevity-regulating activity.

Key words: Polyphenols; *Boletus edulis*; antioxidant activity; lifespan; *Caenorhabditis elegans*; stress resistance

1. Introduction

Edible mushrooms are widely recognized not only for their culinary value but also for their rich content of bioactive compounds with health-promoting properties^[1,2]. Among them, *Boletus edulis*, commonly known as porcini or king bolete-is particularly appreciated for its abundant proteins, vitamins, minerals, and polyphenols^[3]. Polyphenols, especially phenolic acids and flavonoids, are known to act as potent antioxidants, capable of scavenging reactive oxygen species (ROS) and mitigating oxidative stress, a central contributor to aging and the pathogenesis of chronic diseases such as cancer, cardiovascular dysfunction, and neurodegeneration^[4-6]. The antioxidant and anti-inflammatory potential of mushroom-derived polyphenols thus has significant implications for the development of functional foods and nutraceuticals.

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Several species within the *Boletus* genus, such as *Boletus regius*, *B. brunneissimus*, *B. speciosus*, and *Leccinum rugosiceps*, exhibit diverse phenolic profiles and sensory characteristics^[7,8]. However, polyphenol composition can vary not only between species but also between free and bound phenolic forms, which differ in bioavailability and biological activity^[7,9,10]. Despite growing interest in the pharmacological effects of mushroom polyphenols, there is limited comparative analysis of different *Boletus* species in terms of their antioxidant capacity and phenolic composition. More importantly, the relationship between these phenolic constituents and their *in vivo* biological activity remains poorly understood, particularly regarding their ability to modulate oxidative stress in living organisms.

To address this gap, we employed *Caenorhabditis elegans* as a model system for *in vivo* antioxidant evaluation. Owing to its conserved aging-related signaling pathways, short life cycle, and genetic manipulability, *C. elegans* has been widely used to study redox regulation and dietary interventions^[11–14]. This model offers key advantages for elucidating mechanistic insights into how natural compounds influence oxidative stress and lifespan^[15,16]. Importantly, we utilized *daf-16* mutant strains to investigate the role of the DAF-16/FOXO transcription factor, a central regulator of stress resistance and longevity, in mediating the antioxidant effects of mushroom polyphenols^[17]. Since DAF-16 controls the expression of genes such as *sod-3*, *ctl-1*, and *gst-4*, its involvement is essential to distinguish direct enzymatic antioxidant effects from transcriptional regulation^[17,18].

Therefore, the present study aims to compare the polyphenol composition of five wild *Boletus* species from Yunnan Province, evaluate their antioxidant activities using both *in vitro* assays (DPPH, FRAP, ORAC) and *in vivo* endpoints in *C. elegans* model, and explore the potential molecular mechanisms by examining gene expression and enzymatic activities in wild-type and *daf-16* mutant nematodes. By integrating phenolic profiling with *in vitro* and *C. elegans*-based evaluations, this study provides comparative evidence linking polyphenol composition with antioxidant and lifespan-regulating effects in wild edible *Boletus edulis*.

2. Materials and methods

2.1 Chemicals and reagents

Acetone, n-hexane, ethyl acetate, Folin-Ciocalteu reagent, methanol, and acetonitrile were purchased from CNW Technologies GmbH (Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine, FeCl₃, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), superoxide dismutase (SOD) assay kit, 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA), and thiobarbituric acid (TBA) were obtained from Sigma-Aldrich (USA). All reagents were used in analytical grade or higher purity.

Wild-type *C. elegans* (N2) was kindly provided by Prof. Hongyuan Zhou (Southwest University). Mutant strains *daf-2* (*e1370*) and *daf-16* (*mu86*) were obtained from Prof. Jinmin Gao's lab (Nankai University). *Escherichia coli* OP50 strain (uracil auxotroph) used as a food source was supplied by the *C. elegans* Academic Platform.

2.2 Sample preparation

Five wild *B. edulis* samples were collected from Yunnan Province and identified based on macroscopic morphological characteristics, including pileus color and shape, pore characteristics, stipe morphology, and overall fruiting body appearance. The samples were designated as *B. edulis* Bull.: Fr. (BeB), *B. regius* Krombh (BrK), *B. brunneissimus* W.F. Chiu (BbW), *B. speciosus* Frost (BsF), and *L. rugosiceps* (Peck) Singer (LrS). The samples were vacuum freeze-dried using a vacuum freeze dryer (SCIENTZ-20F, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China), subsequently ground into powder using a laboratory grinder (RS-JS612, Royalstar, Hefei Royalstar Small Household Appliance Co., Ltd., Hefei, China), and stored in a desiccator protected from light until further analysis^[8].

2.3 Extraction of polyphenols from *B. edulis*

The extraction of free and bound phenolic compounds was performed based on a previously established method by our research group^[19]. Briefly, freeze-dried powder (1 g) was mixed with 80% acetone and homogenized in an ice-water bath, followed by centrifugation (10000 r/min, 10 min). The process was repeated three times, and the supernatants were combined and concentrated using rotary evaporation, with final adjustment in methanol. The residue was subjected to alkaline hydrolysis, neutralization, and lipid removal, followed by extraction of bound phenols using ethyl acetate. The ethyl acetate extracts were then concentrated and reconstituted in methanol for further analysis.

2.4 Determination of phenolic compound contents in *B. edulis* extracts

The contents of free and bound phenolics were determined using modified methods from Kupina et al.^[20]. A 0.2 mL sample was mixed with 0.8 mL of water and 0.2 mL of Folin-Ciocalteu reagent. After standing for 6 min, 2 mL of Na₂CO₃ solution and 1.6 mL of deionized water were added. The mixture was incubated for 90 min, and the absorbance was measured at 760 nm. Gallic acid served as the standard to construct a calibration curve, and phenolic content was expressed as milligrams of gallic acid equivalents per 100 g of dry weight (mg GAE/ g DW).

Total flavonoid content was determined using the aluminum chloride colorimetric assay. Equal volumes (0.5 mL) of 2% AlCl₃ and extract solution in methanol were mixed and incubated at room temperature for 1 h. Absorbance was measured at 415 nm, and results were expressed as milligrams of rutin equivalents per gram dry weight (mg RE/g DW)^[21].

2.5 Determination of phenolic composition of *B. edulis* varieties based on 600MRM analysis

Samples were thawed on ice and vortexed for 30 seconds to mix thoroughly. A 60 µL aliquot of each sample was transferred to a 1.5 mL EP tube and centrifugally concentrated to dryness over 8 h. The dried samples were reconstituted in 240 µL of 60% acetonitrile (containing internal standards), vortexed for 30 s, followed by sonication and centrifugation at 12,000 rpm for 15 min at 4 °C. A 60 µL aliquot of the supernatant was carefully transferred to an LC vial for analysis, with mixed samples used as quality control (QC) samples.

Liquid Chromatography Conditions: Analysis was performed using an ACQUITY UPLC H-Class (Waters) ultra-performance liquid chromatography system equipped with a Waters Atlantis Premier BEH

Z-HILIC column (1.7 μm , 2.1 mm $\times$ 150 mm)^[22]. Mobile phase A consisted of ultrapure water: acetonitrile (8:2) with 10 mmol/L ammonium acetate, and mobile phase B was acetonitrile: ultrapure water (9:1) with 10 mmol/L ammonium acetate; both phases were adjusted to pH 9 with pure water. The sample temperature was maintained at 8 °C, and the injection volume was 1 μL .

Mass Spectrometry Conditions: A SCIEX 6500 QTRAP+ triple quadrupole mass spectrometer equipped with an IonDrive Turbo V ESI ion source was used for analysis in multiple reaction monitoring (MRM) mode^[23]. The ion source parameters were: Curtain Gas = 35 psi, IonSpray Voltage = +5000 V / -4500 V, Temperature = 500, Ion Source Gas 1 = 50 psi, Ion Source Gas 2 = 50 psi. Mass spectrometric data acquisition and quantitative analysis of target compounds were performed using SCIEX Analyst Work Station Software (1.7.2) and DIA Data Driven Flow (v1.0.1).

2.6 Analysis of antioxidant activity of *B. edulis* polyphenol extracts

The *in vitro* antioxidant activity of *B. edulis* extracts was evaluated using three assays: DPPH radical scavenging assay, FRAP assay, and ORAC assay.

2.6.1. DPPH radical scavenging activity

A 100 μL aliquot of the extract was mixed with 3.9 mL of 0.1 mmol/L DPPH solution and incubated in the dark for 30 min. The absorbance was measured at 517 nm, and the scavenging activity was calculated^[3].

2.6.2. FRAP assay

The FRAP assay involved mixing 200 μL of the extract with 3 mL of FRAP reagent and incubating at 37 °C for 30 min. The absorbance was measured at 593 nm^[7].

2.6.3. ORAC assay

The oxygen radical absorbance capacity (ORAC) assay was performed according to a previously reported method with slight modifications. Briefly, fluorescein was used as the fluorescent probe, and AAPH was used as the peroxy radical generator. The extract was mixed with fluorescein solution, and the reaction was initiated by adding AAPH. The decrease in fluorescence intensity was recorded at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Antioxidant capacity was calculated from the area under the fluorescence decay curve using Trolox as the standard and expressed as μmol Trolox equivalents per g extract ($\mu\text{mol TE/g}$)^[24].

2.7 Effect of *B. edulis* polyphenol extracts on lifespan and antioxidant levels in *C. elegans*

C. elegans was cultured on nematode growth medium (NGM) plates seeded with *E. coli* OP50 at 20 °C. For synchronization, gravid adults were transferred to M9 buffer to lay eggs, which were then collected, washed, and allowed to hatch and develop on NGM plates. The larvae were grown until reaching the L4 stage, typically after 2-3 days. Synchronized L4 stage *C. elegans* were used for assessing lifespan and intracellular reactive oxygen species (ROS) levels^[15].

For the lifespan assay, synchronized L4-stage worms were transferred to NGM plates containing *B. edulis* polyphenol extracts at concentrations of 5 $\mu\text{g/mL}$ for free phenolics and 1.5 $\mu\text{g/mL}$ for bound phenolics,

respectively. The day of worm transfer was defined as day 0. Survival was monitored daily, and the numbers of naturally dead and lost worms were recorded until all worms in the control group had died^[25]. To measure intracellular ROS levels, *C. elegans* were incubated with the extracts and stained with H2DCFDA. Fluorescence intensity was measured using a microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 535 nm^[16].

For mechanistic investigations, ROS levels were also measured in *daf-2 (e1370)* and *daf-16 (mu86)* mutant strains under the same treatment conditions.

2.8 MDA content and antioxidant enzyme levels in *Caenorhabditis elegans*

Malondialdehyde (MDA) content and levels of catalase (CAT) and SOD in *C. elegans* were assessed after treatment with *B. edulis* extracts. MDA content was determined by homogenizing treated *C. elegans* in a lysis buffer, followed by the thiobarbituric acid reactive substances (TBARS) assay. Absorbance was measured at 532 nm, and MDA levels were expressed as nmol MDA per mg protein^[26]. Antioxidant enzyme activities were assessed by homogenizing treated *C. elegans* and measuring CAT and SOD activities using commercial assay kits. CAT activity was determined by monitoring the decomposition of hydrogen peroxide at 240 nm, and SOD activity was measured by the inhibition of the reduction of nitroblue tetrazolium (NBT) at 560 nm^[27].

2.9 Effects of *B. edulis* polyphenol extracts on the expression of anti-aging genes in *C. elegans*

Total RNA was extracted from *C. elegans* using the Trizol method, and reverse transcription was performed with 500 ng of RNA using Maxima Reverse Transcriptase (Thermo Scientific). Primer design was based on the *act-1* housekeeping gene, and sequences are listed in **Table S2**. qPCR was conducted using 2 × SG Fast qPCR Master Mix (High Rox) in an ABI 7500 system. The thermal cycling program included an initial denaturation at 95 °C for 3 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method with *act-1* as the internal reference gene. For wild-type N2 worms, the untreated N2 group was used as the calibrator. For the *daf-16* mutant assay, the untreated N2 group was retained as the common calibrator to compare transcript abundance between wild-type and mutant backgrounds. All reactions were performed in triplicate.

2.10 Western blot analysis

Protein expression in *C. elegans* was analyzed by Western blot as previously described^[28], with minor modifications. After sample denaturation, proteins were separated on 12% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk in TBST, incubated with primary antibodies overnight at 4 °C, and with HRP-conjugated secondary antibodies for 2 h in 37 °C. Protein bands were visualized using ECL chemiluminescence detection.

2.11 Statistical analysis

All data are presented as mean ± standard deviation (SD) from at least three independent replicates. Statistical analyses were performed using SPSS 25.0. Differences among groups were evaluated by one-way

analysis of variance (ANOVA), followed by Tukey's post hoc test. Statistical significance was defined as $P < 0.05$. Lifespan data were analyzed using survival curves and mean lifespan values. Pearson correlation analysis was performed to evaluate the relationships between phenolic compound contents and antioxidant-related indicators. Graphs were generated using GraphPad Prism 8.0, and the correlation heatmap was produced using ChiPlot.

3. Result and Discussion

3.1 Total phenolic content of *B. edulis* extracts

The total phenolic and flavonoid contents, including both free and bound forms, of five *B. edulis* varieties are summarized in **Table 1**. For phenolics, the free phenolic content ranged from highest to lowest as follows: LrS > BsF > BbW > BeB > BrK. LrS exhibited the highest free phenolic content (15.41 ± 0.38 mg GAE/g DW), whereas BrK showed the lowest (13.83 ± 0.13 mg GAE/g DW). The elevated free phenolic content in LrS suggests its potential for immediate antioxidant activity, given that free phenolics are more readily absorbed and actively neutralize free radicals^[7]. In contrast, the bound phenolic content ranked as BbW > BeB > LrS > BsF > BrK. BbW displayed the highest bound phenolic content (9.73 ± 0.27 mg GAE/g DW), implying that a significant portion of its polyphenols are structurally bound and may exert delayed but sustained antioxidant effects upon release during digestion^[29].

Table 1 Total phenolic content and total flavonoid content of free and bound polyphenol fractions from *Boletus edulis* samples.

Varieties	Total phenolics (mg GAE/g DW)						Total flavonoids (mg RE/g DW)					
	Free	Contribution Rate (%)	Bound	Contribution Rate (%)	Total		Free	Contribution Rate (%)	Bound	Contribution Rate (%)	Total	
BeB	14.66 ± 0.11^a	68.26 ± 3.41	6.82 ± 0.24^b	31.74 ± 1.59	21.48 ± 0.35^b		12.09 ± 0.10^a	81.26 ± 4.06	2.79 ± 0.13^d	18.74 ± 0.94	14.88 ± 0.23^b	
BrK	13.83 ± 0.13^b	77.18 ± 3.86	4.09 ± 0.07^c	22.82 ± 1.14	17.92 ± 0.20^c		9.83 ± 0.29^b	72.65 ± 3.63	3.12 ± 0.22^c	27.35 ± 1.37	12.95 ± 0.50^c	
BbW	15.01 ± 0.17^{ab}	60.67 ± 3.03	9.73 ± 0.27^a	39.33 ± 1.97	24.75 ± 0.43^a		8.72 ± 0.20^c	80.42 ± 4.02	8.12 ± 0.24^a	19.58 ± 0.98	16.84 ± 0.44^a	
BsF	15.38 ± 0.32^a	74.81 ± 3.74	5.18 ± 0.16^b	25.19 ± 1.26	20.57 ± 0.49^b		10.25 ± 0.24^b	79.93 ± 4.00	2.58 ± 0.06^d	18.06 ± 0.90	12.81 ± 0.30^c	
LrS	15.41 ± 0.38^a	74.65 ± 3.73	5.23 ± 0.18^b	25.35 ± 1.27	20.64 ± 0.56^b		8.30 ± 0.25^c	55.83 ± 2.79	6.57 ± 0.33^b	44.17 ± 2.21	14.87 ± 0.58^c	

Note: Different letters in the same group in the same column were significantly different ($P < 0.05$).

Similarly, the total flavonoid content varied notably among the varieties. BeB exhibited the highest total flavonoid content (14.88 ± 0.23 mg RE/g DW), primarily due to its abundant free flavonoids (12.09 ± 0.10 mg RE/g DW), followed by LrS and BbW. As key bioactive compounds, flavonoids contribute substantially to antioxidant capacity through mechanisms such as hydrogen donation and metal ion chelation^[3]. In contrast, BbW showed the highest bound flavonoid content (8.12 ± 0.24 mg RE/g DW), further reinforcing its potential for long-term antioxidant effects. Notably, while LrS had the highest free phenolics, its free flavonoid content was relatively lower, suggesting a distinct polyphenolic profile dominated by phenolic acids rather than flavonoids.

Collectively, these compositional differences highlight that both free and bound polyphenols -including flavonoids - play complementary roles in antioxidant activity, with free forms offering rapid protection and bound forms contributing to prolonged physiological effects. The diversity in phenolic and flavonoid distribution among varieties underscores the biochemical heterogeneity within *Boletus edulis*, which may account for differences in biological efficacy observed in subsequent in vitro and in vivo analyses.

3.2 Profiling of phenolic compounds in *B. edulis* extracts using 600MRM

The diversity in phenolic composition among different *B. edulis* varieties can be attributed to their genetic makeup, environmental conditions, and physiological adaptations^[30]. Using a 600MRM-based targeted metabolomics approach, a total of 27 polyphenolic compounds were identified and quantified across both free (–F) and bound (–B) fractions of five *B. edulis* varieties (Table 2).

Table 2 Phenolic compounds identified and quantified in free and bound polyphenol fractions from *Boletus edulis* samples.

Compound name	Formula	Molecular Weight (g/mol)	Concentration (μg/g)									
			BeB-F	BrK-F	BbW-F	BsF-F	LrS-F	BeB-B	BrK-B	BbW-B	BsF-B	LrS-B
Resveratrol	C ₁₄ H ₁₂ O ₃	228.24	742.7	613.86	534.7	721.14	713.36	642.1	852.16	854.95	568.4	752.18
		28	7±28.	±63.51	7±79.	±22.32	±6.86	9±246	±84.78	±210.4	2±138	±313.7
			32		71			.53		6	.88	
3,4-Dihydroxymandelic acid	C ₈ H ₈ O ₅	184.14	346.6	327.40	312.6	298.65	358.54	287.1	291.38	288.29	280.1	286.36
		58	5±1.7	±1.33	7±8.9	±3.99	±2.47	4±4.8	±0.67	±4.83	2±0.0	±1.96
			2		2			9			4	
Curcumin	C ₂₁ H ₂₀ O ₆	368.37	124.0	122.61	124.6	122.58	124.09	122.3	546.91	160.77	124.3	132.44
		91	5±2.2	±0.02	2±0.0	±1.09	±0.52	1±1.5	±71.65	±7.73	2±2.1	±5.34
			6		3			7			7	
Salicylic acid	C ₇ H ₆ O ₃	138.12	40.64	44.68±	47.81	46.86±	43.69±	40.02	44.79±	46.13±	40.85	41.14±
		05	±0.61	0.14	±3.37	4.46	0.28	±0.21	0.41	0.25	±0.21	0.62
2-Hydroxyphenylacetic acid	C ₈ H ₈ O ₃	152.14	53.23	50.23±	76.28	47.14±	55.26±	47.16	47.68±	49.6±0.	46.67	54.58±
		7	±0.95	0.58	±2.49	0.41	0.78	±0.73	0.49	25	±0.26	0.69
Benzoylformic acid	C ₈ H ₆ O ₃	150.13	24.91	97.35±	69.41	15.76±	45.98±	2.29±	11.56±	24.9±3.	1.25±	6.72±0.
		12	±3.52	15.05	±2.22	5.94	2.18	0.25	3.37	97	0.21	88
Naringin	C ₂₇ H ₃₂ O ₁	580.53	11.08	13.23±	15.12	18.01±	12.43±	14.23	14.97±	16.84±	16.92	23.65±
		33	±1.16	0.98	±1.05	4.12	1.82	±3.11	2.1	2.55	±0.18	2.22
trans-Cinnamic acid	C ₉ H ₈ O ₂	148.15	1144.	2316.0	4807.	681.37	1251.7	-	-	-	-	-
		83	28±5	6±64.4	41±75	±50.2	2±65.9					
			5.92	6	5.38		3					
3-Methoxybenzoic acid	C ₈ H ₈ O ₃	152.14	-	-	2.53±	-	-	-	-	-	-	-
		7			0.22							
Benzoic acid	C ₇ H ₆ O ₂	122.12	227.5	3324.0	2091.	2236.4	2216.7	47.15	80.21±	66.74±	64.58	48.72±
		11	2±20.	3±58.3	04±23	4±64.1	5±63.0	±39.0	2.17	11.94	±30.7	13.59
			09	6	0.67	2	3	5				
Orsellinic acid	C ₈ H ₈ O ₄	168.14	24.11	125.87	14.43	-	22.27±	-	1.85±0	-	-	-
		64	±5.22	±7.95	±3.61		0.82		.36			
2,3-Dihydroxybenzoic acid	C ₇ H ₆ O ₄	154.11	6.18±	10.66±	18.03	0.84±0	17.08±	-	11.25±	2.91±0.	0.62±	1.84±0.
		99	0.94	0.42	±1.62	.24	1.52		0.65	48	0.31	01

Anthranilic acid	C ₇ H ₇ NO	137.13 57	-	-	-	2.13 ± 0.24	-	-	24.57 ± 14.49	39.08 ± 1.95	-	5.7 ± 7.04
2-Methoxybenzoic acid	C ₈ H ₈ O ₃	152.14 7	125.0 8 ± 33.45	270.26 ± 6.32	217.3 1 ± 23.37	-	386.73 ± 28.22	-	11.35 ± 0.36	11.67 ± 4.31	-	22.4 ± 3.65
Gentisic acid	C ₇ H ₆ O ₄	154.11 99	-	-	-	-	4.67 ± 3.94	123.9 9 ± 16.0	448.3 ± 25.54	136.6 ± 18.35	121.2 2 ± 6.96	343.1 ± 34.71
3-(3-Hydroxyphenyl)propanoic acid	C ₉ H ₁₀ O ₃	166.17 35	-	132.83 ± 31.49	806.7 8 ± 185.49	-	427.14 ± 246.37	-	-	-	-	-
2,4-Dihydroxybenzoic acid	C ₇ H ₆ O ₄	154.11 99	58.34 ± 2.63	5.12 ± 2.69	0.81 ± 0.13	-	14.14 ± 2.54	0.66 ± 0.05	10.35 ± 2.58	2.28 ± 0.34	-	7.82 ± 1.93
Hydroxyphenyllactic acid	C ₉ H ₁₀ O ₄	182.17 29	60.72 ± 4.83	15.45 ± 0.69	-	280.76 ± 19.64	378.52 ± 16.73	-	-	0.73 ± 0.08	-	-
Vanillylmandelic acid	C ₉ H ₁₀ O ₅	198.17 23	450.3 3 ± 7.34	404.28 ± 3.09	409.6 3 ± 13.93	417.24 ± 21.02	401.64 ± 0.42	400.1 1 ± 0.49	401.48 ± 2.74	401.18 ± 1.79	409.0 8 ± 2.03	406.7 ± 0.26
4-Hydroxybenzoic acid	C ₇ H ₆ O ₃	138.12 05	35.63 ± 7.96	240.01 ± 6.81	486.2 ± 15.02	361.57 ± 86.98	500.54 ± 23.33	583.1 4 ± 17.29	313.49 ± 26.87	702.35 ± 30.07	101.2 8 ± 2.79	789.42 ± 32.03
4-Hydroxyhippuric acid	C ₉ H ₉ NO	195.17 17	4.21 ± 2.59	5.05 ± 0.38	45.38 ± 0.37	-	20.24 ± 2.54	-	-	-	-	-
p-Hydroxymandelic acid	C ₈ H ₈ O ₄	168.14 64	-	-	-	-	-	-	101.94 ± 109.59	35.78 ± 4.41	-	-
Caffeic acid	C ₉ H ₈ O ₄	180.15 71	106.5 3 ± 2.95	104.21 ± 2.21	86.3 ± 0.38	97.61 ± 26.36	87.39 ± 3.12	90.89 ± 10.95	119.46 ± 13.22	117.51 ± 9.55	90.06 ± 5.19	117.3 ± 10.35
3,5-Dihydroxybenzoic acid	C ₇ H ₆ O ₄	154.11 99	27.06 ± 2.44	37.9 ± 2.25	20.22 ± 15.7	-	-	-	273.78 ± 36.22	86.52 ± 9.63	-	-
3-Hydroxyanthranilic acid	C ₇ H ₇ NO	153.13 51	141.3 2 ± 0.21	155.76 ± 4.7	137.2 ± 2.37	150.24 ± 19.92	138.01 ± 1.83	121.4 1 ± 2.36	123.72 ± 4.66	118.63 ± 1.46	131.7 1 ± 4.49	135.27 ± 0.78
Terephthalic acid	C ₈ H ₆ O ₄	166.13 06	-	-	-	-	-	436.0 7 ± 26.48	624.65 ± 32.21	118.78 ± 3.32	138.6 5 ± 17.39	115.16 ± 31.42
3-Hydroxycinnamic acid	C ₉ H ₈ O ₃	164.15 77	47.25 ± 7.26	30.82 ± 4.65	28.96 ± 3.59	27.32 ± 16.46	82.31 ± 11.02	40.12 ± 12.27	73.03 ± 2.61	68.42 ± 29.32	32.32 ± 7.32	54.66 ± 8.62

Among the quantified compounds, resveratrol was ubiquitously detected across all samples and fractions, with relatively stable concentrations (534.77–854.95 µg/g), suggesting its conserved biosynthesis and potential role as a core antioxidant component in *B. edulis*^[3]. 3,4-dihydroxymandelic acid also showed widespread presence, with high levels in both free and bound forms (e.g., 358.54 ± 2.47 µg/g in LrS-F), highlighting its involvement in redox homeostasis.

Marked compositional divergence was observed in compounds like trans-cinnamic acid and benzoic acid. For example, trans-cinnamic acid was most abundant in BbW-F (4807.41 ± 755.38 µg/g), nearly fivefold

higher than in BeB-F ($1144.28 \pm 55.92 \mu\text{g/g}$), suggesting genotype-specific metabolic flux toward phenylpropanoid derivatives. Similarly, benzoic acid content peaked in BrK-F ($3324.03 \pm 58.36 \mu\text{g/g}$), but was markedly lower in BeB-B ($47.15 \pm 39.05 \mu\text{g/g}$), indicating potential differences in shikimate pathway activity across varieties.

Notably, several phenolic acids such as 4-hydroxybenzoic acid, caffeic acid, and 3-hydroxyanthranilic acid were widely distributed but varied in abundance. 4-hydroxybenzoic acid was particularly enriched in LrS-B ($789.42 \pm 32.03 \mu\text{g/g}$) and BbW-B ($702.35 \pm 30.07 \mu\text{g/g}$), supporting their strong antioxidant performance. Caffeic acid, a known ROS scavenger, remained relatively consistent across samples, ranging from $86.30 \pm 0.38 \mu\text{g/g}$ in BbW-F to $119.46 \pm 13.22 \mu\text{g/g}$ in BrK-B.

Among bound phenolics, gentisic acid and terephthalic acid showed significant accumulation, especially in BrK-B ($448.30 \pm 25.54 \mu\text{g/g}$ and $624.65 \pm 32.21 \mu\text{g/g}$, respectively), implying a structural or storage role in the fungal cell wall matrix. In contrast, compounds like 3-hydroxycinnamic acid and naringin showed broad but relatively low to moderate presence, indicating auxiliary roles in antioxidant defense.

Taken together, these data underscore the complex and variable phenolic profiles across *B. edulis* varieties and extract types. Free fractions tend to be enriched in low molecular weight, rapidly absorbable phenolics (e.g., trans-cinnamic acid, caffeic acid), whereas bound fractions harbor higher levels of structural or slowly released compounds (e.g., gentisic acid, terephthalic acid). This duality likely contributes to both immediate and sustained antioxidant effects. The distinct composition patterns observed across varieties reflect not only environmental and genetic factors^[30], but also suggest differential metabolic routing within polyphenol biosynthesis pathways—information that is critical for rational selection of strains in functional food or nutraceutical applications.

3.3 Principal component analysis and Hierarchical clustering analysis

Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were conducted to visualize the differences in polyphenolic profiles among *Boletus edulis* extracts. The PCA plot (**Fig. 1a**) revealed that the first two principal components effectively separated the samples, with BrK-F and BbW-B located at opposite ends of PC1, indicating significant compositional divergence. LrS-F was positioned distantly from other free phenolic extracts, suggesting a distinct polyphenol profile. HCA results (**Fig. 1b**) further supported this classification, grouping BeB-B, LrS-B, and BsF-B into one cluster, likely due to shared bound phenolic components. In contrast, free phenolic extracts such as BeB-F and BbW-F formed separate branches, reflecting their compositional uniqueness. These clustering patterns align with earlier chemical profiling, highlighting both species-specific and fraction-specific polyphenol distributions^[31].

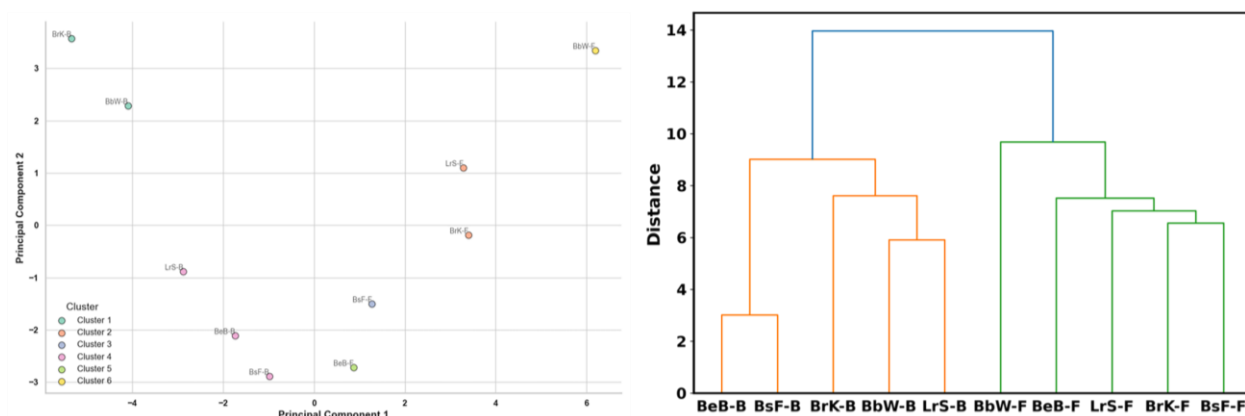


Fig.1 The PCA (a) and HCA (b) of the polyphenolic compounds in *Boletus* extracts.

3.4 In vitro antioxidant activity of *B. edulis* polyphenol extracts

The antioxidant activities of *B. edulis* polyphenol extracts were evaluated using DPPH, FRAP, and ORAC assays, revealing distinct antioxidant patterns between free and bound phenolics (**Fig. 2a–f**). Among free extracts, BrK-F showed the strongest DPPH scavenging activity ($IC_{50} = 36.00 \mu\text{g/mL}$), while BsF-F displayed the highest ferric reducing capacity ($76.87 \mu\text{g eq/mL}$). BbW-F exhibited the strongest oxygen radical absorbance (ORAC = $26.05 \mu\text{mol TE/mg}$), suggesting diverse antioxidant mechanisms across varieties. In the bound phenolic group, BeB-B consistently showed the best performance in both DPPH ($IC_{50} = 27.82 \mu\text{g/mL}$) and ORAC ($24.64 \mu\text{mol TE/g}$), while BsF-B had the highest FRAP value ($177.40 \mu\text{g eq/mL}$), indicating a strong electron-donating capacity.

The relatively strong performance of certain bound phenolic extracts suggests that bound phenolics may contribute to antioxidant activity, although their biological effects cannot be inferred solely from in vitro assays. These results also suggest that phenolic form and extraction method significantly influence radical scavenging and reducing properties, supporting earlier findings on structure–function relationships of phenolics^[10,27].

K-means clustering further classified the extracts into six clusters based on their polyphenolic profiles (**Fig. 2g**). BrK-F and LrS-F grouped together, likely due to similar contents of resveratrol, salicylic acid, and caffeic acid, suggesting comparable antioxidant activities and potential functional interchangeability. In contrast, BeB-F, BbW-F, and BsF-F formed distinct clusters, driven by unique combinations of compounds such as 3,4-dihydroxymandelic acid and gentisaldehyde. Among bound extracts, BeB-B, LrS-B, and BsF-B clustered together, characterized by higher levels of 3-hydroxycinnamic acid and 4-hydroxybenzoic acid, possibly reflecting similar biosynthetic or storage mechanisms^[4,29].

These findings indicate that the antioxidant behavior of *Boletus* polyphenol extracts is strongly influenced by phenolic form, chemical structure, and compositional profile. However, the in vitro antioxidant ranking should be interpreted as chemical reactivity under cell-free conditions rather than as a direct predictor of biological efficacy in vivo. For instance, BsF-B showed the highest FRAP value, suggesting strong electron-donating and ferric-reducing capacity, whereas other extracts showed superior DPPH or ORAC

responses. Because DPPH, FRAP, and ORAC assays reflect different antioxidant mechanisms, including radical scavenging, electron transfer, and peroxy radical quenching, extracts with high activity in one assay may not necessarily show the strongest biological effect in *C. elegans*. Therefore, these assays provide complementary information for screening antioxidant potential, while further in vivo evaluation is required to determine their physiological relevance.

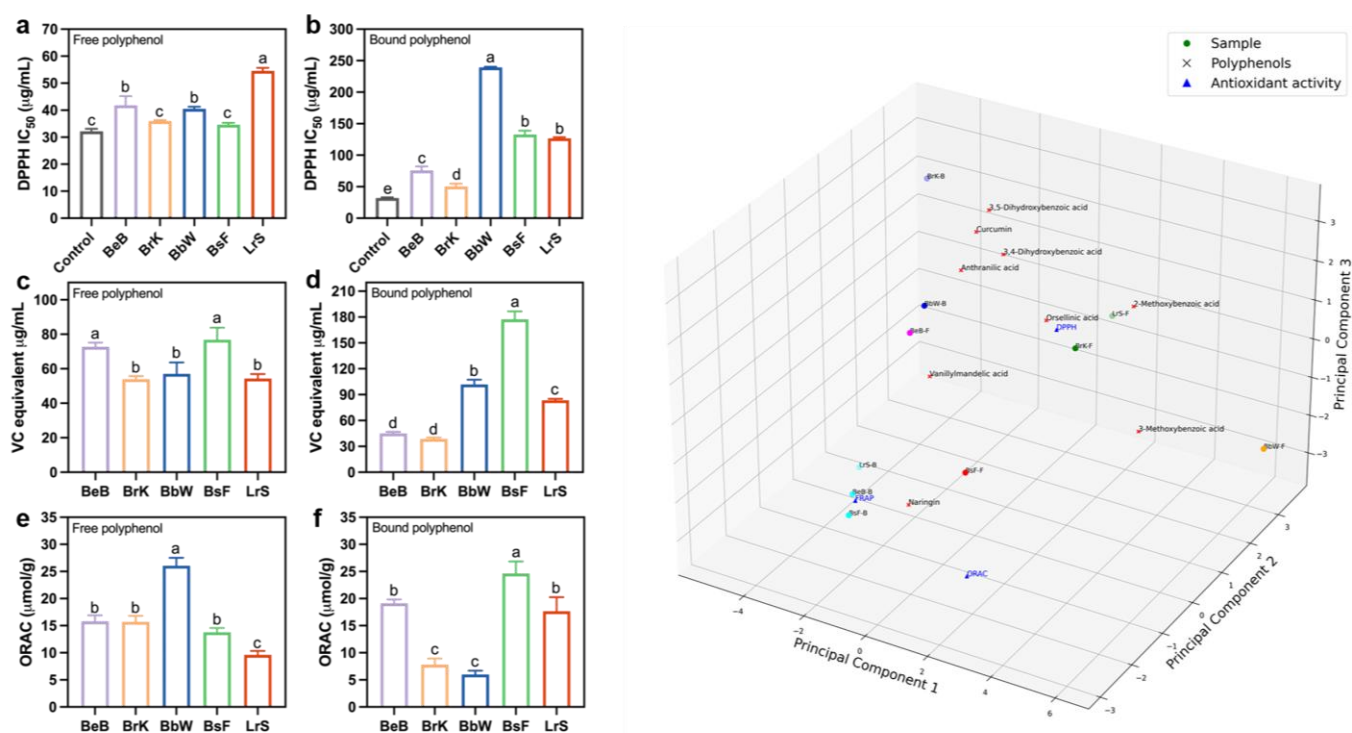


Fig. 2. In vitro antioxidant activity of different *B. edulis* polyphenol extracts assessed by DPPH, FRAP, and ORAC assays (a-f), and K-means clustering based on phenolic composition (g).

3.5 In vivo antioxidant activity

3.5.1 Effects of *B. polyphenol* extracts on the lifespan of *C. elegans*

The polyphenol extracts from *B. edulis* were tested for their ability to extend the lifespan of *C. elegans* as an organism-level indicator of longevity-promoting effects associated with oxidative stress regulation. The control group had an average lifespan of 17.66 ± 1.29 days, while treatment with polyphenol extracts significantly extended the lifespan across different extracts (**Fig. 3** and **Table 3**).

Table 3 The average lifespan of *C. elegans* treated with polyphenol extracts from *Boletus edulis*.

Lifespan (days)	Control	BeB	BrK	BbW	BsF	LrS
Free phenolic(-F)	17.66 ± 1.29^c	22.49 ± 1.28^a	19.80 ± 1.09^b	18.88 ± 1.21^b	20.79 ± 1.76^a	22.07 ± 1.35^a
Increase percentage(%)(-F)	-	$27.35 \pm 6.84\%$	$12.16 \pm 3.04\%$	$6.90 \pm 1.72\%$	$17.73 \pm 4.43\%$	$25.00 \pm 6.25\%$
Bound phenolic(-B)	17.66 ± 1.29^c	20.12 ± 0.92^a	20.45 ± 1.18^a	20.66 ± 0.99^a	18.86 ± 1.03^b	20.14 ± 1.14^a
Increase percentage(%)(-B)	-	$13.97 \pm 3.49\%$	$15.82 \pm 3.96\%$	$17.01 \pm 4.25\%$	$6.81 \pm 1.70\%$	$14.05 \pm 3.51\%$

Note: Data are presented as mean $\pm$ SD (days) for both free (F) and bound (B) phenolic extracts; The percentage increase relative to the control group is also shown as mean $\pm$ SD (%); Different superscript letters within the same row indicate significant differences ($P < 0.05$).

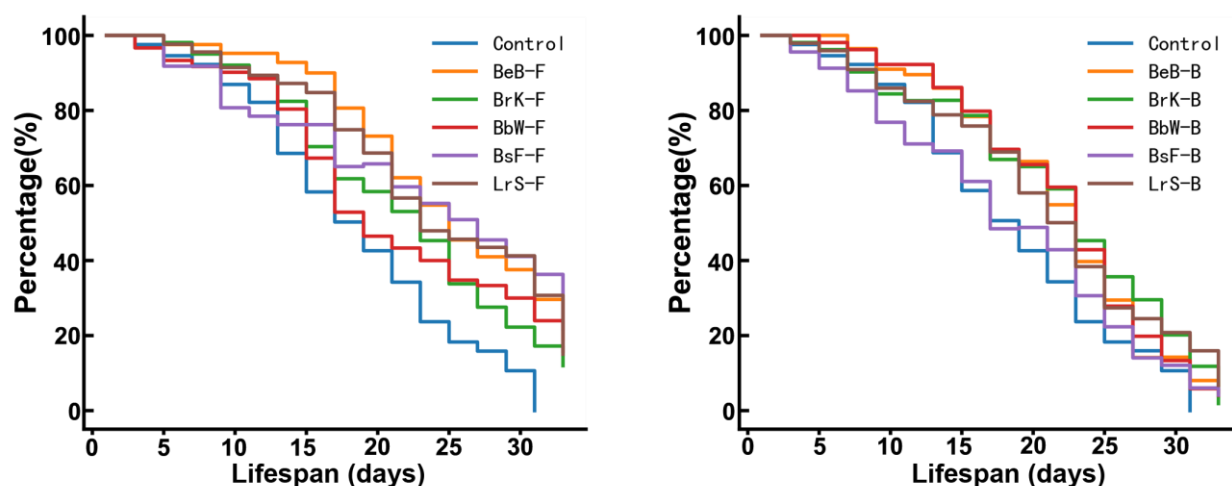


Fig.3 The average lifespan of *Caenorhabditis elegans* affected by *Boletus* polyphenol extracts.

Among the free phenolic extracts, BeB-F had the most pronounced effect, extending the average lifespan to 22.49 ± 1.28 days ($27.35 \pm 6.84\%$ increase), followed by LrS-F at 22.07 ± 1.350 days ($25.0 \pm 6.25\%$ increase). The ranking of lifespan extension for other varieties was BsF-F (20.79 ± 1.76 days, $17.73 \pm 4.43\%$), BrK-F (19.80 ± 1.09 days, $12.16 \pm 3.04\%$), and BbW-F (18.88 ± 1.21 days, $6.90 \pm 1.72\%$). These results suggest that BeB-F and LrS-F contained phenolic profiles favorable for lifespan extension, possibly involving compounds such as resveratrol and 3,4-dihydroxymandelic acid. However, because these extracts contained complex mixtures of phenolics, the contribution of individual compounds requires further validation.^[32]

In comparison, bound phenolic extracts demonstrated a relatively lesser effect on lifespan extension. BbW-B had the best result (20.66 ± 0.99 days, $17.01 \pm 4.25\%$ increase), followed by BrK-B (20.45 ± 1.18 days, $15.82 \pm 3.96\%$), LrS-B (20.14 ± 1.14 days, $14.05 \pm 3.51\%$), and BeB-B (20.12 ± 0.92 days, $13.97 \pm 3.49\%$). BsF-B had the least effect, extending the lifespan to 18.86 ± 1.03 days ($6.81 \pm 1.70\%$). Although bound phenolics are generally considered less immediately bioaccessible than free phenolics, the bound extracts still showed lifespan-promoting effects, suggesting that their biological activity may be associated with gradual release, metabolic transformation, or activation of endogenous stress-response systems.^[13]

3.5.2 Effects of *Boletus* polyphenol extracts on the antioxidant activities in *C. elegans*

To further determine whether the chemical antioxidant capacity observed in vitro could be translated into biological redox regulation, intracellular ROS levels, MDA content, and antioxidant enzyme activities were measured in *C. elegans*. It should be noted that these in vivo endpoints do not necessarily follow the same ranking as DPPH, FRAP, or ORAC values, because they are influenced by compound uptake, metabolic transformation, release behavior, and activation of endogenous stress-response systems. As shown in **Fig. 4a** and **4e**, all extracts significantly reduced ROS accumulation compared to the control ($P < 0.05$), indicating effective ROS-scavenging capacity. Among the free phenolic treatments, LrS-F showed the strongest reduction (fluorescence intensity: 78.17), followed closely by BrK-F (79.41) and BeB-F (82.62). In the bound phenolic group, BbW-B and BeB-B both exhibited marked reductions (77), suggesting sustained ROS suppression likely due to gradual release during digestion, despite the typically lower bioavailability of bound

phenolics. These findings were further corroborated by fluorescence microscopy (**Fig. 4i**), where treated worms displayed markedly reduced green fluorescence compared to controls, confirming intracellular ROS reduction. The results demonstrate that both free and bound polyphenols attenuate oxidative stress at the cellular level, consistent with previous reports^[13,32].

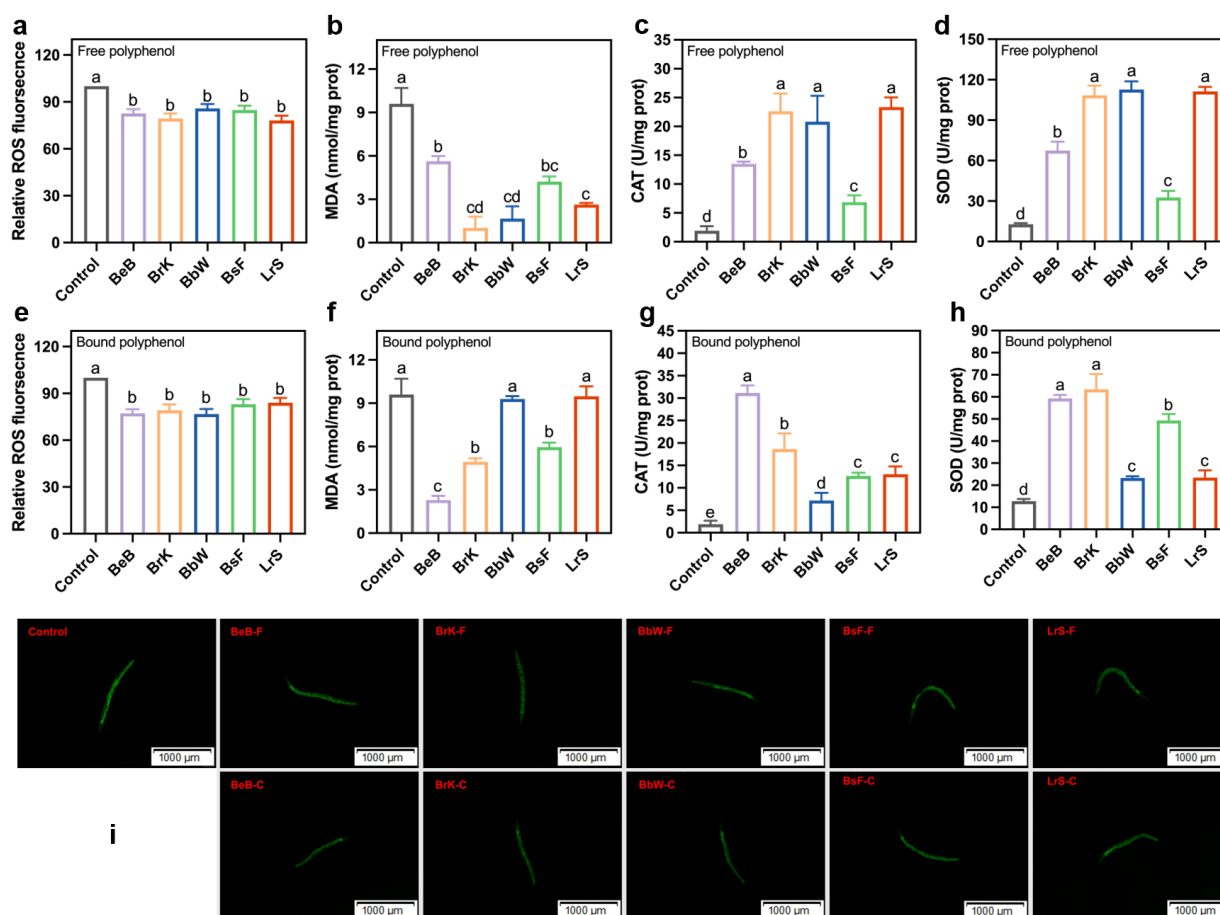


Fig.4 Effects of *Boletus edulis* polyphenol extracts on in vivo antioxidant and stress-related indicators in *C. elegans*: ROS Level (a, e), MDA Content (b, f), CAT Activity (c, g), SOD Activity (d, h) and fluorescence microscopy images of ROS distribution in nematodes (i).

Consistent with this, *Boletus* polyphenol extracts significantly decreased MDA levels compared to the control group (9.601 nmol/mg), indicating strong protection against lipid peroxidation (**Fig. 4b** and **4f**). BrK-F showed the most pronounced effect (1.016 nmol/mg), followed by BbW-F (1.800 nmol/mg) and LrS-F (2.318 nmol/mg), with BsF-F and BeB-F also exerting moderate reductions. Bound phenolics similarly reduced MDA levels, with BeB-B decreasing MDA to 2.291 nmol/mg, and BrK-B showing a moderate effect (4.938 nmol/mg), underscoring the ability of both free and bound forms to mitigate lipid oxidative damage despite their differing bioavailability^[3,33]. These findings confirm that *Boletus* polyphenols, particularly from BrK and BeB, effectively protect cellular membranes from oxidative damage, supporting their potential in oxidative stress-related interventions^[11,13].

To further evaluate antioxidant mechanisms, CAT and SOD activities were measured (**Fig. 4c, d, g, h**). In the control group, CAT activity was 1.907 U/mg. Among free phenolics, LrS-F exhibited the highest activity (23.311 U/mg), followed by BrK-F (22.615 U/mg) and BbW-F (20.797 U/mg), corresponding well

with their ROS and MDA-reducing capacities and suggesting that CAT upregulation is a key mechanism^[11]. In the bound group, BeB-B showed the most pronounced increase (31.144 U/mg), over 15-fold higher than the control, while BrK-B also significantly enhanced CAT activity (18.558 U/mg), highlighting the capacity of bioavailable bound phenolics to strengthen oxidative defense^[34].

SOD activity analysis supported this trend, with BbW-F (63.150 U/mg), LrS-F (61.282 U/mg), and BrK-F (59.481 U/mg) showing the strongest effects among free phenolics. BeB-B (54.080 U/mg) and BrK-B (49.870 U/mg) also significantly increased SOD levels compared to the control (10.324 U/mg), indicating meaningful activation of enzymatic antioxidant systems even by bound compounds^[35]. These findings indicate that both free and bound bolete polyphenol extracts can enhance endogenous antioxidant enzyme activities under the present experimental conditions, with LrS-F, BbW-F, and BeB-B demonstrating the most consistent upregulation of CAT and SOD. Notably, the *in vivo* antioxidant responses did not completely parallel the *in vitro* antioxidant ranking. BsF-B exhibited the highest FRAP value *in vitro*, but it did not produce the greatest lifespan extension. This suggests that strong chemical reducing power alone is insufficient to predict organismal longevity. Conversely, BbW-B showed the most pronounced lifespan extension, although its effects on individual antioxidant biomarkers such as CAT and SOD were not always the strongest. This apparent discrepancy may be explained by the fact that lifespan extension is an integrated physiological outcome rather than a single antioxidant endpoint. In *C. elegans*, longevity is regulated by multiple factors, including redox balance, metabolic adaptation, stress-response signaling, reproductive allocation, and hormetic responses. Therefore, BbW-B may extend lifespan through coordinated regulation of oxidative stress and longevity-associated pathways rather than through maximal activation of each antioxidant enzyme. These results indicate that chemical antioxidant capacity, biochemical antioxidant markers, and lifespan extension represent different but complementary layers of biological evaluation.

3.6 Correlation analysis

To better understand the relationship between specific polyphenols and antioxidant performance, we conducted a correlation analysis combining *in vitro* antioxidant capacity and *in vivo* indicators (**Fig. 5**). Several compounds, particularly 3,4-dihydroxybenzoic acid, ferulic acid, and 3,5-dihydroxybenzoic acid, exhibited strong positive correlations with DPPH ($r = 0.82, 0.79, 0.65$), FRAP, and ROS, MDA, CAT and SOD activities in *C. elegans*, suggesting that these phenolics may be key contributors to the observed antioxidant effects. Notably, 3,4-dihydroxybenzoic acid correlated strongly with CAT ($r = 0.78$) and SOD ($r = 0.75$), and ferulic acid showed significant inverse correlations with MDA ($r = -0.73$) and ROS ($r = -0.76$), indicating their role in enzymatic defense and lipid peroxidation inhibition. These compounds were enriched in BrK-F, BbW-F, and BeB-B, which also showed strong biological performance across ROS, MDA, and antioxidant enzyme assays.

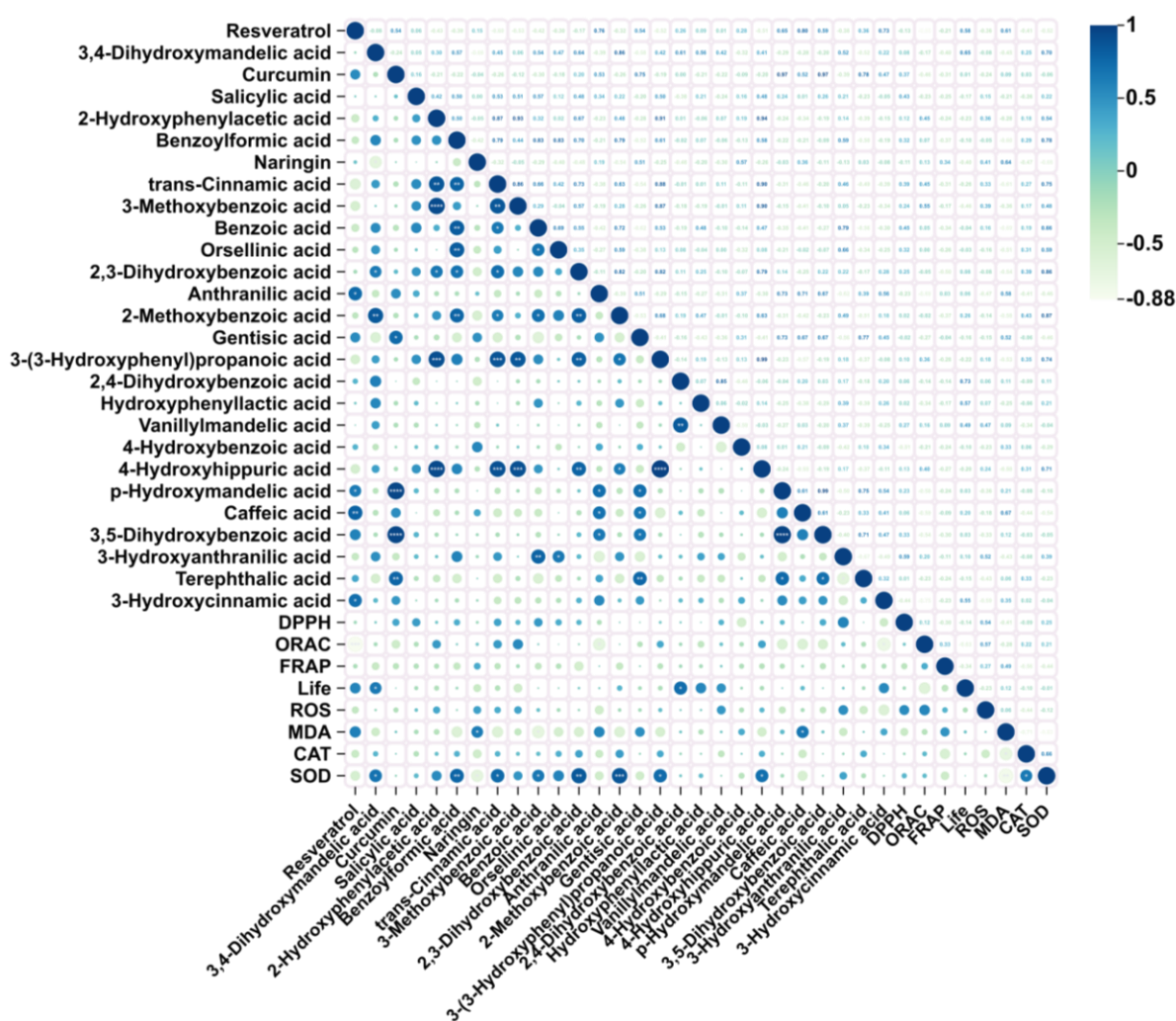


Fig.5 Correlation analysis between several polyphenols and antioxidant activities in vivo and in vitro. The size of each circle corresponds to the strength of the correlation, and statistical significance is denoted by asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

For instance, BrK-F contained high levels of 3,4-dihydroxybenzoic acid and showed the lowest MDA content (1.02 nmol/mg), high CAT and SOD activities, and effective ROS reduction. Similarly, BbW-F, rich in ferulic acid and phenylpyruvic acid, demonstrated the highest ORAC (26.05 $\mu\text{mol TE/mg}$) and strong enzymatic antioxidant activity. BeB-B, despite being a bound extract, exhibited high CAT activity (31.14 U/mg) and was associated with higher levels of ferulic acid and 3-hydroxycinnamic acid, indicating its sustained antioxidant potential upon release.

These correlations highlight the biological relevance of specific phenolic compounds and support the antioxidant performance observed in representative samples. Overall, the results suggest that not all phenolics contribute equally to antioxidant activity, and the presence of specific polyphenols, especially dihydroxybenzoic acid derivatives and ferulic acid, plays a key role in determining the antioxidant effectiveness of different *Boletus* extracts in vivo.

3.7 Mechanistic insights into the antioxidant and anti-aging effects of *Boletus* polyphenols

To elucidate the underlying mechanisms of the antioxidant and anti-aging effects observed in the preceding experiments, two representative polyphenol fractions were selected for in-depth investigation.

These were the free phenolic extract from LrS-F and the bound phenolic extract from BeB-B, both of which demonstrated superior antioxidant activities and significant lifespan extension in *C. elegans*.

3.7.1. Effect of *Boletus* polyphenols on lifespan of wild-type and mutant *C. elegans* strains

To determine whether the lifespan-extending effects of LrS-F and BeB-B involve conserved longevity-related signaling pathways, lifespan assays were performed in *C. elegans* wild-type (N2) and two mutant strains, *daf-2* (e1370) and *daf-16* (mu86). As shown in **Fig. 6a–c**, both LrS-F and BeB-B significantly extended the lifespan of N2 worms compared to controls ($P < 0.01$). The mean lifespan was increased by approximately 24–27% following treatment.

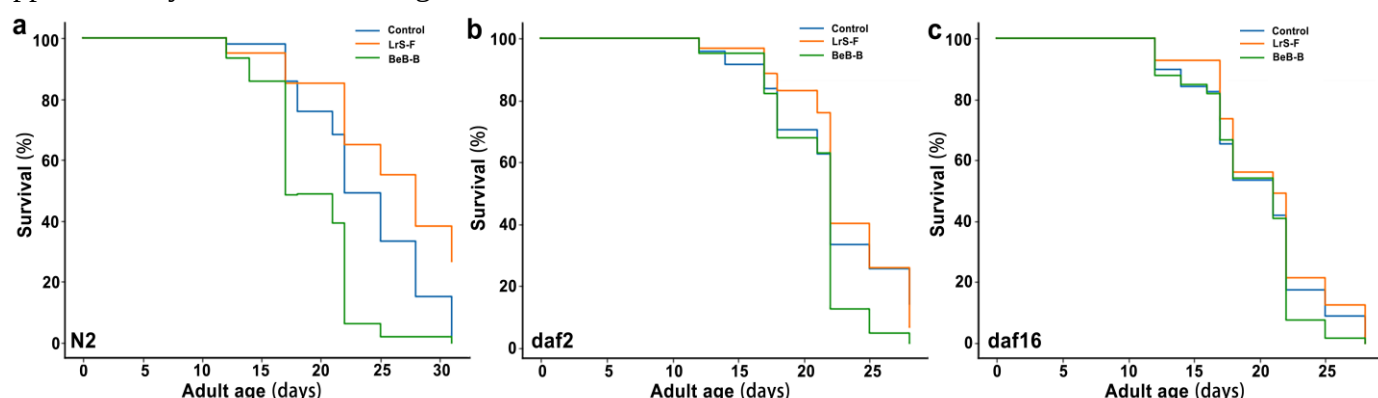


Fig. 6 Effects of *Boletus* polyphenol extracts on lifespan of *Caenorhabditis elegans* strains. Lifespan (a) wild-type N2, (b) mutant *daf-2* (e1370), and (c) mutant *daf-16* (mu86).

In contrast, the lifespan-extending effects of LrS-F and BeB-B were markedly diminished in *daf-2* mutants and completely abolished in *daf-16* mutants, suggesting that their activity depends on an intact insulin/IGF-1-like signaling (IIS) pathway, particularly the downstream transcription factor *daf-16*.

DAF-2 encodes an insulin/IGF-1 receptor homolog that inhibits *daf-16* via AKT-mediated phosphorylation under nutrient-rich conditions. Inhibition of *DAF-2* signaling, or activation of stress responses, allows *daf-16* to translocate to the nucleus and activate genes involved in longevity, oxidative defense, and detoxification processes^[12]. The absence of any lifespan extension in *daf-16* mutants indicates that *daf-16* is essential for mediating the effects of LrS-F and BeB-B. Similar *daf-16* dependence has been observed with other polyphenols^[36], suggesting that *Boletus* polyphenols may exert conserved anti-aging actions through modulation of this pathway.

3.7.2 *Boletus* polyphenols enhance endogenous antioxidant enzyme activities

To further evaluate whether the lifespan-extending effects of *Boletus* polyphenols are functionally linked to oxidative stress defense, we measured the activities of three key endogenous antioxidant enzymes - SOD, CAT, and total antioxidant capacity (T-AOC) - in *C. elegans* N2, *daf-2*, and *daf-16* strains. As shown in **Fig. 7 a–c**, both LrS-F and BeB-B treatments significantly increased SOD, CAT, and T-AOC activities in wild-type worms ($P < 0.01$), consistent with a robust enhancement of endogenous antioxidant defenses.

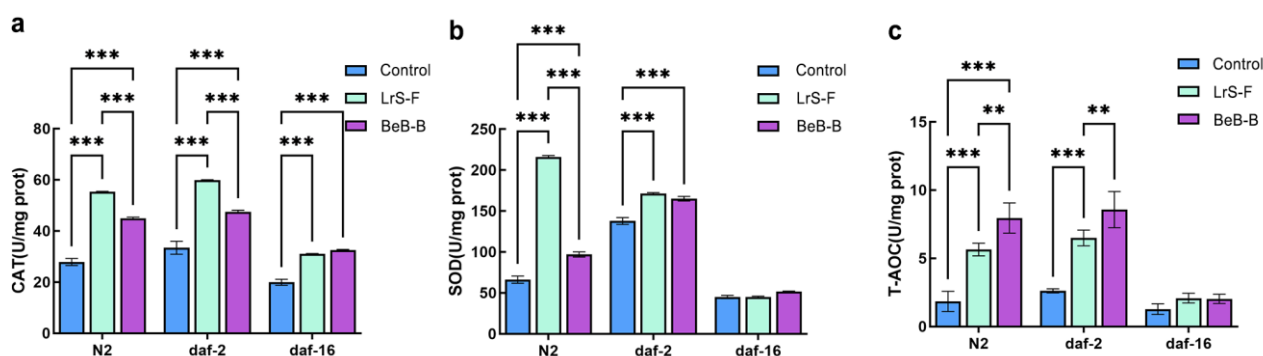


Fig.7 Polyphenol extracts enhance antioxidant enzyme activities in *C. elegans*. CAT (a), SOD (b), and T-AOC (c).

Similar increases were also observed in the *daf-2* mutants, though the magnitude of elevation was slightly reduced. In contrast, no significant enzymatic changes were detected in *daf-16* mutants following LrS-F or BeB-B treatment, indicating that *daf-16* is essential for mediating the polyphenol-induced activation of antioxidant enzymes.

These results provide functional evidence that *Boletus* polyphenols confer oxidative stress resistance through upregulation of enzymatic antioxidant systems, which likely contributes to reduced ROS accumulation and delayed aging. The dependence on *daf-16* is consistent with its established role in regulating genes encoding phase II detoxifying enzymes and oxidative defense proteins^[17,37]. Notably, the retention of partial enzyme activation in *daf-2* worms suggests that polyphenol-induced antioxidant activation can bypass upstream insulin signaling inhibition, but remains strictly dependent on the downstream transcriptional activator *daf-16*.

3.7.3 Effects of *Boletus* polyphenols on gene expression in *C. elegans*

To investigate the transcriptional regulation underlying the antioxidant and longevity-promoting effects of *Boletus edulis* polyphenols, qRT-PCR was conducted to assess the expression of key stress- and aging-related genes in wild-type (*C. elegans* N2) and *daf-16* mutant strains. As shown in **Fig. 8a**, both LrS-F and BeB-B treatments significantly upregulated the expression of *sod-3*, *hsp-16.2*, *jnk-1*, and *sir-2.1* in wild-type worms compared to the control group ($p < 0.05$). *sod-3*, a well-established *daf-16* target gene encoding the mitochondrial antioxidant enzyme SOD-3, exhibited the most pronounced induction, especially under BeB-B treatment, consistent with enhanced reactive oxygen species (ROS) detoxification. Similarly, *hsp-16.2*, encoding a small heat shock protein, and *sir-2.1*, a gene involved in stress resistance and lifespan regulation, were markedly elevated. Moderate but significant increases were also observed in the expression of *daf-16*, *jnk-1*, and *pmk-1*, suggesting potential involvement of the MAPK signaling cascade.

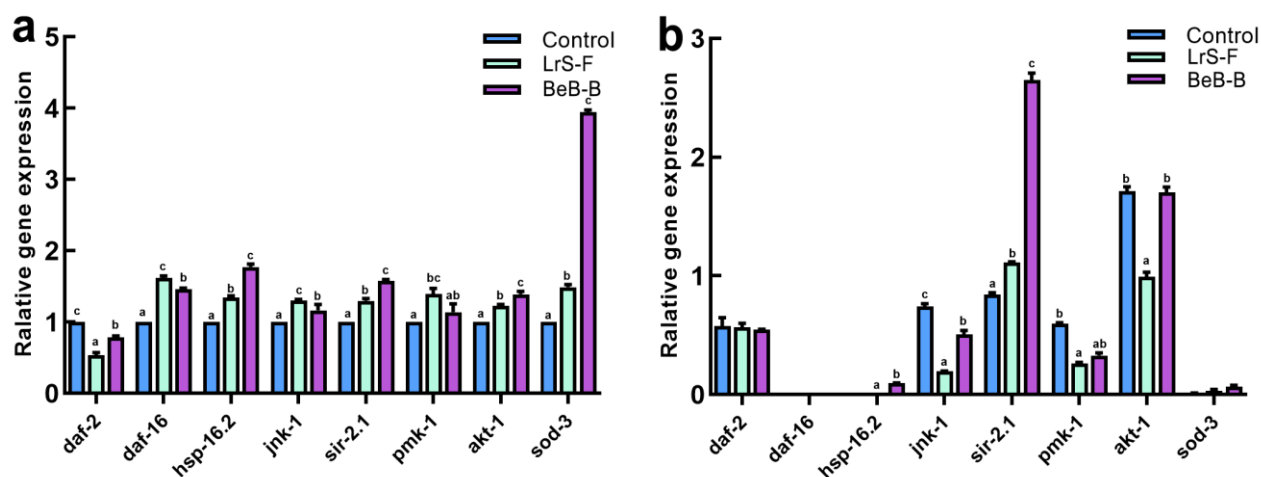


Fig. 8 Relative mRNA expression of stress- and longevity-related genes in *C. elegans*. (a) Gene expression in N2 worms; (b) gene expression in *daf-16* mutants. Expression levels in (b) were normalized to the untreated N2 group to facilitate comparison between wild-type and mutant backgrounds.

In *daf-16* mutants (**Fig. 8b**), the expression of *sod-3* was nearly abolished and did not respond to either treatment, confirming its strict dependence on DAF-16 for transcriptional activation. However, the expression levels of *jnk-1*, *sir-2.1*, and *pmk-1* still showed moderate induction following polyphenol exposure, implying that *Boletus* polyphenols may also activate stress response pathways independent of DAF-16, such as those involving JNK-1 and PMK-1.

Collectively, these results suggest that both free (LrS-F) and bound (BeB-B) polyphenol fractions modulate a network of longevity-related genes. While many of the observed effects are mediated through the DAF-16 axis, parallel activation of DAF-16-independent pathways may also contribute to the overall antioxidant and pro-longevity effects. These findings are consistent with previous research indicating that polyphenols can modulate both *daf-16*-dependent and -independent gene networks to promote oxidative stress resistance and lifespan extension in *C. elegans*^[36].

3.7.4 *Boletus* polyphenols activate antioxidant signaling at the protein level

To further validate the molecular effects of *Boletus* polyphenols, Western blot analysis was performed to assess the expression of key signaling proteins related to oxidative stress and longevity in *C. elegans*. As shown in **Fig. 9a–d**, both LrS-F and BeB-B treatments led to notable changes in several protein targets.

DAF-16 protein levels were significantly elevated in both treatment groups compared to the wild-type control (**Fig. 9a, b**), in agreement with the qRT-PCR results showing transcriptional upregulation of *daf-16* (**Fig. 8a**). DAF-2 expression exhibited a slight decrease following treatment, consistent with a possible release of inhibitory signaling on DAF-16 via the insulin/IGF-1 pathway^[12,38]. As expected, DAF-16 protein was undetectable in the *daf-16* mutant strain. HSP-16.2 expression was strongly upregulated by both LrS-F and BeB-B, with a more pronounced effect in the BeB-B group (**Fig. 9c**), echoing the transcriptional increase observed in **Fig. 8a**. Notably, HSP-16.2 protein was absent in the *daf-16* mutant, confirming its status as a DAF-16-dependent target. JNK-1 levels also increased in both treatment groups (**Fig. 9c**), consistent with qPCR data showing transcriptional upregulation of *jnk-1* (**Fig. 8a–b**). Importantly, this induction was preserved in *daf-16* mutants, suggesting that JNK-1 activation occurs via a DAF-16-independent route, possibly mediated by stress-responsive MAPK cascades^[39].

SKN-1 protein levels were significantly elevated following both LrS-F and BeB-B treatment (**Fig. 9d**), indicating activation of SKN-1-mediated oxidative stress response pathways. This response was retained in *daf-16* mutants, suggesting that SKN-1 may act independently or in parallel with DAF-16, potentially through PMK-1/p38 MAPK signaling^[17]. SIR-2.1 protein expression was mildly but significantly elevated in BeB-B-treated worms (**Fig. 9d**), corroborating the moderate transcriptional upregulation observed in both wild-type and *daf-16* backgrounds (**Fig. 8a–b**). These results suggest that LrS-F and BeB-B may regulate oxidative stress resistance through both *daf-16*-associated responses and additional JNK-1/SKN-1-related pathways^[40].

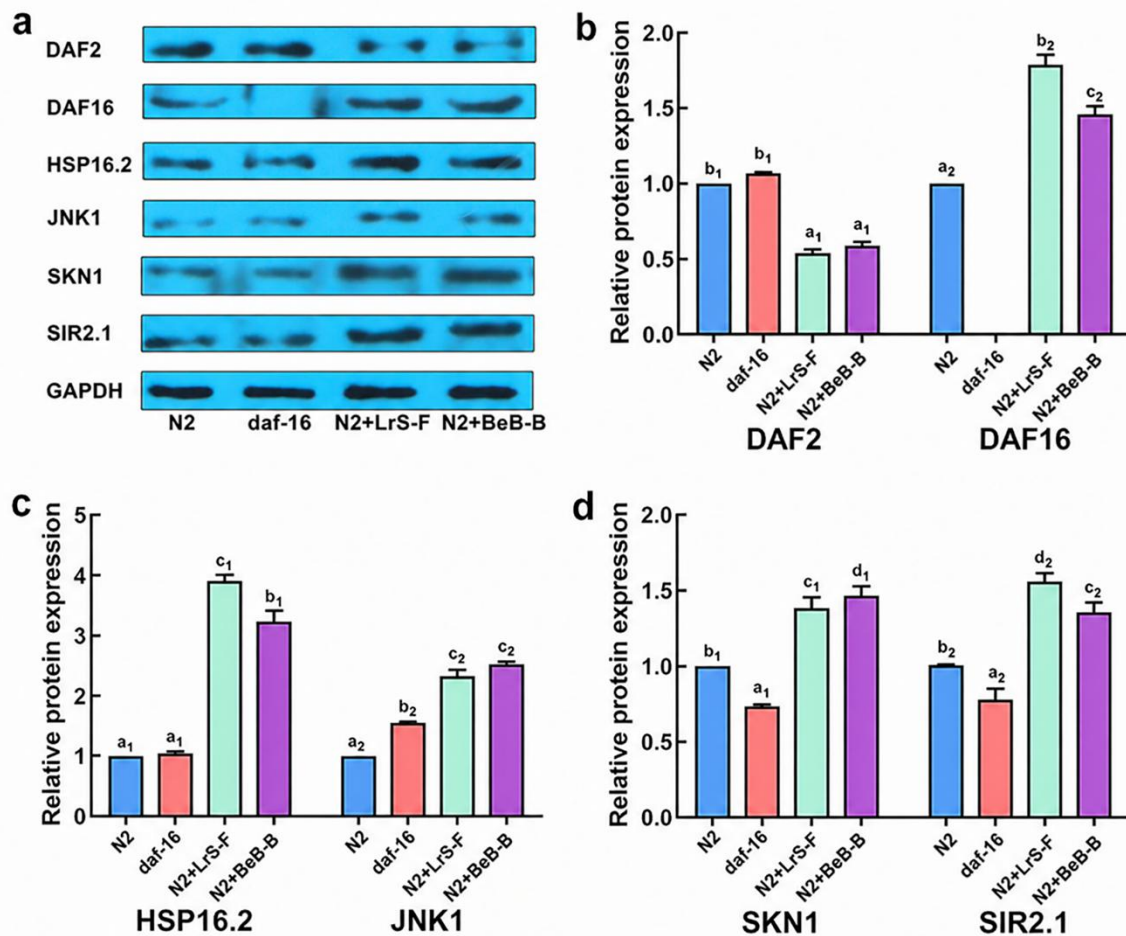


Fig.9 Effects of *Boletus* polyphenols on protein expression in *C. elegans*. (a) Western blot analysis of DAF-2, DAF-16, HSP-16.2, JNK-1, SKN-1, and SIR-2.1 proteins in N2, *daf-16*, and N2 worms treated with LrS-F or BeB-B; Relative quantification of DAF-2 and DAF-16 (b), HSP-16.2 and JNK-1 (c) and SKN1 and SIR2.1 (d) protein expression.

Taken together, these findings suggest that LrS-F and BeB-B modulated stress- and longevity-related targets at both transcriptional and protein levels. The reduced responsiveness of *sod-3* and *hsp-16.2* transcripts in *daf-16* mutants supported the involvement of DAF-16-dependent transcriptional regulation, whereas the maintained induction of JNK-1 and SKN-1 proteins suggested possible participation of additional DAF-16-independent stress-response pathways. However, these results mainly reflect downstream expression changes and do not fully resolve upstream signaling events. In addition, although *C. elegans* is a useful model for evaluating oxidative stress resistance and lifespan regulation, it cannot fully represent mammalian digestion, absorption, metabolism, or tissue-specific responses. The present findings should therefore be

interpreted as preliminary in vivo evidence, and future studies should include bioaccessibility analysis, broader *Boletus edulis* resources, and mammalian cell or animal models to further validate the physiological relevance of *B. edulis* polyphenols.

4. Conclusion

This study revealed marked differences in the composition and biological activities of free and bound polyphenol extracts from five wild edible *Boletus* samples. Among the tested fractions, LrS-F and BeB-B showed relatively strong overall antioxidant and lifespan-promoting effects, as indicated by reduced intracellular ROS and MDA levels, enhanced CAT and SOD activities, and extended lifespan in *C. elegans*. Further mutant-based, transcriptional, and protein-level analyses suggested that these effects were associated with the regulation of stress-response pathways. In particular, the reduced responsiveness of *sod-3* and *hsp-16.2* transcripts in *daf-16* mutants supported the involvement of DAF-16-dependent transcriptional regulation, whereas the maintained induction of JNK-1 and SKN-1 proteins indicated possible participation of additional DAF-16-independent responses. Overall, these findings provide comparative evidence that *B. edulis* polyphenols can modulate oxidative stress-related responses in *C. elegans* and may serve as natural antioxidant resources for further functional food research.

Acknowledgments

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