



Antiaging effects of *Amaranthus tricolor* extract in H₂O₂-induced yeast model, *Schizosaccharomyces pombe*

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ABSTRACT

Oxidative stress accelerates aging and contributes to various degenerative diseases. *Amaranthus tricolor* (red spinach) is known for its strong antioxidant properties. However, its cellular mechanisms in modulating aging remain unclear. This study utilized the fission yeast *Schizosaccharomyces pombe* as a model to evaluate the protective and pro-longevity effects of ethanol-derived *A. tricolor* leaf extract. Treatment with 49 µg/ml extract enhanced yeast survival under H₂O₂-induced oxidative stress and extended chronological lifespan compared to untreated and calorie-restriction controls. The extract also increased mitochondrial membrane potential (ΔΨ_m), as assessed by Rhodamine staining. Flow cytometry revealed an elevated proportion of cells in the G0/G1 phase, indicating delayed cell cycle progression. Gene expression analysis showed upregulation of aging-related (*sir2*⁺) and oxidative stress-response genes (*sod2*⁺, *ctl1*⁺). Liquid chromatography-high resolution mass spectrometry profiling identified major metabolites, including (2E)-3-(3,4-dimethoxyphenyl)acrylic acid, 2-O-caffeoylglucaric acid, and (10E,15Z)-9,12,13-trihydroxy-10,15-octadecadienoic acid. Overall, the findings demonstrate that *A. tricolor* extract enhances oxidative stress resistance and promotes cellular longevity in yeast, supporting its potential as a natural source for nutraceutical development.

1. INTRODUCTION

Aging is an intrinsic biological process driven by the gradual accumulation of cellular damage, leading to the progressive loss of homeostasis and function [1]. One of the major contributors to aging is the overproduction of reactive oxygen species (ROS), which disrupts redox balance and causes oxidative damage to DNA, proteins, and lipids [2]. Excessive ROS also triggers cellular senescence by inhibiting cell proliferation [3]. Antioxidants mitigate these effects by neutralizing ROS and maintaining cellular redox homeostasis [4]. However, endogenous antioxidant defenses are often inadequate, underscoring the importance of identifying plant-derived antioxidant sources that can support cellular protection

and delay oxidative stress-induced aging. A promising approach to counteract oxidative stress-induced aging is the use of natural antioxidants or antiaging compounds.

Amaranthus tricolor L., commonly known as red spinach, is a fast-growing leafy vegetable with a C₄ photosynthetic pathway. It is widely cultivated across Asia, Africa, Australia, and the Americas [5]. The leaves, stems, and seeds of this plant are edible in both raw and cooked forms, making significant contributions to dietary nutrition. This affordable crop is rich in proteins (14.25%), dietary fiber (8.18%), lipids (4.04%), and essential minerals such as magnesium, calcium, potassium, phosphorus, and iron [6–9]. Its red pigmentation originates from bioactive compounds, including betalains, betacyanins, betaxanthins, carotenoids, and anthocyanins, which exhibit strong antioxidant properties [10,11].

Previous studies have shown that *A. tricolor* possesses potent antioxidant activity, with an IC₅₀ value of 19.42 ± 0.91 µg/ml against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. It also demonstrates antibacterial effects at 63 µg/ml against

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Escherichia coli, *Penicillium oxalicum*, and *Staphylococcus aureus*, outperforming tetracycline at 250 µg/ml [12]. In addition, *A. tricolor* hydroalcoholic leaf extract has shown neuroprotective effects in scopolamine-induced and other neurodegenerative rat models, improving memory, stress tolerance, and biochemical markers in a dose-dependent manner [13]. *Amaranthus tricolor* also exhibits anti-inflammatory, nitric oxide-scavenging, and anticancer properties, with IC_{50} values of 72.49 ± 3.38 , 88.13 ± 3.60 , and 96.12 ± 6.20 µg/ml, respectively [14]. These findings have confirmed that *A. tricolor* has been shown to possess various pharmacological effects, such as antioxidant, antimicrobial, neuroprotective, anti-inflammatory, and anticancer activities.

Despite extensive reports on its antioxidant potential, the cellular mechanisms underlying the biological activity of *A. tricolor* remain unclear. Yeast models provide a useful approach for such studies. *Schizosaccharomyces pombe* is a well-established eukaryotic model for aging research, often described as a “micromammal” due to its similarities with higher organisms. This fission yeast divides symmetrically, grows optimally at 25°–36°C, and completes a generation cycle within 2–4 hours [15]. Notably, its mitochondrial DNA closely resembles that of humans in size, structure, and gene content, making it an excellent system for studying mitochondrial function and cellular aging [16]. *Schizosaccharomyces pombe* exhibits several conserved mechanisms that regulate cellular aging through oxidative stress response pathways. Many of the molecular mechanisms involved in aging in *S. pombe* are homologous to those in multicellular eukaryotes, including humans [17]. These include the involvement of sirtuin proteins, mitogen-activated protein kinase (MAP kinase), autophagy, and mitochondrial activity [18,19]. Furthermore, calorie restriction is a conserved mechanism across yeasts and humans [20,21]. It has been reported that calorie restriction can extend the lifespan of *S. pombe* by suppressing the Target of Rapamycin (TOR) pathway and enhancing the regulation of the SIR2 histone deacetylase pathway [22]. These interconnected pathways promote mitochondrial activity, which subsequently enhances the expression of antioxidant enzymes such as superoxide dismutase (*sod2*) and catalase (*ctl1*), thereby increasing cellular resistance to ROS [23].

We hypothesize that the *A. tricolor* leaf extract enhances cellular longevity in *S. pombe* by increasing mitochondrial activity and activating conserved oxidative-stress response pathways, including the upregulation of *sir2*⁺, *sod2*⁺, and *ctl1*⁺, as well as promoting G0/G1 cell-cycle arrest. Therefore, this study aimed to evaluate the antioxidant and antiaging effects of *A. tricolor* extract in *S. pombe* and to identify phytochemical constituents potentially associated with these biological activities.

2. MATERIALS AND METHODS

2.1. Sample preparation

Leaves of *A. tricolor* were collected from the Agribusiness and Technology Park, IPB University, Bogor, Indonesia (GPS coordinates: 6°32'53.885"S, 106°43'58.712"E) at 25–30 days after planting, with an average height of 30 cm. The species was taxonomically identified and deposited at the

Herbarium Bandungense (Herbarium Code: 933/IT1.C11.2/TA.00/2024).

2.2. Sample extraction

Extraction was performed using a modified remaceration method [24]. Fresh leaves were washed, oven-dried at 50°C for 24 hours, ground, and sieved through an 80-mesh filter. A total of 20 g of powder was extracted with 50% ethanol (1:10 w/v) for 30 hours and then concentrated using a rotary evaporator at 60°C.

2.3. Antioxidant activity assay

Antioxidant capacity was determined using the DPPH method [25]. The extract (31.25–250 µg/ml) was dissolved in distilled water, and 100 µl of each solution was mixed with 100 µl of 125 µM DPPH (Himedia) (prepared by dissolving 2.465 mg in 50 ml of 96% ethanol) in a 96-well microplate. Ascorbic acid (Merck) served as the positive control. The reaction mixtures were incubated for 30 minutes at room temperature (27°C) in the dark, and absorbance was measured at 517 nm using an ELISA microplate reader (Epoch BioTek). IC_{50} values were calculated from regression analysis of inhibition percentages.

2.4. Yeast culture preparation

Schizosaccharomyces pombe strain ARC039 (*h⁻ leu1-32 ura4-294*) was used as a model organism [26]. Cells were grown in YES medium containing 3% glucose. For calorie restriction, YES medium with 0.3% glucose was used.

2.5. Spot assay for antiaging and oxidative tolerance assays

Antiaging activity was evaluated using a spot assay method [27]. Overnight cultures were grown in YES medium at 30°C, 150 rpm, and then transferred to fresh YES medium containing *A. tricolor* extract at 0.5×, 1×, 2.5×, and 5× IC_{50} concentrations (initial $OD_{600} = 0.05$). Untreated cultures (extract-free) served as the negative control, while yeast grown under calorie-restricted conditions served as the positive control. Cultures were incubated at 30°C, 150 rpm, and sampled on days 1, 7, and 11. Each culture was adjusted to $OD_{600} = 1$, serially diluted, and 2.5 µl of each dilution was spotted onto YES agar (3% glucose). Plates were incubated at 30°C for 72 hours. For oxidative stress tolerance, cells from day 1 cultures were spotted onto YES agar containing H₂O₂ (1, 2, and 3 mM) following serial dilutions (up to 10⁻⁴). Plates were incubated at 30°C for 3 days [26].

2.6. Mitochondrial activity assay

Mitochondrial activity was evaluated in *S. pombe* cells using Rhodamine B, a cationic fluorescent dye that accumulates in active mitochondria in a membrane potential-dependent manner, followed by fluorescence microscopy [28]. Cultures were incubated for 18 hours and pelleted by centrifugation. Cells were washed with 0.1 M phosphate-buffered saline (PBS, pH 7.4), stained with 100 nM Rhodamine B (Sigma-Aldrich), and incubated for 30 minutes before observation under a fluorescence microscope (Olympus BX51). Untreated cultures (extract-free) served as the negative control, while yeast grown

under calorie-restricted conditions served as the positive control to compare mitochondrial fluorescence intensity.

2.7. Cell cycle analysis

Cell cycle distribution was measured with flow cytometry [29]. Cells were treated with 49 and 97 $\mu\text{g/ml}$ of *A. tricolor* extract and cultured in YES medium (3% glucose) for 48 hours. Cells without extract treatment were used as the control. Pellets obtained by centrifugation (4,000 rpm, 15 minutes) were washed with PBS (0.1 M, pH 7.4) and fixed in 300 μl PBS with 800 μl of 96% ethanol at 4°C for 16 hours. Fixed cells were washed twice with PBS 1X, added RNase (110,000 U. ml^{-1}) and stained with 50 μl propidium iodide (1 mg/ml) for 20 minutes in the dark. Samples were analyzed using flow cytometry (BD FACSCalibur™) with the FL2-H channel (excitation 535 nm, emission 617 nm). At least 15,000 events were collected per sample after excluding debris based on forward and side scatter parameters. Gating was applied to the main population to ensure analysis of single-cell events. Cell cycle distribution (G0/G1, S, and G2/M phases) was determined using BD CellQuest Pro software.

2.8. Gene expression analysis

Gene expression was analyzed using quantitative reverse transcription PCR (qRT-PCR) [21]. *Amaranthus tricolor* extracts at the optimal concentrations (49 and 97 $\mu\text{g/ml}$) were added to *S. pombe* cultures grown in liquid YES medium with an initial OD600 of 0.05, and incubated for 24 hours at 27°C with shaking (300 rpm). Untreated yeast cultures were used as the control. For oxidative stress induction, 1 mM H_2O_2 was added to the culture 1 hour prior to harvesting. Cells were collected by centrifugation at 12,000 $\times g$ for 1 minute, and total RNA was extracted using the RNA Plant Kit Plus (Tiangen, China). cDNA was synthesized from 500 ng RNA using the ReverTra Ace™ qPCR RT Kit (Toyobo, Japan). qRT-PCR was performed using the QuantStudio™ 5 Real-Time PCR System (Thermo Fisher, USA) and Thunderbird SYBR® qPCR Master Mix (Toyobo, Japan). The amplification program consisted of 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Primers targeting oxidative stress-related genes (*sod2*⁺, *ctt1*⁺), the cell-cycle regulator (*sir2*⁺), and the reference gene (*act1*⁺) are listed in Table 1. The fold changes ($2^{-\Delta\Delta\text{Ct}}$) of the targeted gene expression (*sod2*⁺, *ctt1*⁺, and *sir2*⁺) were quantified by comparing Ct values of the target genes in treatment sample relative to control sample, and normalized to a reference gene (*act1*⁺).

2.9. Liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis

Extract *A. tricolor* metabolites profiling was carried out using LC-HRMS [29]. Separation was performed on a UHPLC Vanquish Tandem Q Exactive Plus Orbitrap system (Thermo Scientific) with an Accucore C18 column (1.5 μm , 2.1 \times 100 mm). The mobile phase consisted of (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid under a gradient of 0–1 minute (5% B), 1–25 minutes (5–95% B), 25–28 minutes (95% B), and 28–30 minutes (5% B). The column temperature was maintained at 30°C with a 2 μl injection volume, 100–1500

Table 1. qRT-PCR primers used in this study.

Type of modulation	Target gene	Primer
Reference gene [17]	<i>act1</i> ⁺ (<i>Actin</i>)	Forward (F): 5'CGGTCGTGACTTGACTGACT 3'
		Reverse (R): 5' ATTCACGTTTCGGCGGTAGT 3'
Oxidative stress response gene [17]	<i>sod2</i> ⁺ (<i>Superoxide dismutase 2</i>)	Forward (F): 5' ATTTGGAGGGAGAGGTTGCC 3'
		Reverse(R): 5' GATTGATGTGACCACCGCCA 3'
Cell cycle [21]	<i>ctt1</i> ⁺ (<i>Catalase</i>)	Forward (F): 5' TCGTGACGGCCCTATGAATG 3'
		Reverse(R): 5' AGCAAGTGGTCGGATTGAGG 3'
Cell cycle [21]	<i>Sir2</i> (Sirtuin 2)	Forward (F): 5'ACTCCTGTTTCGCATACCCA 3'
		Reverse(R): 5'ACGTTTACAAATCTCCACAATAACC 3'

m/z scan range, and positive ion mode. Spectral data were collected in data-dependent acquisition mode to obtain both MS¹ and MS² fragmentation spectra. Methanol was used as the blank solvent to subtract background ions. Data processing was carried out using Compound Discoverer™ 3.2 (Thermo Fisher Scientific) with library matching against mzCloud, ChemSpider, PubChem, and HMDB databases. Compound annotations were based on accurate mass (< 5 ppm error), isotopic pattern, and MS² fragmentation matching. Relative abundances were estimated from total ion chromatogram peak areas.

2.10. Statistical analysis

Data from the antioxidant (DPPH) and gene expression assays are expressed as means \pm standard errors of the mean ($n = 3$), while data from the antiaging and oxidative stress assays, mitochondrial activity assays, and cell cycle analysis were obtained in duplicate. Statistical significance among groups was assessed using one-way analysis of variance at a 95% confidence level. Post hoc comparisons were conducted using Duncan's multiple range test (DMRT). Differences were considered statistically significant at $p \leq 0.05$. All statistical analyses were performed using R software (v 4.3.1, Beagle Scouts).

3. RESULTS

3.1. Antioxidant activity of *A. tricolor* extract *in vitro*

The antioxidant activity was evaluated *in vitro* using the DPPH assay. The leaf extract of *A. tricolor* exhibited strong antioxidant potential, with an IC₅₀ value of $97.196 \pm 4.35 \mu\text{g/ml}$, compared with $2.201 \pm 0.03 \mu\text{g/ml}$ for ascorbic acid. This IC₅₀

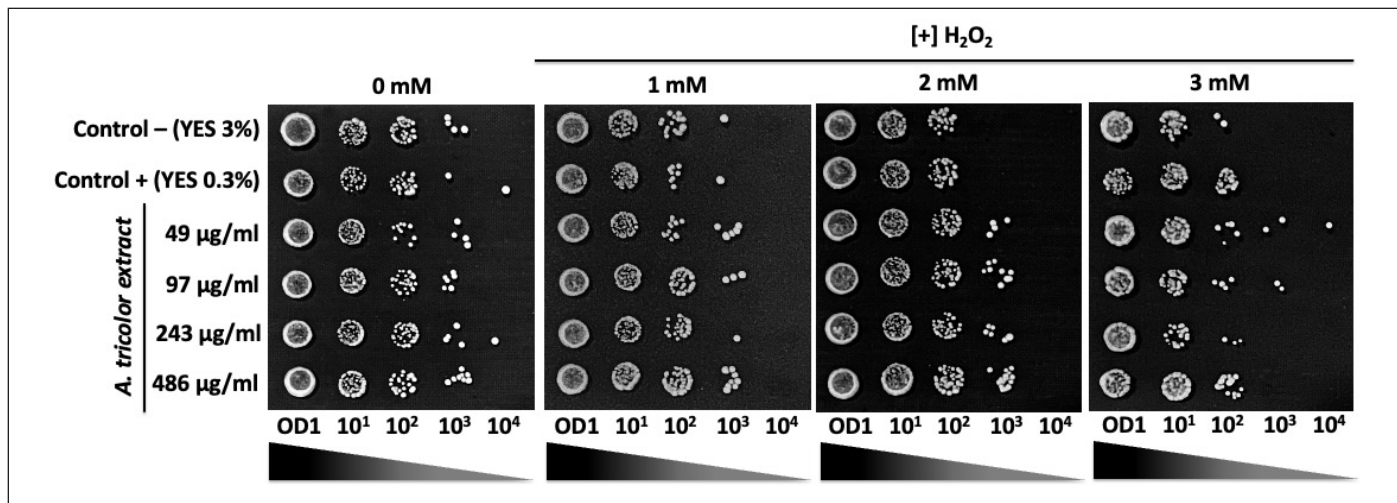


Figure 1. The effect of ethanol derived-*A. tricolor* extracts on the viability of yeast *S. pombe* toward 1, 2 and mM of H_2O_2 -induced oxidative stress treatment. Yeast grown in YES medium with normal glucose concentration (3% w/v) without extract supplementation is designated as negative control. Yeast grown in YES medium with low glucose concentration (0.3%w/v) or calorie restriction treatment without extract supplementation is assigned as positive control..

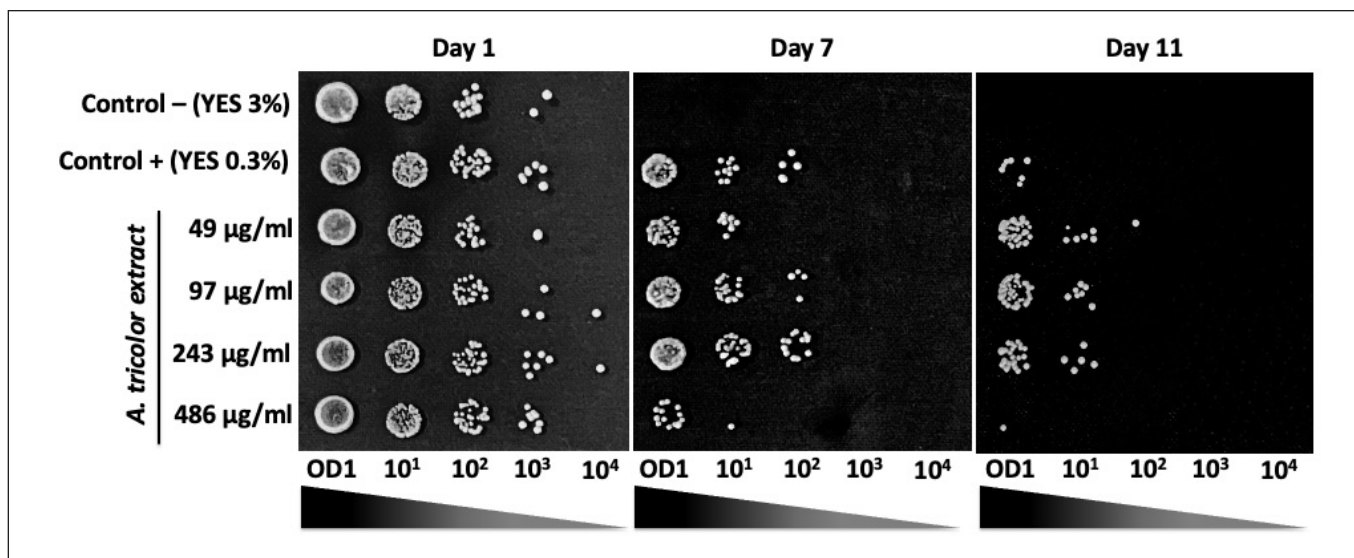


Figure 2. The effect of ethanol derived-*A. tricolor* extracts on the life span of yeast *S. pombe* for up to 11 days of incubation. Yeast grown in YES medium with normal glucose concentration (3%w/v) without extract supplementation is designated as negative control. Yeast grown in YES medium with low glucose concentration (0.3%w/v) or calorie restriction treatment without extract supplementation is assigned as positive control.

value was subsequently employed as a reference concentration for cellular-level applications in *S. pombe*.

3.2. Antioxidant activity of *A. tricolor* extract on *S. pombe*

Spot assay results demonstrated that *A. tricolor* extract at concentrations of 49 and 97 $\mu\text{g/ml}$ conferred enhanced yeast growth under oxidative stress induced by 3 mM H_2O_2 , compared with other tested concentrations (Fig. 1). These findings suggest that these concentrations are optimal for supporting yeast viability under oxidative stress conditions.

3.3. Antiaging activity of *A. tricolor* extract

The chronological lifespan (CLS) assay was employed to evaluate the potential of *A. tricolor* extract in extending yeast cell viability. Treatment with *A. tricolor* extract at a concentration of 49 $\mu\text{g/ml}$ appeared to maintain higher cell

viability of *S. pombe* up to day 11 compared to both the positive control (cells cultured in YES medium under caloric restriction (CR), 0.3% glucose) and the negative control (cells grown in YES medium with 3% glucose) (Fig. 2). This observation suggests a potential lifespan-extending effect of the extract.

3.4. *Schizosaccharomyces pombe* mitochondrial activity after administration *A. tricolor* extract

Mitochondrial function was qualitatively assessed using Rhodamine B, a cationic dye that accumulates within mitochondria in proportion to membrane potential ($\Delta\psi\text{m}$). Among the tested concentrations, cells treated with 49 $\mu\text{g/ml}$ *A. tricolor* extract displayed the most intense red fluorescence signal (Fig. 3), indicating enhanced mitochondrial polarization compared with controls.

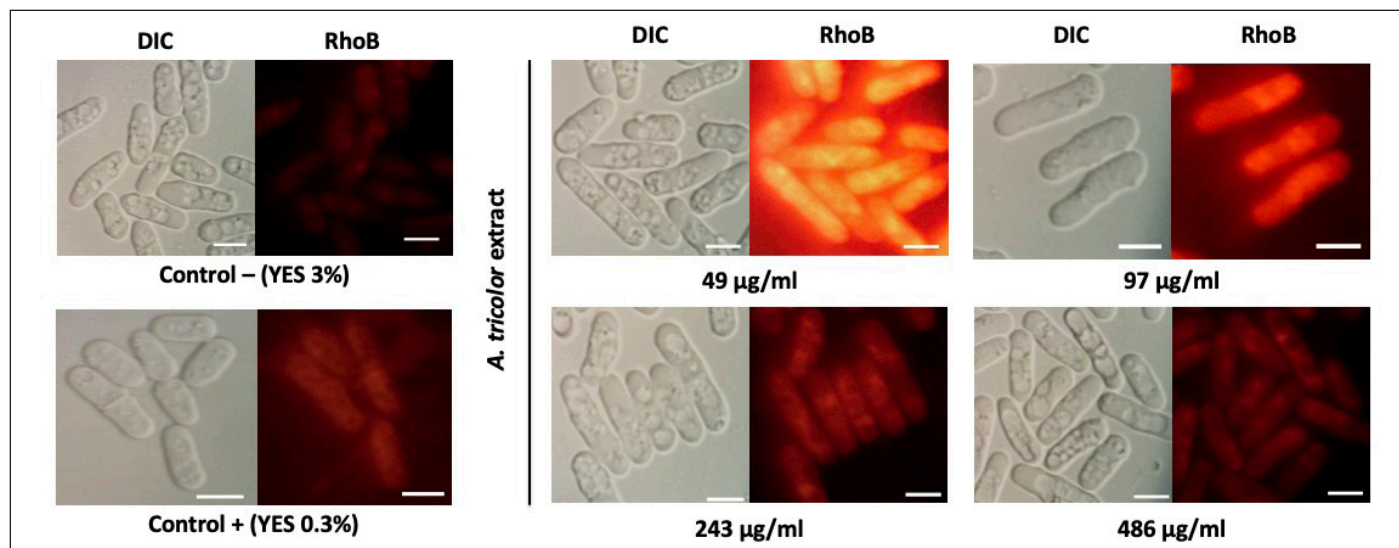


Figure 3. The effect of ethanol derived-*A. tricolor* extracts at various concentrations on the mitochondrial activity of yeast *S. pombe*. Red fluorescence signals, which are visualized by using Rhodamine B (RhoB) staining indicate active mitochondria. DIC = Differential Interference Contrast. Cells grown in YES medium with 3% glucose were used as the negative control, while those cultured in YES medium with 0.3% glucose (calorie restriction) served as the positive control. Scale bar = 5 μm .

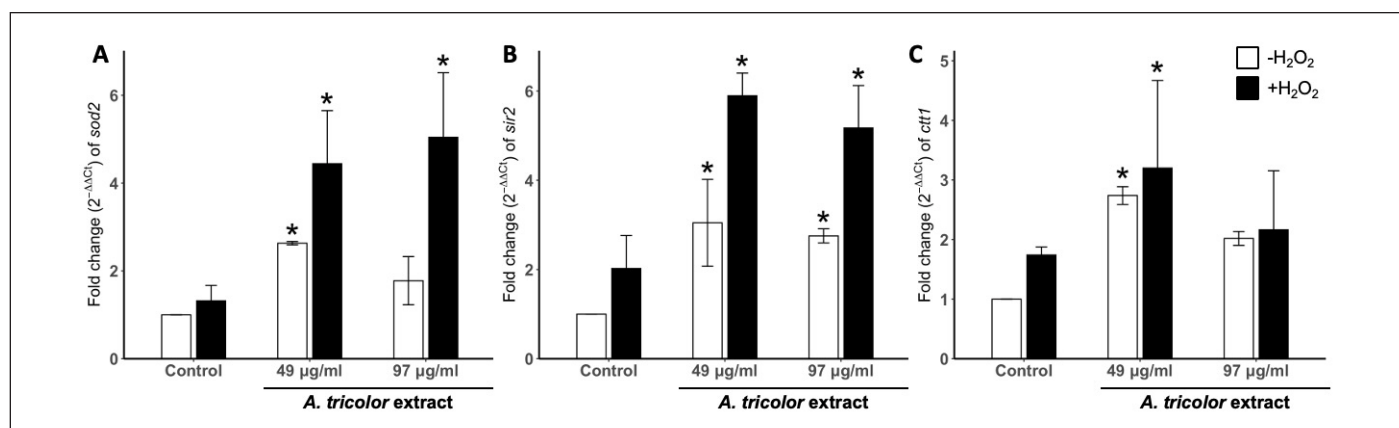


Figure 4. Fold change on the expression gene level of (A) *sod2* (superoxide dismutase), (B) *sir2* (sirtuin), and (C) *ctt1* (catalase) genes in *S. pombe* after administration ethanol derived-*A. tricolor* extracts (49 and 97 $\mu\text{g/ml}$). Oxidative stress was induced by adding hydrogen peroxide (H_2O_2) at a final concentration of 1 mM one hour prior to harvesting. Asterisks (*) denote statistically significant differences compared to the untreated control ($p < 0.05$), as determined by DMRT.

3.5. Fold change on the expression of the gene involved in oxidative stress tolerance and aging regulation

Gene expression analysis was performed to evaluate the impact of *A. tricolor* extract on the transcriptional regulation of antioxidant-associated genes (*sod2* and *ctt1*) and the aging-related gene *sir2*. Treatment with the extract resulted in a marked upregulation of all three genes compared to the untreated control, indicating that *A. tricolor* enhances both antioxidant defense and longevity-associated pathways (Fig. 4).

3.6. Cell cycle distribution *S. pombe* after administration *A. tricolor*

Cell cycle distribution in *S. pombe* was analyzed using flow cytometry, dividing the cell population into G0/G1

(growth phase 1), S (DNA synthesis), and G2 (growth phase 2) phases. The results demonstrated that treatment with *A. tricolor* extract at a concentration of 49 $\mu\text{g/ml}$ significantly increased the proportion of cells in the G0/G1 phase compared to the untreated control, with values of 91.33% and 87.54%, respectively (Fig. 5).

3.7. Putative compounds in the extract of *A. tricolor*

The LC-HRMS chromatogram displayed a wide range of retention times with diverse peaks, reflecting the chemical complexity of the *A. tricolor* extract. Three dominant compounds were identified: (2E)-3-(3,4-dimethoxyphenyl)acrylic acid (11.77 min, m/z 208.0736), 2-O-caffeoylglucuronic acid (12.33 min, m/z 372.0693), and (10E,15Z)-9,12,13-trihydroxy-10,15-octadecadienoic acid (12.39 min, m/z 328.2250) (Fig. 6).

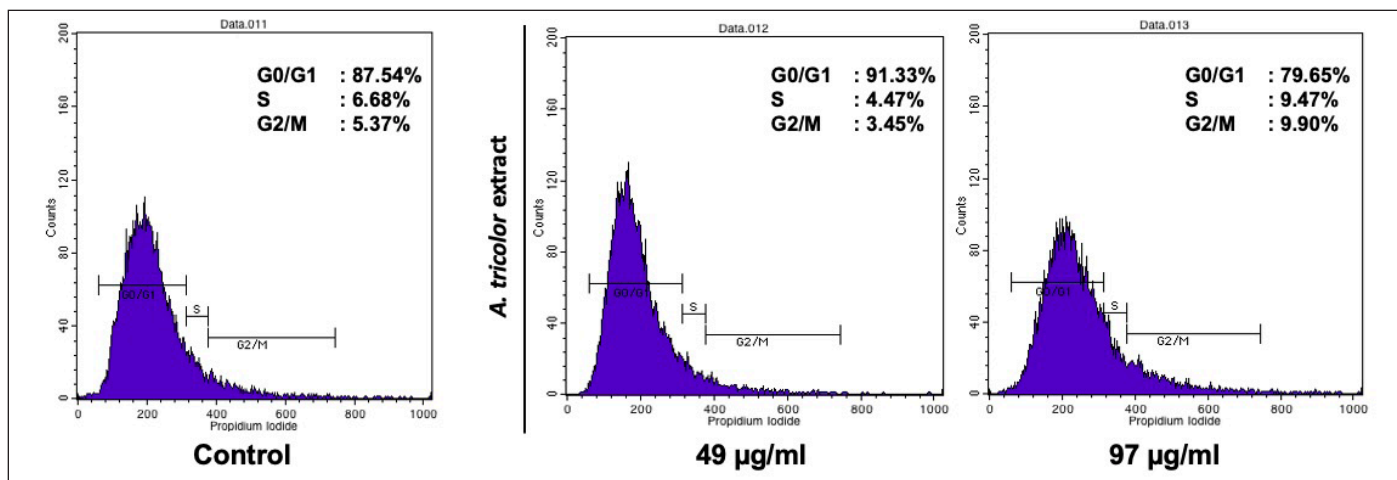


Figure 5. Effect of the ethanol-derived *A. tricolor* extracts (49 and 97 µg/ml) on the cell cycle of *S. pombe*. The total amount of each yeast cell phase was mentioned in the right corner of each figure.

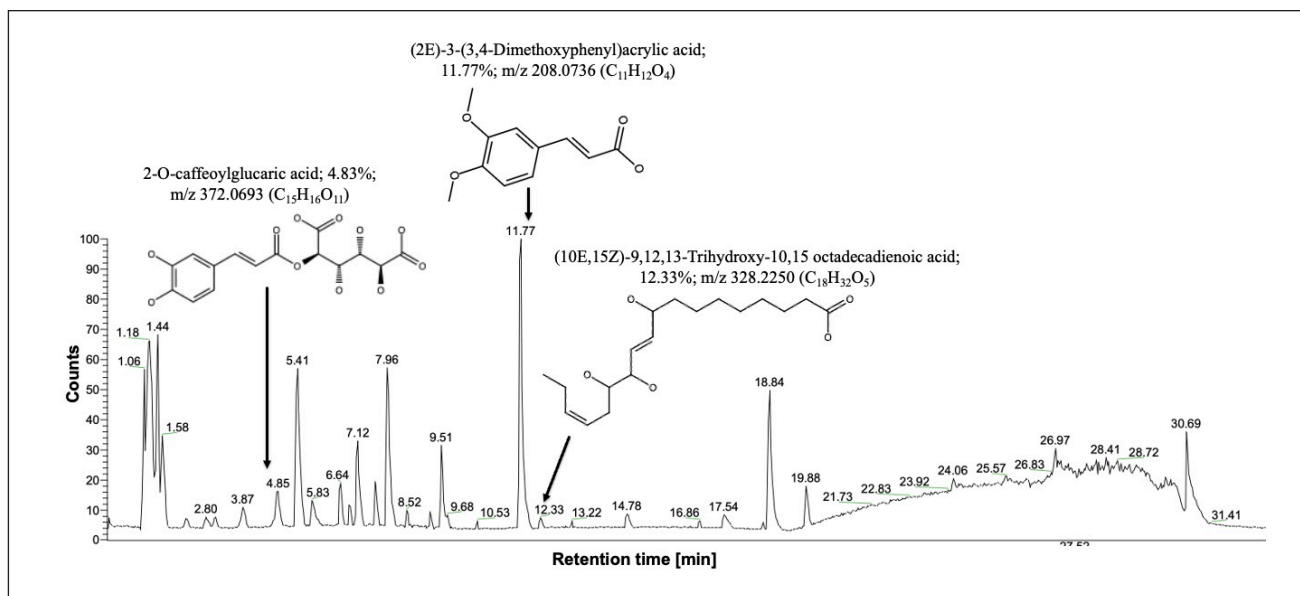


Figure 6. LC-HRMS chromatogram analysis of the ethanol-derived *A. tricolor* extracts.

4. DISCUSSION

Amaranthus tricolor is recognized as an important plant with notable antioxidant potential. In the present study, ethanol leaf extracts of *A. tricolor* demonstrated strong antioxidant and anti-aging activities. Although the extract was approximately 100-fold less potent than ascorbic acid, which served as the positive control, the observed IC_{50} value (97.19 ± 4.35 µg/ml) falls within the 50–100 µg/ml range, a category classified as strong antioxidant capacity [30]. In comparison, extracts of *Adenostemma lavenia* obtained using water and chloroform exhibited much weaker antioxidant activity, with IC_{50} values of 252.02 ± 3.23 µg/ml and 222.37 ± 1.16 µg/ml, respectively [22]. This indicates that *A. tricolor* demonstrates a substantially stronger free radical scavenging capacity than several other reported plant extracts. The high antioxidant

capacity of *A. tricolor* is attributed to its rich pigmentation, including betalains, anthocyanins, betacyanins, carotenoids, betaxanthins, and chlorophyll, which are known to effectively scavenge free radicals—major contributors to cellular aging [31]. Additionally, the presence of phytochemical constituents such as phenolic compounds and flavonoids further enhances its antioxidant efficacy [32].

In this study, 50% ethanol was used as the extraction solvent due to its safety and broad extraction efficiency for food applications. Ethanol is a class 3 solvent with low toxicity and is widely applied for isolating plant bioactives [33]. Solvent polarity strongly influences phytochemical yield and antioxidant activity; highly polar solvents (e.g., water) extract hydrophilic compounds, while nonpolar solvents (e.g., chloroform) favor lipophilic molecules. Semi-polar solvents

such as 50% ethanol enable the co-extraction of both polar and nonpolar constituents, particularly phenolics and flavonoids, which are key contributors to antioxidant activity [34]. This likely explains the strong antioxidant potential and relatively low IC_{50} value observed for the *A. tricolor* extract.

Amaranthus tricolor demonstrated strong protective effects against oxidative stress, particularly at concentrations of 49 and 97 $\mu\text{g/ml}$, where yeast survival under 3 mM H_2O_2 challenge was markedly improved. The concentration range used in this study was determined based on the IC_{50} value obtained from antioxidant screening, representing approximately 0.5 \times , 1 \times , 2.5 \times , and 5 \times IC_{50} levels. These concentrations are near or below the extract's IC_{50} value (97 $\mu\text{g/ml}$), suggesting that the extract exerts effective antioxidant activity at sub-cytotoxic levels. However, higher concentrations appeared to reduce yeast growth, which may be associated with dose-dependent cytotoxic effects of phenolic compounds. Previous studies have shown excessive concentrations of plant-derived antioxidants can induce oxidative imbalance or interfere with mitochondrial metabolism, thereby limiting cell proliferation [35]. The observed growth advantage indicates that *A. tricolor* extract confers cellular protection under oxidative conditions. This antioxidant capacity is consistent with a previous report demonstrating that ethanol and hexane fractions from clove (bud and leaf) extract preserved *S. pombe* viability under the same stress level of 3 mM H_2O_2 [29]. Since oxidative stress is a key contributor to cellular aging [7], these results highlight the potential of *A. tricolor* extract as a protective agent against ROS-induced damage in eukaryotic cells. A previous study ethanol-derived clove leaf extract at a concentration of 100 $\mu\text{g/ml}$ has also been shown to improve yeast cell viability under oxidative stress induced by 3 mM H_2O_2 [20]. In our study, yeast cultures treated with *A. tricolor* extract also showed better growth compared to those subjected to CR. CR is known to delay cellular aging by suppressing oxidative damage and improving mitochondrial efficiency through conserved pathways such as SIR2 activation and TOR inhibition [36,37]. The observation that *A. tricolor* extract enhanced cell viability and mitochondrial activity while upregulating antioxidative genes (*sod2* and *ctt1*) suggests that it may exert a CR-mimetic effect. Similar to CR, the extract likely reduces intracellular ROS accumulation and modulates stress response signaling to maintain redox homeostasis. *Schizosaccharomyces pombe* responds to H_2O_2 -induced oxidative stress through several regulatory pathways, particularly involving the Sty1 and Pap1 signaling cascades. At low concentrations of H_2O_2 (<1 mM), Pap1 is activated via Tpx1, a peroxidase sensor that neutralizes ROS [38]. However, at higher concentrations of H_2O_2 , the MAP kinase pathway is triggered through the activation of Sty1. This kinase plays a critical role in oxidative stress adaptation by phosphorylating and stabilizing Atf1, a transcription factor orthologous to mammalian SAPK [39]. In this study, the elevated H_2O_2 concentration likely engaged the Sty1-mediated MAPK pathway, indicating that *A. tricolor* extract may be involved in modulating stress-response signaling under oxidative conditions.

Amaranthus tricolor also exhibited a pronounced ability to extend cellular lifespan, as evidenced by prolonged survival of *S. pombe* up to day 11 at a concentration of 49 $\mu\text{g/ml}$. This effect was superior to both the caloric restriction control (0.3% glucose) and the standard growth condition (3% glucose), underscoring the extract's capacity to delay chronological aging and sustain cellular viability under stress. These findings suggest that it is the first report to demonstrate the potential of *A. tricolor* extract as a novel anti-aging agent at low concentrations. Notably, viability assessments on days 7 and 11 were chosen as these time points represent the stationary phase in the growth curve of *S. pombe*, during which aging-related phenotypes typically emerge, and cell death normally occurs in untreated cultures [40]. Therefore, the ability of the extract to sustain cell viability during this period highlights its antiaging efficacy. A similar phenomenon was observed in a previous study, where the aqueous (10,000 $\mu\text{g/ml}$) and chloroform (2505 $\mu\text{g/ml}$) fractions of *Dichrocephala integrifolia*, as well as the aqueous fraction (10,000 $\mu\text{g/ml}$) of *Galinsoga parviflora*, extended yeast viability up to day 11 [17]. A similar finding was made, where the addition of *A. lavenia* extract using distilled water as a solvent at concentrations of 1,260 and 888 $\mu\text{g/ml}$ successfully prolonged yeast cell viability up to day 11 [26]. A previous study also demonstrated that ethanol-derived clove leaf extract at a concentration of 100 $\mu\text{g/ml}$ was capable of extending yeast cell viability for up to 9 days [20]. These findings suggest that *A. tricolor* extract holds promise as a novel antiaging agent at low concentrations. This effect is likely attributed to its strong antioxidant activity, which may modulate physiological and cellular processes in *S. pombe*, particularly by reducing the accumulation of ROS during the stationary phase. Excessive ROS can damage DNA and proteins, ultimately triggering cellular senescence, often associated with telomere shortening [41]. However, further studies using colony-forming unit counting or quantitative survival analysis are needed to confirm these findings.

Schizosaccharomyces pombe possesses the intrinsic ability to extend its cellular viability under nutrient-limited conditions [42]. Caloric restriction has been shown to prolong yeast lifespan by inhibiting nutrient-sensing signal transduction pathways such as Pka1, Sck2, and the TOR pathway, all of which are known to reduce intracellular levels of ROS [43]. In addition, calorie restriction enhances the expression of Sir2 (sirtuins), a family of proteins that help maintain genomic stability by repressing ribosomal DNA transcription [44]. Therefore, the inclusion of calorie restriction as a positive control in this study is biologically important because it provides a benchmark for a well-established, nongenetic longevity mechanism. This comparison enables evaluation of whether *A. tricolor* extract can mimic or enhance the lifespan-extending and oxidative stress-reducing effects conferred by calorie restriction.

The cellular impact of *A. tricolor* extract was further assessed through the evaluation of mitochondrial activity in *S. pombe* using Rhodamine B staining. This dye selectively accumulates within mitochondria in response to the transmembrane potential ($\Delta\psi\text{m}$), thus serving as a reliable

indicator of mitochondrial integrity and activity [45,46]. The presence of red fluorescence reflects the formation of mitochondrial membrane potential, which is closely associated with cellular energy status and ROS signaling. Among the tested concentrations, treatment with 49 $\mu\text{g/ml}$ of *A. tricolor* extract produced the strongest fluorescence signal, suggesting enhanced mitochondrial activity at this level. The fluorescence increase may also indicate the activation of adaptive mitochondrial ROS signaling mechanisms, which play a crucial role in maintaining redox homeostasis under mild oxidative stress [29]. A similar mitochondrial activation was observed in a previous study, where the compound 11 α -hydroxy-15-oxo-kaur-16-en-19-oic acid (11 α OH-KA) isolated from *A. lavenia* stimulated mitochondrial activity at a relatively low concentration of 45 $\mu\text{g/ml}$ [26]. Although these observations are qualitative, the visible increase in fluorescence suggests improved mitochondrial integrity at this concentration. Future studies incorporating quantitative $\Delta\psi\text{m}$ will be required to validate these findings.

Mitochondrial activation is commonly associated with the generation of ROS. These mild ROS levels are known to trigger the expression of stress-responsive genes that contribute to increased cell survival and resilience under oxidative conditions [47,48]. Based on these findings, we propose that *A. tricolor* extract may enhance mitochondrial function and modulate cellular redox status by inducing low-level ROS production, which subsequently activates protective mechanisms within *S. pombe* cells. This hypothesis is further supported by previous work, which demonstrates that extracts from *Bacillus* sp. SAB E-41 also increased ROS levels moderately in *S. pombe*, functioning as a pro-oxidant while still promoting cellular defense and longevity [26].

Amaranthus tricolor extract significantly modulated the transcriptional response of antioxidant- and aging-related genes in *S. pombe*. Treatment with the extract led to marked upregulation of *sod2* (superoxide dismutase) and *ctt1* (catalase), both of which are critical enzymes in mitigating oxidative stress by detoxifying ROS, as well as *sir2* (sirtuin), a key regulator of lifespan and genomic stability. This coordinated induction of antioxidant defenses and longevity-associated pathways suggests that *A. tricolor* not only enhances cellular resistance to oxidative stress but may also contribute to the delay of cellular aging processes. These findings are the same as with a previous study, where aqueous and chloroform fractions of *Synedrella nodiflora* extract were shown to double the expression of *sod2* and *ctt1* [17].

The upregulation of *sod2*, *sir2*, and *ctt1* by *A. tricolor* extract suggests a close relationship between its cellular antiaging effects and the oxidative stress response in *S. pombe*. Antioxidant defense genes such as *sod2* and *ctt1* play essential roles in mitigating intracellular ROS accumulation, thereby the maintenance of redox homeostasis under stress conditions [39]. The *sod2* gene encodes a mitochondrial superoxide dismutase and is transcriptionally regulated by the Pap1 protein under low oxidative stress [46]. Meanwhile, *ctt1*, which encodes catalase, functions downstream in the Central Environmental Stress Response pathway, activated through Tpx1-Pap1 and Sty1-Atf1 signaling, and has been directly implicated in lifespan extension

in *S. pombe* [17]. Notably, catalase is essential for detoxifying H_2O_2 , thereby enhancing resistance to oxidative stress. This antioxidant mechanism is consistent with the increased mitochondrial activity observed following treatment with 49 and 97 $\mu\text{g/ml}$ *A. tricolor* extract (Fig. 3). The *sir2* gene encodes the NAD^+ -dependent deacetylase sirtuin, a protein known for its involvement in DNA repair and neuroprotection [49]. Upregulation of *sir2* at a relatively low extract concentration (49 $\mu\text{g/ml}$) may contribute to cellular longevity in *S. pombe*, potentially by modulating chromatin structure and genome stability. Similar effects were observed, where it was found moderate overexpression of Sir2 in the *Saccharomyces cerevisiae* BY4743 strain suppressed cell proliferation, indicating a shift toward an extended lifespan [50].

Treatment with *A. tricolor* extract at 49 $\mu\text{g/ml}$ induced a marked G0/G1 arrest in *S. pombe*, indicating a protective mechanism against replicative stress that underlies its antiaging potential. In contrast, treatment with a higher concentration (97 $\mu\text{g/ml}$) resulted in a lower G0/G1 population (79.65%), suggesting a dose-dependent response. Compared to the untreated control, treatment with 49 $\mu\text{g/ml}$ *A. tricolor* extract increased the G0/G1 cell population, suggesting delayed S-phase entry and enhanced genomic stability that may contribute to its antiaging effect. This phenomenon aligns with previous findings suggesting that the ability to maintain cells in G0/G1 is associated with prolonged lifespan and enhanced cellular quiescence. For instance, treatment with *Pseudomonas* sp. PTR-08 extract was reported to arrest *S. pombe* in G1 phase, delaying progression into S phase and mimicking cellular aging deceleration [21]. Downregulation of G1-phase-specific genes such as *cdc18*⁺, *cdc22*⁺, *cdt1*⁺, *cdt2*⁺, and *dfp1*⁺ helps maintain genomic stability by delaying G1/S progression, reducing replication stress, and promoting cellular quiescence [51]. Although the observed G0/G1 arrest may contribute to improved cellular longevity, it is likely a secondary adaptive response associated with enhanced stress resistance, as similarly observed under nutrient limitation or TOR inhibition conditions [52,53].

The observed increase in G0/G1 cell population was modest (~4%); such shifts have been previously linked to biologically meaningful extensions in CLS and stress tolerance. A previous study showed that even a modest 4%–10% increase in the proportion of cells in the G0/G1 phase was sufficient to significantly extend CLS and improve oxidative stress resistance in *Schizosaccharomyces pombe* strains lacking the *sch9* gene (*sch9* Δ) [54]. Similar outcomes have been reported for natural compounds such as resveratrol and wild pink bayberry extract, which induced G0/G1 arrest in malignant NK cells and MDA-MB-231 cancer cells, contributing to reduced proliferation and activation of stress defense pathways [55,56]. Taken together with increased mitochondrial activity, improved viability, and upregulation of aging-associated genes, our findings suggest that *A. tricolor* extract may exert its antiaging effects by modulating the cell cycle to favor G0/G1 arrest, in line with previously proposed mechanisms of cellular longevity.

The LC-HRMS chromatogram revealed a broad retention time profile with multiple peaks, highlighting

the chemical complexity of the *A. tricolor* extract. Among these, three major compounds were identified: (2E)-3-(3,4-dimethoxyphenyl)acrylic acid, 2-O-caffeoylglucaric acid, and (10E,15Z)-9,12,13-trihydroxy-10,15-octadecadienoic acid. These identifications were based on exact mass and known molecular formulas. (2E)-3-(3,4-dimethoxyphenyl)acrylic acid and (10E,15Z)-9,12,13-trihydroxy-10,15-octadecadienoic acid were found in the highest relative abundance, accounting for 11.7% and 12.3%, respectively.

The majority of annotated metabolites consisted of carboxylic acids and phenolic acid derivatives, many of which are known for their antioxidant properties. Phenolic acids exert their antioxidant effects mainly through hydrogen atom or electron donation, metal ion chelation, and resonance stabilization of free radicals [57]. Carboxylic acids demonstrate structure-activity relationships where carboxyl groups support metal ion chelation and enhance free radical scavenging [58]. For instance, (2E)-3-(3,4-dimethoxyphenyl)acrylic acid, previously isolated from *Rubus urticifolius*, has been reported to possess potent antioxidant activity and potential as a safe compound for protecting against skin hyperpigmentation, oxidative stress, inflammation, and aging-related conditions [59]. Likewise, 9,12,13-trihydroxy-10,15-octadecadienoic acid, a unique compound found in *Prosthechea karwinskii*, exhibits free radical scavenging activity and contributes to cellular defense against ROS [60]. Furthermore, 2-O-caffeoylglucaric acid, frequently detected in *Amaranthus* species, has also been highlighted as a key contributor to antioxidant potential [61]. To the best of our knowledge, this is the first report demonstrating that *A. tricolor* possesses antiaging activity in an organism model. The presence of phenolic compounds in the extract likely contributes to its strong antioxidant potential, which may underlie its ability to prolong cell viability, enhance mitochondrial activity, and scavenge free radicals through the upregulation of antioxidative genes (*sod2* and *ctt1*) as well as the aging-related regulatory gene *sir2*. These compounds may activate conserved stress-response pathways such as MAPK and Nrf2-like signaling, enhance mitochondrial resilience, and reduce ROS accumulation, thereby contributing to improved cellular protection and longevity [62]. However, our findings should be interpreted in the context of certain limitations. Although *S. pombe* shares many conserved aging-related pathways with higher eukaryotes, its unicellular biology limits its ability to emulate multicellular processes such as tissue-specific aging, intercellular signaling, and systemic metabolism. Future studies should evaluate *A. tricolor* extract in mammalian cell lines or animal models to investigate its effects at the cellular, tissue, and organ levels under realistic physiological conditions.

5. CONCLUSION

This study demonstrates, for the first time, that *A. tricolor* extract exhibits antiaging and antioxidant activity in *S. pombe*. The extract (49 µg/ml) extended CLS, enhanced tolerance to oxidative stress, increased mitochondrial activity, and promoted G1-phase arrest while upregulating *sod2*⁺, *ctt1*⁺, and *sir2*⁺. LC-HRMS revealed phenolic and carboxylic acid

derivatives known for antioxidant and antiaging properties. These findings support the potential of *A. tricolor* as a promising candidate for nutraceutical development.

6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All data generated and analyzed are included in this research article.

11. PUBLISHER’S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used AI-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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