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Antiaging Properties of the Ethanol Fractions of Clove (*Syzygium aromaticum* L.) Bud and Leaf at the Cellular Levels: Study in Yeast *Schizosaccharomyces pombe*

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Abstract: The exposure of reactive oxygen species is one of the aging triggers at cellular level. The antioxidants have been used as strategic efforts in overcoming the accumulation of ROS. Previous research using crude extracts of clove bud and leaves showed its potential as an antioxidant agent. However, no data were available regarding the antioxidant and antiaging activities of subsequent fractions of clove extracts. Therefore, this study aimed to analyze the antioxidant and antiaging activities of the n-hexane and ethanol fractions from clove bud and leaves. Antioxidant and antiaging activities were tested at the cellular level using the yeast model *Schizosaccharomyces pombe*. The highest flavonoid content was shown by clove leaf n-*hexane* fraction (25.6 mgQE·g^{-1}). However, ethanol fraction of clove bud (FEB) showed the highest antioxidant activity based on TBA and antiglycation assays. FEB (8 µg·mL^{-1}) and leaf ethanol fraction (FEL) (10 µg·mL^{-1}) were able to induce yeast tolerance against oxidative stress. In addition, FEB could induce mitochondrial activity and delay the G1 phase of the cell cycle. FEB was found to be rich in gallic acid and (15Z)-9,12,13-trihydroxy-15-octadecenoic. Based on the data, FEB shows the potential antiaging activity, which is promising for further development as biopharmaceutical product formulations.

Keywords: antioxidant; aging; clove; chronological life span; cell cycle; mitochondria; *Schizosaccharomyces pombe*; gallic acid; phenolic compounds

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1. Introduction

The pharmaceutical industry has produced various antioxidant-based antiaging cosmetic and supplement products. The current trend in the cosmetics industry is an application of natural resources, including natural-based antioxidant compounds over synthetic ones as cosmetic ingredients. Such a strategy may potentially reduce the production cost and avoid adverse effects of the synthetic materials used [1]. Indeed, the United States Food and Drug Administration (US FDA) reported an increase in cases of adverse health effects on cosmetic products from 2015 as many as 706 to 1591 cases in 2016, and more than 35% of skincare products caused severe health problems [2].

Aging at the cellular level is one of the inducers of a decline in the function of tissues and organs that can increase the prevalence of diseases, one of which is degenerative diseases. Cellular aging can occur due to exposure and accumulation of reactive oxygen species (ROS) from UV lights, cigarette smoke, pollutants, and chemicals in cosmetic products. The exposure of ROS sources can accelerate cell aging, for example, in skin cells. The aging of skin cells is characterized by facial wrinkles, dull skin color, thickening of the skin, gradual reduction in skin elasticity, slowing of epidermal turnover, which causes a decrease in one's aesthetics and appearance [3]. The continuous accumulation of ROS

molecules with an unbalanced intracellular antioxidative mechanism will induce oxidative stress. Oxidative stress can damage the activity of essential macromolecules such as DNA, proteins, carbohydrates, and lipids that can induce cell aging [4]. Antioxidant activity plays a vital role in helping to slow down the cellular aging process [3]).

Natural cosmetic ingredients are one solution in providing cosmetic products based on safe antioxidant and antiaging activities. One of the plants reported to have antioxidant activity is clove (*Syzygium aromaticum* L.). Previous studies reported that clove extract could extend the life span of model organisms, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, and increase yeast resistance to hydrogen peroxide (H_2O_2) oxidative stress [5,6]. However, the information regarding the activity of clove leaf and bud fractions as antioxidant and antiaging agents at the cellular level is not yet available. Therefore, this study was aimed to analyze the antioxidant activity and antiglycation activity of the n-hexane and ethanol fractions from clove bud and leaf extract in vitro and their potential in delaying aging at the cellular level. *S. pombe* as a model of organism was used to study the potential mode of action of clove fraction in delivering antiaging properties.

2. Materials and Methods

2.1. Yeast Cell Culture

Fission yeast *Schizosaccharomyces pombe* ARC039 (h-leu1-32 ura4-294) was routinely maintained in the Yeast Extract with Supplement (YES) medium at 30 °C. Yeast was also cultured in calorie restriction treatment by using YES liquid medium containing lower glucose concentration (0.3%, *w*/*v*). Unless stated differently, the 1 L of YES medium was composed by 5 g yeast extract, 30 g glucose, 0.128 histidine, 0.128 leucine, 0.128 adenine, 0.01 uracil, 0.128 arginine. Agar (20 $g \cdot L^{-1}$) was used to make solid medium.

2.2. Fractionations of Clove Extract

The ethanol extracts of clove buds and leaves were prepared as described previously [6]. Furthermore, 81.6 g of clove bud 70% ethanol extract and 46.6 g clove leaf 70% ethanol extract were fractionated with 200 mL of n-hexane and 100 mL of 70% ethanol respectively into a separating funnel. To obtain sample fractions, the n-hexane (Sigma-Aldrich, St. Louis, MO, USA) and ethanol (Merck, Billerica, MA, USA) phases were separated and concentrated in a vacuum rotary evaporator at 45 °C. The resulted fractions included clove bud-derived n-hexane fractions (FHB), clove leaves derived n-*hexane* fractions (FHL), clove bud-derived ethanol 70% fractions (FEB), clove leaves derived ethanol 70% fractions (FEL) were then used as sample fraction for further analysis.

2.3. Total Flavonoid Content

Total flavonoid was quantified based on the previous method [7]. Each sample fraction was adjusted to 1000 $\mu g \cdot mL^{-1}$. 0.5 mL sample fraction solution was then mixed with 0.5 mL 2% $AlCl_3$ (Merck, Billerica, MA, USA), and incubated at room temperature for 30 min. The absorbance was measured at 415 nm with spectrophotometer UV-Vis. Blank solution containing sample without the addition of $AlCl_3$ 2% was treated the same as samples. Quercetin (Sigma-Aldrich, St. Louis, MO, USA) was used as standard for calibration curve. Total flavonoid content was expressed as mg $QE \cdot g^{-1}$ fraction.

2.4. Antioxidant Activity Based on 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

Sample fractions were added with 0.4 mM of DPPH (Sigma-Aldrich, Steinheim, Germany) solution, 20% methanol, and 0.2 M of 2-(N-morpholino) ethanesulfonic acid (MES) buffer solution, respectively [8]. Next, the sample solution was incubated for 30 min in a dark room and measured using a spectrophotometer at a wavelength of 520 nm. Ascorbic acid was used as a positive control. Antioxidant activity is expressed in percentage of DPPH reduction by calculation: % reduction DPPH = $(1 - (X_1/X_0)) \times 100\%$.

X_1: sample absorbance, X_0: blank absorbance.

2.5. Antioxidant Activity Based on 2,2′-Azino-Bis(3-Ethylbenzothiazoline-6-Sulphonic Acid) (ABTS) Assay

Sample fractions were dissolved in 99.8% ethanol (1 $\mu g \cdot mL^{-1}$) and mixed with oxidized ABTS radicals (Carbosynth Ltd., Compton, UK) (7.46 mM ABTS solution, and potassium persulfate ($K_2S_2O_8$) 2.45 mM). Measurements were made at a wavelength of 734 nm [9]. Antioxidant activity is expressed in percentage reduction of ABTS with the calculation: % reduction ABTS = $(1 - (X_1/X_0)) \times 100\%$.

X_1: sample absorbance, X_0: blank absorbance.

2.6. Antioxidant Activity Based on Thiobarbituric Acid (TBA) Assay

Each sample fractions were added with 50 mM linoleic acid (Aldrich, Buchs, Switzerland) in 99.8% ethanol, and 0.1 M phosphate buffer pH 7.0 [10]. Then, solutions were incubated in a water bath at 40 °C for 7 days. Each sample solution was added with 20% trichloroacetic Acid (TCA) (Sigma-Aldrich, Burlington, MA, USA) solution, and 1% 2-thiobarbituric acid (TBA) (Sigma-Aldrich, Darmstadt, Germany) solution after 7th day of incubation. Subsequently, the sample was heated at 100 °C for 10 min and centrifuged at 3000 rpm for 15 min. Sample was then measured using a spectrophotometer with a wavelength of 532 nm. Antioxidant activity is expressed in percentage of malondialdehyde formation (MDA) by calculation: % reduction MDA = $(1 - (X_1/X_0)) \times 100\%$.

X_1: MDA concentration with sample, X_0: MDA concentration without sample.

2.7. Antiglycation Assay

Antiglycation assay was done by using previous method [11]. Four different solutions were prepared prior quantification. Solution A was made by mixing 40 µL glucose 235 mM, 40 µL fructose 235 mM, 80 µL BSA 20 $\mu g \cdot mL^{-1}$, 80 µL each sample fraction and diluted in 200 µL 0.2 M phosphate buffer solution (pH 7.4). For positive control, aminoguanidin (Sigma-Aldrich, St. Louis, MO, USA) was added instead of sample fraction. Solution A_0 was designed as correction solution for solution A, which contained similar ingredients as solution A, yet 80 µL aquadest was substituted for fructose or glucose. Solution B or solution control was made by mixing 40 µL glucose 235 mM, 40 µL fructose 235 mM, 80 µL BSA 20 $\mu g \cdot mL^{-1}$, 80 µL aquadest and 200 µL 0.2 M phosphate buffer solution (pH 7.4). Solution B_0 was prepared as correction solution for solution B, by which 80 µL aquadest was used to substitute glucose and fructose. Each solution was then incubated for 40 h at 60 °C. After incubation, 100 µL from each solution was transferred to microplate well (Nunc 96). Fluorescence intensity was then measured by using fluorometer (FluoroSTAR BMG LABTECH, USA) at excitation and emission wavelength of 330 nm and 440 nm, respectively. IC50 was then quantified by using the following formula, % Inhibition = $1 - ((A - A_0)/(B - B_0)) \times 100\%$.

A: fluorescent intensity of sample, A_0: correction fluorescent intensity of sample, B: fluorescent intensity of control, B_0: correction fluorescent intensity of control.

2.8. Yeast Viability Assay (Spot Test Assay)

Yeast *S. pombe* was pre-cultured in YES liquid medium for 18 h at 30 °C. Pre-culture was then transferred to new YES liquid medium at initial OD600 = 0.05 and further used as main culture. Each sample fraction at various concentration was added into each main culture and further be incubated at 30 °C. Concentration of sample fraction used in this assay was based on the IC_{50} value of DPPH assay including 0.25×, 1×, 4×, 8×, and 12× IC_{50}. Following 7 and 11 days of incubation, each culture was harvested and adjusted to OD600 = 1 using sterile medium. The particular solution was then serially diluted and each dilution suspension was then spot on solid YES medium and incubated for three days at 30 °C. Yeast grown in YES medium with lower glucose concentration (0.3% *w*/*v*) was used as positive control or commonly known as calorie restriction treatment [12], while yeast grown in YES medium without sample fraction was designed as a negative control.

2.9. Yeast Oxidative Stress Response Assay

Yeast culture was prepared as described previously on yeast viability assay. Yet, only the best concentration of sample fraction was used in this assay. Calorie-restricted yeast culture was used as positive control in addition to 0.1 $\mu g \cdot mL^{-1}$ ascorbic acid treatment. Each yeast culture was then serially diluted and each dilution suspension was spot on solid YES medium containing 1.2 and 3 mM H_2O_2 as oxidative stress treatment [13]. Agar medium was then incubated for three days at 30 °C.

2.10. Chronological Lifespan (CLS) Assay

Fission yeast cells were cultured as described previously in oxidative stress response assay. Following 1, 5, 10, and 15 day of incubation, each yeast culture was then serially diluted and spread in solid YES agar medium. Each YES agar was then incubated for 3 days at 30 °C and grown yeast colony was calculated.

2.11. Mitochondria Activity Assay

Mitochondria activity was assayed by using rhodamine B [14]. Yeast main cultures that have been incubated for 18 h at 30 °C with sample fraction treatment were harvested by centrifugation at 5000 rpm for 1 min. The suspension was rinsed using 0.1 M phosphate buffer pH 7 and subsequently added with 100 nM Rhodamine B (Sigma-Aldrich, St. Louis, MO, USA). After that, fluorescence in correspond to mitochondria activity was observed by using a fluorescence microscope Olympus BX51.

2.12. Cell Cycle Assay

Cell cycle assay was carried out by using Propidium Iodide Flow Cytometry Kit (Abcam, Cambridge, UK) protocol. Fission yeast cultures were treated with sample fractions and incubated for 18 h at 30 °C. Yeast cultures were then harvested by centrifugation and rinsed twice with 1× phosphate buffer saline solution. The cell fixation process was carried out by using 70% ethanol. Then, DNA staining was carried out with a mixture of propidium iodide dye (a mixture of PBS 1X solution, propidium iodide (1 mg/mL), and RNAse (110,000 $U \cdot mL^{-1}$)). Cell cycle analysis was performed using NovoCyte Flow Cytometer (Agilent, Santa Clara, CA, USA).

2.13. Liquid Chromatography-Mass Spectrometry Analysis

Fraction was prepared as described previously. The LC-MS data were obtained by using UHPLC Vanquish Tandem Q Exactive Plus Orbitrap HRMS (ThermoScientific Waltham, MA, USA). LC separation was done by using Accucore C18, 100 × 2.1 mm, 1.5 µm particle size (ThermoScientific, Waltham, MA, USA). H_2O with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid were used as mobile phase. The gradient elution was designed from 0–1 min (5% B), 1–25 min (5–95% B), 25–28 min (95% B), 28–30 min (5% B) and a 0.2 $mL \cdot min^{-1}$ flow rate. Electrospray ionization (HESI) was used. Each sample was injected once (10 µL) with the ESI, operated in both negative and positive ionization mode. Nitrogen was used as the carrier. The mass spectrometer was operated in full scan mode with a scan range of 500–1500 m/z and automatic data-dependent MS/MS fragmentation scans. Moreover, raw LC-MS data were analyzed by Compound Discoverer 2.1 software (Thermo Fisher Scientific, Waltham, MA, USA). The corresponding software was integrated into the mzCloud and ChemSpider for matching fragmentation spectra and compounds.

3. Results

3.1. Total Flavonoid, Antioxidant, and Antiglycation Activities

The n-hexane fraction (FHB and FHL) showed a higher total flavonoid value than the ethanol fraction (FEB and FEL) (Table 1). Interestingly, four sample fractions (FHB, FEB, FHL, FEL) had antioxidant activity against DPPH radicals, which were significantly different from the positive control. The most potent antioxidant activity was shown by FHB (IC_{50} = 6.9 $\mu g \cdot mL^{-1}$) (Table 1). In comparison, the strongest antioxidant activity

in the ABTS method was shown by FHL (IC_{50} = 24.2·μg·mL^{-1}). FEB has the strongest antioxidant activity in the TBA method with IC_{50} value = 2.6 μg·mL^{-1}. In addition, FEB has the strongest IC_{50} antiglycation activity (35.6 μg·mL^{-1}) than other fractions.

Table 1. Antioxidant activities and antiglycation activity of n-*hexane* and ethanol fractions from clove buds and leaves, in vitro.

No	Sample	Total Flavonoid (mgQE·g^{-1} Fractions)	IC50 (μg·mL^{-1})			
			DPPH	ABTS	TBA	Antiglycation
1	Ascorbic acid	NA	6.25 ± 0.13 ^{a}	11.07 ± 0.18 ^{a}	NA	NA
2	α-Tocoferol	NA	NA	NA	36.75 ± 6.20 ^{c}	NA
3	Aminoguanidine	NA	NA	NA	NA	2.35 ± 0.47 ^{a}
4	FHB	7.58 ± 0.08 ^{b}	6.88 ± 0.20 ^{ab}	41.81 ± 0.88 ^{e}	13.38 ± 1.98 ^{b}	41.69 ± 1.26 ^{b}
5	FEB	2.90 ± 0.31 ^{a}	8.37 ± 0.99 ^{bc}	30.64 ± 2.26 ^{d}	2.61 ± 0.80 ^{a}	35.64 ± 2.83 ^{b}
6	FHL	25.65 ± 0.09 ^{b}	8.87 ± 0.75 ^{c}	24.24 ± 0.79 ^{b}	8.99 ± 2.40 ^{bc}	54.12 ± 3.13 ^{c}
7	FEL	7.31 ± 0.23 ^{c}	9.80 ± 0.62 ^{c}	35.43 ± 0.69 ^{c}	11.07± 3.40 ^{bc}	37.15 ± 2.42 ^{b}

Note: Samples with the same letter in each column are not significantly different based on the Tukey HSD test ($p < 0.05$). NA = Not available. Ascorbic acid, α-Tocoferol, Aminoguanidine were used as positive controls for DPPH and ABTS, TBA and Antiglycation assay, respectively. FHB: clove bud n-hexane fraction, FHL: clove leaf n-hexane fraction, FEB: clove bud ethanol fraction and FEL: clove leaf ethanol fraction

Our study indicates that the flavonoid content of four sample fractions had negative correlation with ABTS (IC_{50}). However, it is worth noting that total flavonoid content was positively correlated with antiglycation activity R^2 = 0.94 (Table 2).

Table 2. Correlation coefficient of total flavonoid content, antioxidant and antiglycation activities.

	Flavonoid	DPPH	ABTS	TBA	Antiglycation
Flavonoid	1.00	-	-	-	-
DPPH	0.18	1.00	-	-	-
ABTS	−0.65	−0.40	1.00	-	-
TBA	0.19	−0.16	0.53	1.00	-
Antiglycation	0.94	−0.09	−0.55	0.22	1.00

3.2. Yeast Viability Assay

The four fractions (FHB, FEB, FHL, FEL) could promote yeast cell viability up until day 11 compared to negative controls. The ethanol fraction (FEB 8 μg·mL^{-1} and FEL 10 μg·mL^{-1}) showed higher viability on day 11 with a lower concentration than the n-hexane fraction (FHB and FHL) (Figure 1).

3.3. Antiaging Analysis Based on Chronological Lifespan (CLS)

Based on CLS assay, FEB with a concentration of 8 μ μg·mL^{-1} could maintain the chronological age of yeast cells up to day 20 compared to negative controls. However, the FEB treatment was not better than the positive control and 0.1 μg·mL^{-1} ascorbic acid (Figure 2). A decrease in glucose content (calorie restriction) from 3% to 0.3% in *S. pombe* grown on synthetic defined (SD) medium can increase the chronological age up to 23 days [15].

3.4. Oxidative Stress Response Assay

The selected concentration of sample fractions, especially FEB promoted cells survival against H_2O_2-induced oxidative stress. Yeast cells treated with ethanol-based fractions (FEB and FEL) could grow well under 3 mM H_2O_2 oxidative stress on day 11 (Figures 3 and 4), whilst n-hexane-fraction could not promote yeast viability as compared to the ethanol-derived fractions.

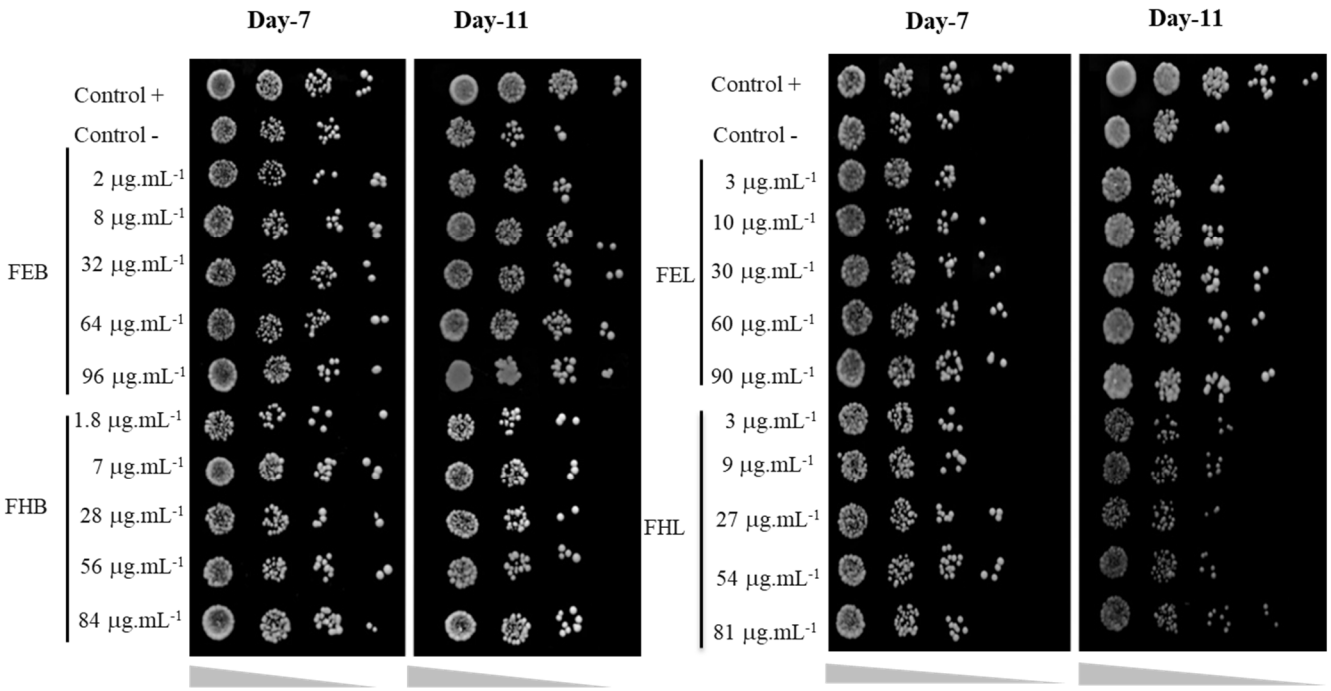

Figure 1. Effect of clove fraction treatment at various concentrations on the viability of *S. pombe* yeast grown on solid YES medium on the 7th and 11th days of incubation. FHB: clove bud n-hexane fraction, FHL: clove bud n-hexane fraction, FEB: clove bud ethanol fraction and FEL: clove leaf ethanol fraction. Yeasts grown on 0.3% and 3% glucose media without clove fraction were used as positive and negative controls, respectively.

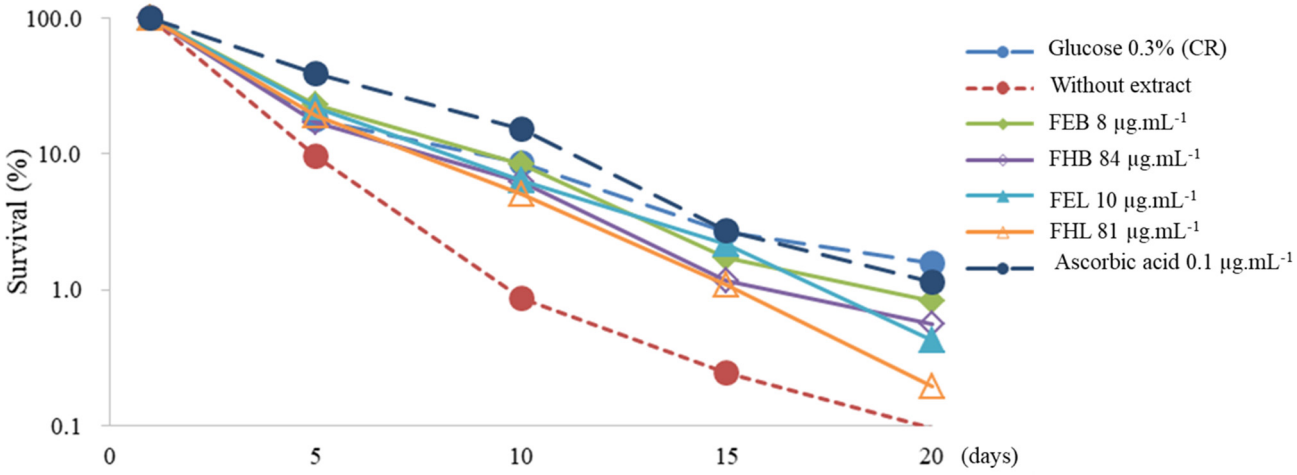

Figure 2. Effect of treatment of selected fractions grown on YES medium on the chronological age of *S. pombe* yeast cells. FHB: clove bud n-hexane fraction, FHL: clove bud n-hexane fraction, FEB: clove bud ethanol fraction and FEL: clove leaf ethanol fraction. Ascorbic acid as a control antioxidant compound. Ascorbic acid as a control treatment of antioxidant compounds. Yeasts grown on 0.3% and 3% glucose media without clove fraction were used as positive and negative controls, respectively.

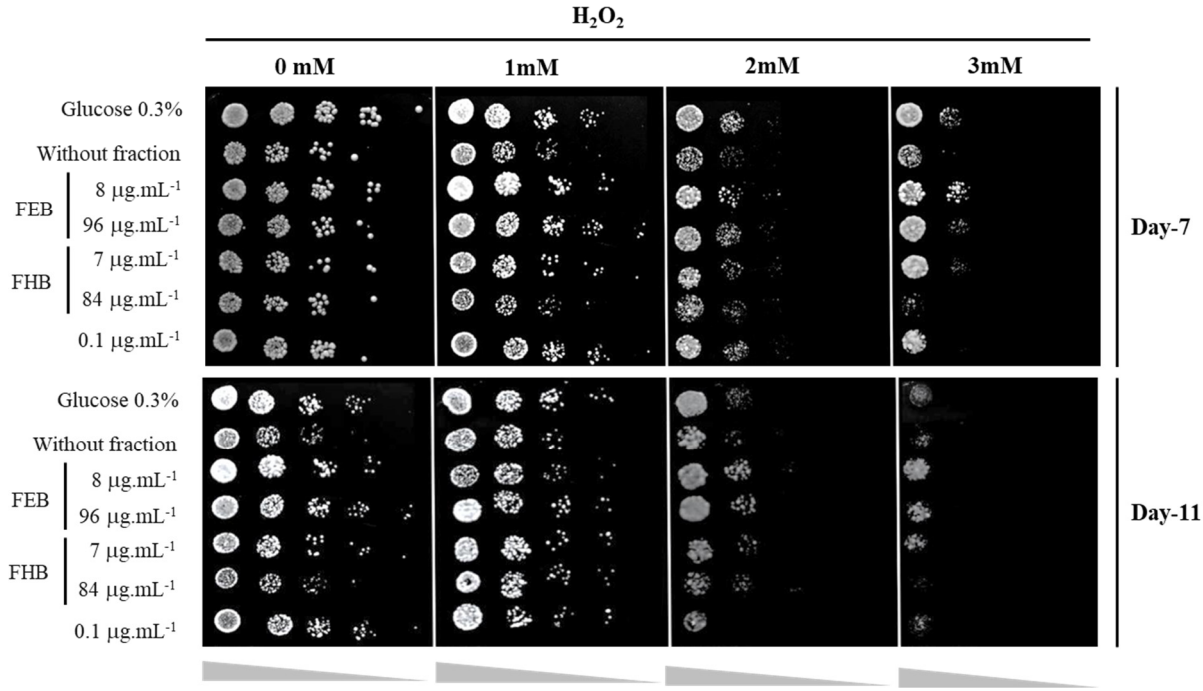

Figure 3. The effect of the treatment of two selected concentrations of each clove bud fraction on the viability of *S. pombe* against H_2O_2-induced oxidative stress conditions after 7 and 11 days of incubation. FHB: clove bud n-hexane fraction, FEB: clove bud ethanol fraction. Yeast grown in low glucose (0.3%) was designed as positive longevity control, while yeast grown in normal YES medium withour fraction was used as a negative control. Ascorbic acid was used as a control of antioxidant treatment.

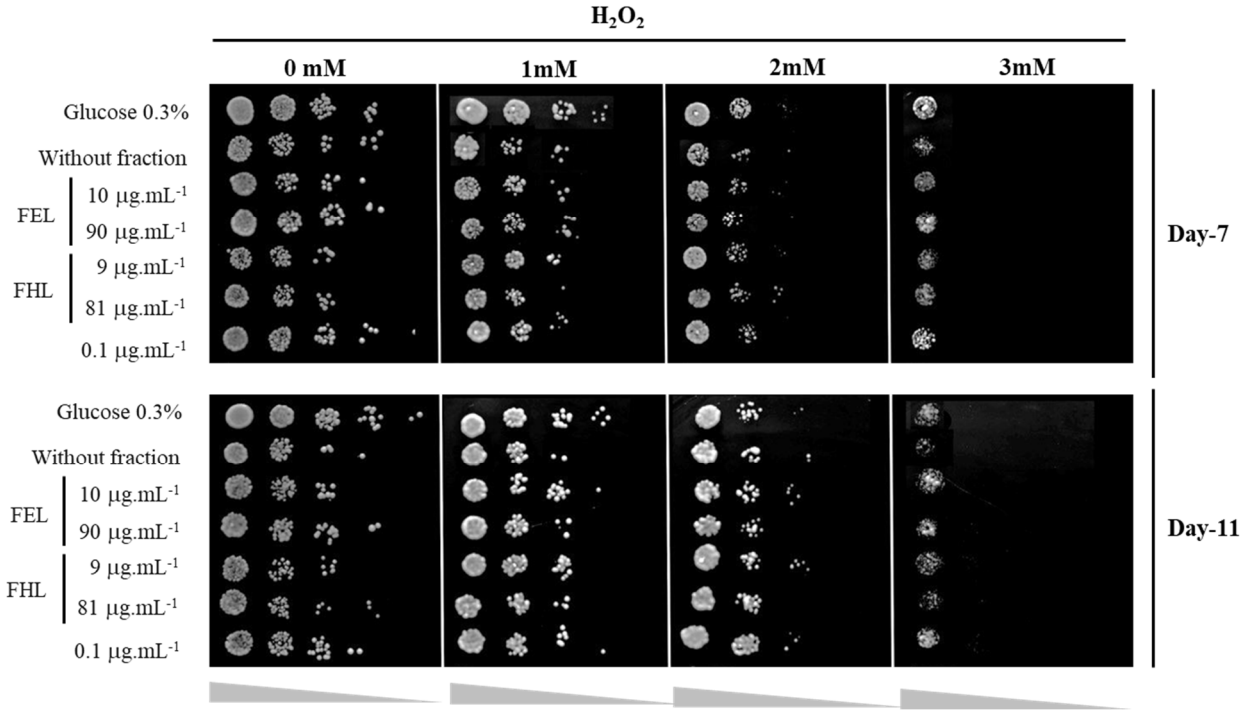

Figure 4. The effect of the treatment of two selected concentrations of each leave fraction on the viability of *S. pombe* against H_2O_2-induced oxidative stress conditions after 7 and 11 days of incubation. FHL: clove leaf n-hexane fraction, FEL: clove leaf ethanol fraction. Yeast grown in low glucose (0.3%) was designed as positive longevity control, while yeast grown in normal YES medium withour fraction was used as negative control. Ascorbic acid was used as a control of antioxidant treatment.

3.5. Mitochondrial Activity Assay

As expected, treatment of FEB and FEL could induce mitochondria activity as shown by fluorescence assay (Figure 5). Similar results were shown in the ascorbic acid and calorie restriction treatments as favorable control treatments. In contrast, no fluorescence was observed in negative control. Yeast cells were not seen to glow in the negative control.

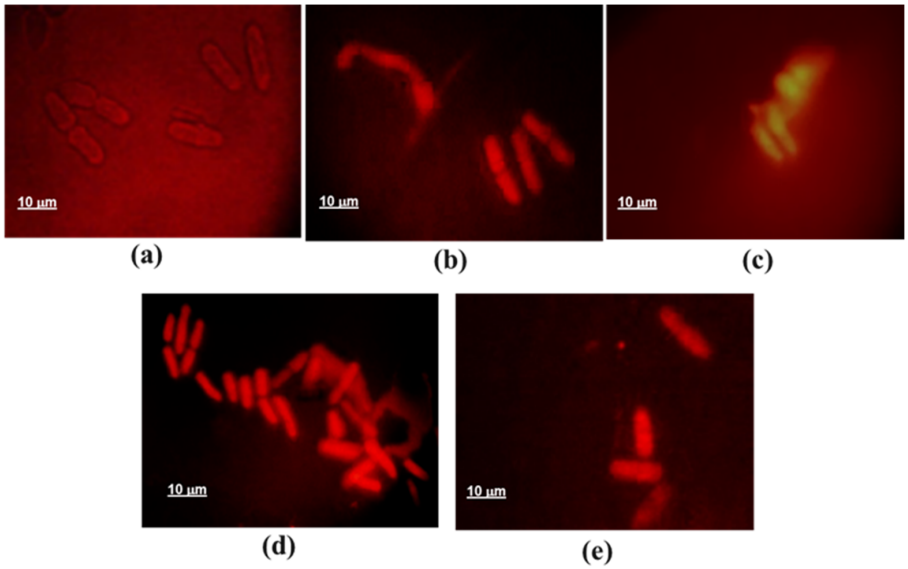

Figure 5. Effect of clove fraction on mitochondrial activity of *S. pombe*: (**a**) negative control (3% glucose of YES medium, 0 µg·mL^{-1} of fraction), (**b**) positive control (0.3% glucose of YES medium, 0 µg·mL^{-1} of fraction), (**c**) Ascorbic acid 0.1 µg·mL^{-1}, (**d**) FEB 8 µg·mL^{-1}, (**e**) FEL 10 µg·mL^{-1}. FEB: clove bud ethanol fraction and FEL: clove leaf ethanol fraction.

3.6. Cell Cycle Analysis

Based on CLS assay, FEB showed strong antiaging activity than other fractions. Indeed, cell cycle analysis revealed that FEB could cause cell cycle delay in *S.pombe*. For instance, FEB treatment can suppress the growth rate of cells from the G1 phase to the next phase, the S and G2 phases. FEB is thought to have an antiaging activity that prevents cells from aging more quickly into the G2 phase and maintains cells longer in the G1 phase (Figure 6).

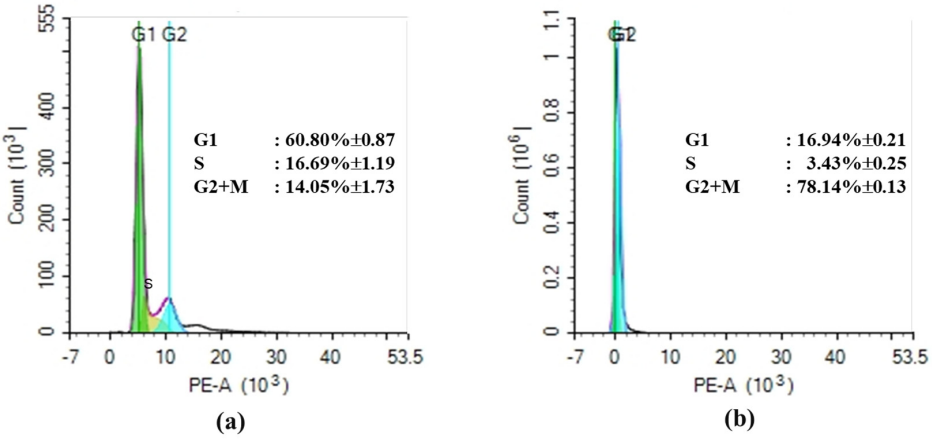

Figure 6. Effect of the ethanol fraction of clove bud on the cell cycle of *S. pombe.* Yeast cells were treated with (**a**) FEB (8 µg·mL^{-1}) and (**b**) without fraction. The total amount of each yeast cell phase was mentioned in the right corner of each figure. All figures and data are means and representative from three independent experiments.

3.7. LC-MS Data Analysis

As FEB showed the strongest antiaging potential, thus we further analyzed the chemical content of this particular fraction. Based on LC-MS data, FEB was found to have gallic acid (C7H6O5) (*m*/*z* 169.0) and (15Z)-9,12,13-Trihydroxy-15-octadecenoic acid at higher concentrations (C18H34O5) (*m*/*z* 329.2) than other compounds (Figure 7).

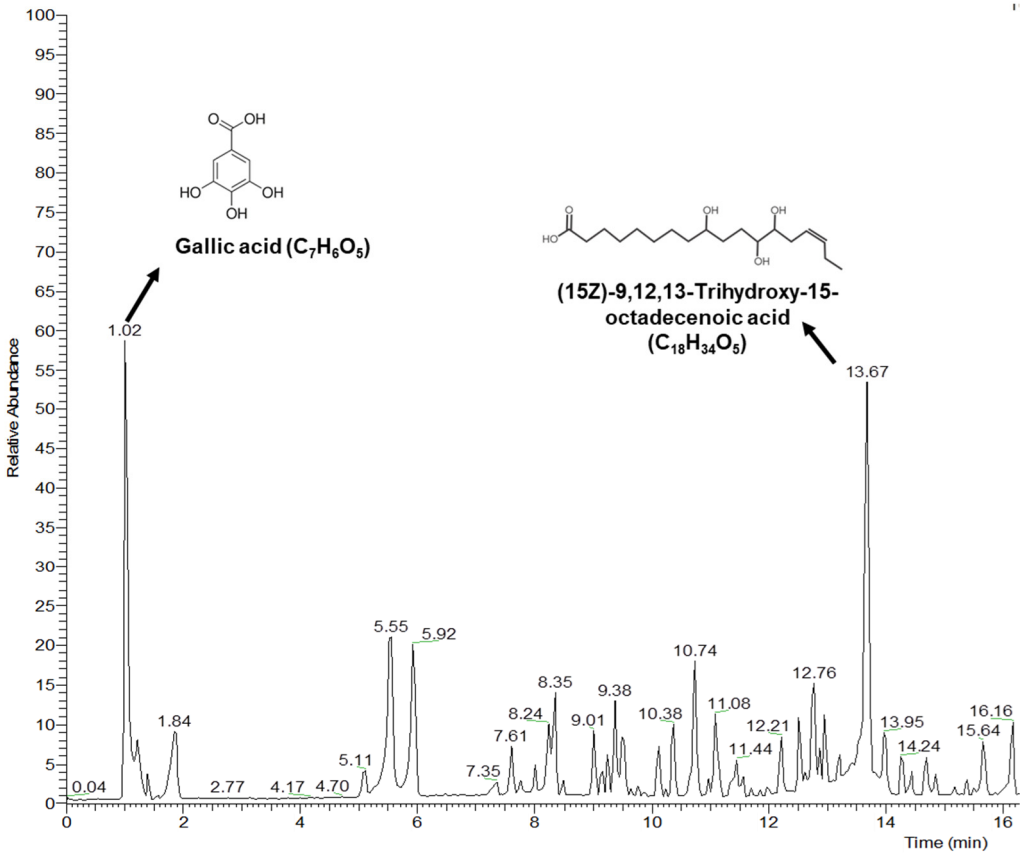

Figure 7. LC-MS analysis of ethanol fraction of clove bud (FEB), which obtained two dominant compounds of gallic acid and (15Z)-9,12,13-Trihydroxy-15-octadecenoic acid. The dominant peaks were identified using the particular mass identity through European (EU) Massbank.com to determine the identities of the detected compounds.

4. Discussion

Clove is one of the important spices that are commonly used in food additives. Further research on clove shows its potential application especially on pharmaceutical use, especially the volatile compound eugenol [16]. For instance, eugenol has been reported as an antimicrobial and antioxidant agent [17–19]. Our research, however, focuses on the ethanol and n-hexane fractions of both clove bud and leaves. The total flavonoid content was found high in FHL (25.65 ± 0.09 mgQE·g^{-1}), while low in FEB (7.58 ± 0.08 mgQE^{-1} g). Previous reports described that the total flavonoid content in the clove bud fraction was found higher by using 80% ethanol solvent and water solvent than n-hexane and ethyl acetate solvents [20]. Such variation on the quality and quantity of flavonoid content on phytoextracts may occur due to the biological and environmental background of the plants, including genetics, geographical elevation, and ecological conditions [21,22]. In fact, different techniques of extraction, although using the same solvent, may also affect flavonoid content on the phytoextract [23].

FHB (IC_{50} = 6.88 ± 0.20 µg·mL^{-1}) and FHL (IC_{50} = 24.24 ± 0.79 µg·mL^{-1}) showed the most potent antioxidant activity based on DPPH and ABTS assay, respectively. A previous study indicated that higher concentration of extract was required to deliver relatively similar antioxidant activity. For instance, 100 times higher concentration of the clove bud

ethanol extract (IC_{50} = 0.41 μg·mL^{-1}) had the strongest scavenging activity in the DPPH method, and the n-hexane extract (IC_{50} = 0.37 μg·mL^{-1}) had the strongest scavenging activity at the ABTS method [24]. Such different results may occur due to the purity of the sample, as in this study, we used a fraction sample.

Interestingly, FEB (IC_{50} = 2.61 ± 0.80 μg·mL^{-1}) showed strong antioxidant activity against TBA, which indicates its ability to inhibit the lipid peroxidation of linoleic acid. In addition, FEB showed the strongest antiglycation activity than other fractions. As compared to clove oil (15 μg·mL^{-1}), FEB relatively showed strong antioxidant activity toward TBA [25]. It has been reported that clove bud water extract at a concentration of 250-1000 μg·mL^{-1} can significantly inhibit the formation of advanced glycation end products (AGEs) and non-flourescent AGEs (Nε-(carboxymethyl) lysine (CML)) [26]. Our data indicate that further fractionation of clove extract may significantly reduce the active concentration with significant antioxidant and antiglycation bioactivities.

By using Pearson correlation analysis, we found that the flavonoid content of four sample fractions had a negative correlation with ABTS-based antioxidant. Thus, it is likely that other bioactive compounds may exhibit antioxidant activity in addition to flavonoid compounds. A previous study reported that clove is mainly composed by phenolic compounds including flavonoids, hidroxibenzoic acids, hidroxicinamic acids, and hidroxiphenyl propens. Amongst these compounds, eugenol obtained from clove oil is the main bioactive compound that is found in concentrations ranging from 9381.70 to 14,650.00 mg per 100 g of fresh plant material [27]. Other phytoextracts may also have phenolic compounds with high radical scavenging activity despite of the low flavonoid content [24,28]. For instance, ethyl acetate-derived clove extract contained oleanic acid in high concentration which considered has antitumor activity [29]. As in our study, FEB sample was enriched by gallic acid, one of the phenolic, a non-flavonoid compound, but belong to hidroxibenzoic acid group instead. In addition, 15Z)-9,12,13-trihydroxy-15-octadecenoic acid, which belong to fatty acid compounds, was found as second major constituent in FEB.

Our data indicate that lower concentration of fractions promoted yeast viability on days 9-11 of incubation than that of previously reported using clove leave extract (100 μg·mL^{-1}) [6]. Previous studies reported that plant extract or fractions could essentially promote yeast life span. In instance, bioactive compound 11αOH-KA (7.5 μg/mL), extracted from *Adenostema lavenia* prolonged *S. pombe* life span in much lower concentration than the corresponding chroroform (888 μg/mL^{-1}) and water fractions (1260 μg·mL^{-1}) [30]. Thus, further purification of FEB is required for further development of the clove-derived bioactive compound as antiaging agent.

Our data indicate that FEB and FEL show the most potential antioxidant agent at cellular levels. *S. pombe* has been reported to induce stress response system in combating oxidative stress. S. pombe will activate intracellular response depending on the severity of H_2O_2-induced oxidative stress via mitogen-activated protein kinase (MAPK). Indeed, at low concentration of H_2O_2 (below 1 mM), MAPK-Pap1 serves as a transcription factor that regulate the downstream pathway of antistress response, the central environmental stress response (CESR). As in this study, we used extreme conditions of oxidative stress toward *S. pombe* cells. Thus, the activity of transcriptional factor Sty1 potentially activates the CESR, which further results in oxidative stress tolerance phenotype [31]. Further study is required to clarify the direct or indirect activity of FEB and FEL toward the Sty1-dependent oxidative stress response pathway on *S. pombe*.

In addition to that MAPK-CESR pathway, nitric oxide has also been reported to regulate oxidative stress response in *S.pombe* [32]. The activity of mitochondria via adaptive ROS signaling has also been suggested to induce oxidative stress response pathway in yeast [33]. In this regard, we further analyze the mitochondria activity following treatment of the FEB and FEL fractions.

FEB likely regulate mitochondria activity which in turn induces oxidative stress response. From LC/MS analysis, gallic acid was majorly present in FEB. Gallic acid

belongs to phenolic compounds, especially the hydroxybenzoates group. Gallic acid has been previously reported to be one of the major constituents of ethanolic phytoextract including green teas, dried leaves of raspberry, grape seeds, and fresh hazelnuts [34]. Previous reports showed that phytoextract and bioactive compounds, especially gallic, acid might induce yeast life span potentially due to mitochondrial activation [6,30,35–39]. Induction of mitochondria activity may result in low oxidative stress conditions, which facilitate activation of adaptive oxidative stress response signaling [33,40]. However, little is known regarding the bioactivity of 15Z)-9,12,13-Trihydroxy-15-octadecenoic acid. It is worth noting that octadecanoic acids has been reported as constituents on the tea leaves of *Coreopsis* cultivars which exhibit antioxidant activity [41].

The suppression on G1 phase on *S. pombe* cell cycle by FEB treatment strongly suggest the antiaging mode of action of the particular fraction. In this regard, previous studies reported that cell cycle delay may link to the prolonged life span. Indeed, previous studies reported that checkpoint in G1 for cell cycle arrest and entry into a quiescent state causes cell cycle delay [42]. However, little is known regarding activity of gallic acid in delaying cell cycle. Previous study reported that gallic acid deliver anticancer activity on human bladder transitional carcinoma cell line by induces G2/M phase cell cycle arrest [43]. Cell cycle arrest on G1 has also been reported to involve in the calorie restriction-dependent longevity of yeast [42].

5. Conclusions

Ethanol fractions of clove bud exhibit antiaging properties in vitro and at cellular level. The particular fractions are predominately rich in gallic acid and 15Z)-9,12,13-Trihydroxy-15-octadecenoic acid compounds. FEB potentially promotes yeast longevity by inducing intracellular oxidative stress response, mitochondria activity, and cell cycle delay. FEB arrests yeast cell cycle at G1 phase that may result in yeast longevity. The potential antiaging properties of FEB promotes its further potential application in cosmetics products equipped with pharmaceutical effect.

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