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Modulation of Aging in Yeast *Saccharomyces cerevisiae* by Roselle Petal Extract (*Hibiscus sabdariffa* L.)

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Abstract: Cellular aging drives diseases. Free radicals are called the main cause of cellular aging. Antioxidants can protect the body from free radical attack by reducing its negative effects. An antioxidant source can be obtained from plants, such as roselle (*Hibiscus sabdariffa.* L). This research was conducted to analyze the ability of roselle petals extract to increase the life span of model organisms *Saccharomyces cerevisiae* BY4741. The IC_{50} value of extract-derived antioxidant activity based on DPPH and ABTS assays were 367,6 ppm and 921,4 ppm, respectively. Antioxidant and antiaging tests were performed *in vivo* via spot assay. The result showed that the roselle petals extract-treated groups had the ability to extend the life span of yeast BY4741 under normal and oxidative stress conditions. This is also supported by the mitochondrial activity of BY4741 with roselle petals extract-treated group higher than those without the extract treatment. The 300 ppm concentration was chosen as the best concentration of roselle petals extract to increase the life span in yeast. Interestingly, important gene to regulate aging in yeast (*SIR2*) as well as oxidative stress response genes (*GPX1* and *GLR1*) were significantly up-regulated in yeast cells treated with 300 ppm roselle extract, as revealed via Real-time quantitative analysis. These results suggest that roselle petals extract exhibits antiaging effects likely via regulation of anti-oxidative stress and aging genes.

Keywords: Antioxidant, Antiglycation, Antiaging, *Hibiscus sabdariffa*. L, *Saccharomyces cerevisiae*

Introduction

Aging defines as a decline in physiological functions gradually, thereby reducing the growth rate (Young, 1997). Hayflick (1976) asserted that the lifespan of cells is limited. In normal condition, a cellular division occurred until reaching a limit, called as cellular aging. However, it comes earlier with presence of cellular distractors. In instance, accumulation of Reactive Oxygen Species (ROS) is reported to the factors that promote cellular aging (Gladyshev, 2014). As reported, cellular aging is responsible for a rising risk of some degenerative diseases such as Alzheimer, Parkinson and cancer (Niccoli and Linda, 2012).

ROS can be generated through normal metabolic process in human body, but in excessive level, it may induce oxidative stress. As free radicals, ROS had the ability to invade major molecules inside the cell such as DNA, lipid and protein (Dat *et al*., 2000). Various approaches been done to combat cellular aging. Among them, application antioxidant agents is considered as one of the promising way to reduce deleterious effect of ROS and AGEs molecules.

Currently, roselle (*Hibiscus sabdariffa L.*) tea drink has been believed to have positive effect to the human body. Indeed, some people are urged to consume the particular tea drink in daily basis. It is reported that roselle petals contain plenty of chemicals including water, protein, fat, fiber, calcium, phosphorous, iron, β-carotene, thiamine, riboflavin, niacin, ascorbic acid, vitamins (B1, B2 and D), anthocyanine, flavonoid and polyphenol (Liu *et al*., 2002; Lin *et al*., 2003; Zuraida *et al*., 2015). Several studies have showed that extracts of roselle could prevent diseases like cancer, obesity and cardiovascular diseases (atherosclerosis and coronary heart disease) (Tseng *et al*., 2000; Ochani and D'Mello, 2009; Alarcon-Aguilar *et al*., 2007; Carvajal-Zarrabal *et al*., 2009). Several groups of compounds in the extract, such as

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anthocyanins and protocatechuic acid, have been implicated as responsible for these effects (Da-Costa-Rocha *et al*., 2014). Hibiscus extract also demonstrated antibacterial, antifungal, antiparasitic, anti-inflammatory and antioxidant effect (Al-Hashimi, 2012; Mardiah *et al*., 2015). Protocatechuic acid compound in extract inhibited the growth of methicillin-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Liu *et al*., 2005). Moreover, roselle extract showed therapeutic promise in decreasing and preventing the development of atherosclerosis and possible related cardiovascular pathologies linked with diabetes (Da-Costa-Rocha *et al*., 2014). Although chemical properties of roselle are known, yet, the effects of roselle to cellular systems remain elusive. Indeed, their anti-aging activity has remained a great opportunity for investigation.

In this study, *Saccharomyces cerevisiae* BY4741 was used as model to evaluate the effects of roselle petal extract on aging pathways. Aging process in yeasts is complex and involves many cellular components conserved to that in mammalian systems. *SIR2* gene is found to be one of the key factors related to the cellular aging. The gene is conserved in both yeasts and higher organisms and enables to regulate the aging processes such as regulation of stress defense, metabolism and glucose tolerance (Roux *et al*., 2010). The higher activity of *SIR2* would prolong yeast's lifespan (Lin *et al*., 2016). Furthermore, mitochondrial activities are also involved in the cellular aging process. Strong mitochondrial membrane potential is also associated with extension of yeast's lifespan (Baracca *et al*., 2003). Indeed, high mitochondrial activity may induce mitochondrial-ROS adaptive signaling which culminate in the up-regulation of genes encoding cellular antioxidant enzymes such as glutathione peroxidase (*GPX*1) and glutathione reductase (*GLR*1), superoxide dismutase (*SOD*1) and catalase (*CTT*1). Their enzymatic activities are essential in scavenging ROS reactivity within cells, leading to extension of cell's lifespan (Bai *et al*., 2016).

This present work aimed to observe the antiaging activity of roselle petal extract applied in yeast *S. cerevisiae* as model and investigate its mechanism on how the aging process was delayed. Interestingly, we found that roselle extract is a potential antiaging agent that increases mitochondrial activity as well as gene involved in oxidative stress in yeast thus prolong yeast life span.

Materials and Methods

Cultures and Medium

The medium for culture of *S. cerevisiae* BY4741 was YPD (yeast extract peptone dextrose) containing 2% glucose, 1% yeast extract, 2% peptone and 2% agar (Jarolim *et al*., 2004). Yeast in the room temperature was sub-cultured in a liquid YPD medium for stock culture and working culture, then incubated at room temperature (25-28°C).

Extraction of Roselle Petal

The roselle petals were collected from Roselle Plantation in Madiun, East Java, Indonesia. The procedure for extraction was adopted from previous method (Zuraida *et al*., 2015). The roselle petals were macerated using ethanol 70% at ratio of 1:5 (sample:solvent). Sample was soaked for 3×24 h and stirred twice a day to dissolve bioactive compounds in the sample. After macerated substance was obtained, the process was repeated twice using the same volume of solvent. The substance was collected and concentrated using rotary evaporator (<60°C) to produce crude extract of roselle petals. The ethanol-derived extract of roselle petals showed the following characteristics: Paste, brownish red in color.

Antioxidant Activity using DPPH and ABTS Assays

The *1,1-diphenil-2-pikrilhidrazil* (DPPH) procedure was conducted as described by previous study (Salazar *et al*., 2009). Briefly, extract was dissolved in absolute ethanol and the solution was made in 5 concentrations, including 150 ppm, 300 ppm, 450 ppm, 600 ppm and 750 ppm. Sample (100 μL) from each solution was transferred into a 96-well microplate (triplicates for each sample), added with 100 μl of 125 μM DPPH in ethanol solutions and incubated at 37°C for 30 min. The absorbance was measured at 514 nm using spectrophotometer.

The *2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)* (ABTS) procedure was performed according to previous study (Re *et al*., 1999). Briefly, ABTS solution was oxidized with $K_2S_2O_8$ to produce radicals. ABTS radicals (180 μL) were transferred into 96-well microplate and added with sample solution (20 μL) at triplicate. After incubation at dark room (25-28°C) for 30 min, the absorbance was measured using spectrophotometer at 734 nm. Ascorbic acid was used as positive control. The IC_{50}, defined as the concentration of substance that generates 50% reduction of free radicals, was determined. Percent inhibition was calculated using the following formula:

$$Inhibition(\%) = 1 - \left(\frac{Abs\,sample - Abs\,control}{Abs\,blank - Abs\,control} \right) \times 100\%$$

Where:

Abs sample = Absorbance of sample (the extracts + DPPH/ABTS)

Abs blank = Ethanol + DPPH/ABTS Abs control is ethanol

Table 1: Primer pairs for Real Time PCR used in this study

Gene	Forward primer	Reverse primer
SIR2	CTTCCACTATGCCCGTACTGT	CGACATTGAACCCTGTGATGC
GPX1	CAGTAAGCGGGAAGTCTGGAA	ACCACCTTCCCATTTCGGTC
GLR1	CTATTGCAGCGGGCAGAAAG	GCTGGGGACGTTCTCGTAAT
ACT1	GGTGTTACTCACGTCGTTCCA	CAGTCAAATCTCTACCGGCCA

Aging Assay

Aging assay was performed by using spot test, as described previously (Stephan *et al*., 2013). BY4741 was cultured in 3 ml of liquid YPD for 24 h started at Optical Density (OD) of 0.1. Assay was performed in five different treatments (150, 300, 450, 600 and 750 ppm). Yeast culture without extract supplementation was used as negative control, while yeast cultured in low glucose (0.5%) was used as positive control. A low glucose treatment is considered as Calorie Restriction (CR) conditions. Such CR conditions will induce yeast cellular mechanisms including mitochondrial activities respons to oxidative stress and autophagy, which contribute to the yeast's lifespan extension (Kaeberlein, 2010). Each culture was incubated for 15 days where spot test was conducted at each 10 and 15 days of incubations, Each culture was adjusted to OD = 1 then serially diluted (up to 10-4). About 2 µL from each dilution suspension was then spotted on solid YPD medium. Spotted medium were incubated for 3 days at room temperature.

Oxidative Stress Tolerance Assay

The spot assay was carried out as above mentioned. Yet, about 2 µL from each dilution suspension was spotted on solid YPD medium containing H_2O_2-induced oxidative stress in various concentrations (7, 8 and 9 mM).

Active Mitochondrial Staining

The procedure was performed according to the previous study (Marchi and Cavalieri, 2008). Yeast BY4741 was incubated in liquid YPD medium supplemented with roselle petal extracts (150, 300, 450, 600 and 750 ppm). The yeast suspension (1 mL) was centrifuged and the pellet obtained was suspended using phosphate buffer and added with 100 nM rhodamine B. Yeast suspensions were then incubated for 30 min at 25°C. Light exposure was avoided throughout experiment. Mitochondrial activity was observed under fluorescence microscope (Olympus BX51).

Gene Expression Assay using Real Time-Polymerase Chain Reaction (RT-PCR)

Yeast BY4741 was treated with a selected concentration of roselle petal extract (300 ppm). Yeast was cultured in YPD medium and incubated in a shaking incubator for overnight at 30°C. Positive and negative controls treatments were prepared as previously described in aging assay. Furthermore, mRNA extraction was conducted using RNeasy Mini Kit (Qiagen, USA). The total mRNA was used as template for cDNA synthesis using iSript cDNA Synthesis Kit (Bio-Rad, USA). RT-PCR experiment was performed using the particular cDNA template with Thunderbird SYBR qPCR Mix (Toyobo, Japan). RT-PCR analysis was conducted to investigate the effects of roselle petal extract on the expression of that aging-related gene n, i.e., *SIR2* gene and response genes to the oxidative stress (glutathione peroxidase *GPX1*, glutathione reductase *GLR1*), using specific primers to each respective genes (Table 1). Data obtained from RT-PCR were analyzed using relative quantification method $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) and normalized towards reference genes *ACT1.*

Results and Discussion

Antioxidant Activities

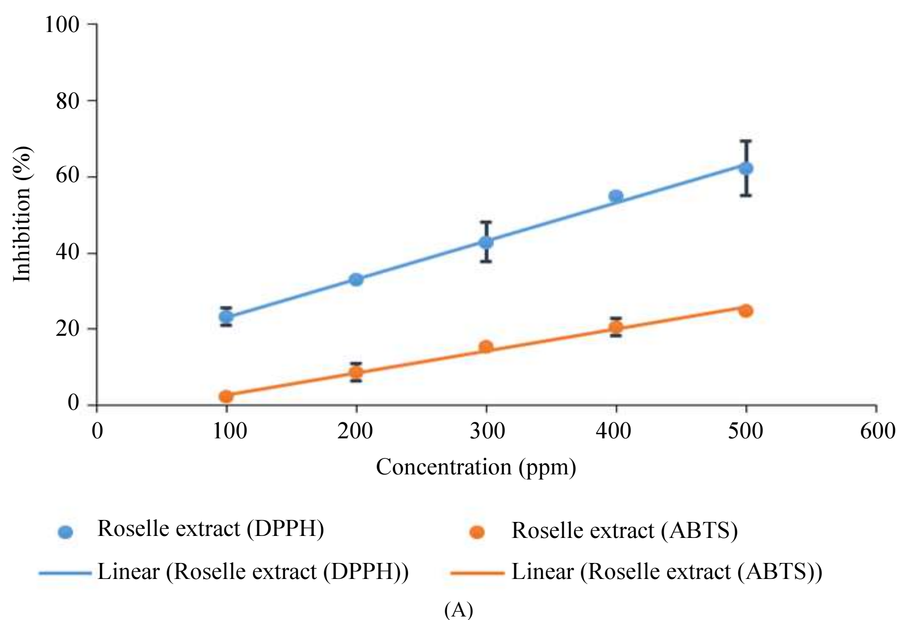
Antioxidant activity resulted from DPPH and ABTS assay was represented as IC_{50}, resulting in value of 367.6 ppm and 921.5 ppm, respectively (Fig. 1A, Table 2). The IC_{50} for positive control (ascorbic acid) was much lower than that roselle petal extract (Fig. 1B and 1C). Antioxidant of the extract was low since it is a crude extract or not purified. It is worth noting that the roselle extract inhibited the activity of radical DPPH and ABTS in dose dependent manner (Fig. 1A). Compared to other plant extracts, IC_{50} of roselle petal extract (based on DPPH assay) was higher than citronella extract (*Cymbopogon citratus*), i.e., 1998 ppm (Lu *et al*., 2014). Meanwhile, IC_{50} of roselle petal extract (ABTS assay) was lower than flower extract of *Helichrysum obconicum,* i.e., 687.9 ppm (Figueira *et al*., 2014). Our results indicated that IC_{50} value based on DPPH and ABTS assays was dissimilar. It is due to different radicals used, which might elicit diverse responses of the antioxidative compounds. Similarly, the discrepancy on IC_{50} between DPPH and ABTS assay was also reported by former studies that investigated antioxidant activity of 50 foods (Floegel *et al*., 2011). This confirmed that each sample could exert various mechanisms on scavenging free radicals.

Based on *in vitro* study of antioxidant activities, the crude extract of roselle petals showed the highest antioxidant activity on scavenging DPPH radicals. This indicates that the extract is promising as antioxidant agent. Furthermore, we investigated antiaging activity of the extract by evaluating the lifespan extension of yeast *S. cerevisiae* BY4741.

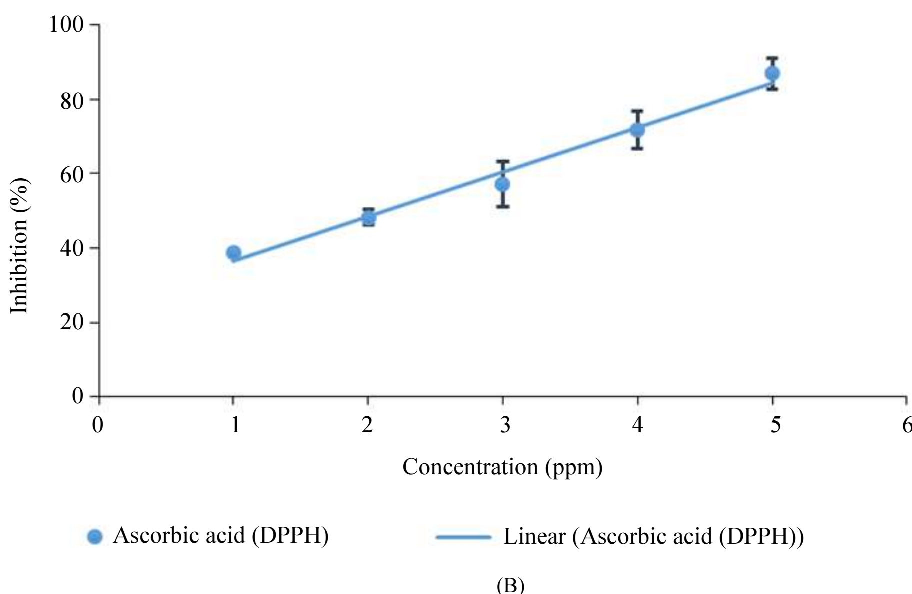
Yeast Lifespan Analysis

The result showed that Roselle petal extract (150, 300 and 450 ppm) could extend yeast's lifespan (Fig. 2) as observed in the effects of extracts on viability of yeast, compared with that without extract addition. Presence of the extract could produce better viability for 15-days culture in comparison with absence of the extract. The viability was even similar to the culture treated in CR conditions (positive control). In our experiment, administration of Roselle petal extract could extend the lifespan of yeast even though in non-CR condition, i.e., culture containing 2% glucose. This finding indicates that the extract may mimic regulation mechanism on the physiological system to that treated with CR condition, leading to lifespan extension.

To our knowledge, this is the first experiment to show the potential properties of Roselle petals as antiaging agent. Previously, a study on antiaging properties of herb plant *Gastrodia elata* (Orchidaceae) was reported (Lin *et al*., 2016). The proposed mechanism of antiaging properties is the improved cellular activity on neutralizing oxidative stress. Therefore, further experiment is carried out, focusing mainly on how the extract would induces oxidative stress tolerance mechanisms in yeast.



(A)



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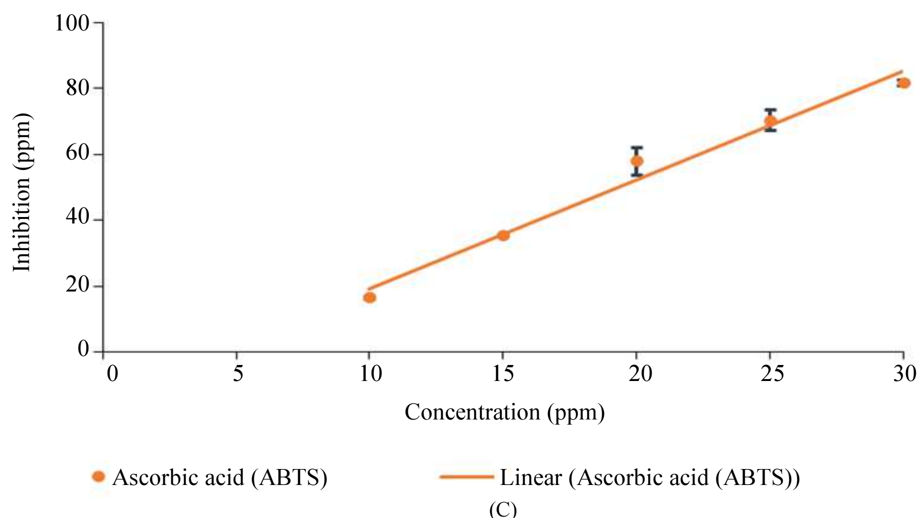


Fig. 1: Anti-oxidant activity of ethanol-derived roselle petal extract is lower than that control, ascorbic acid. (A) The anti-oxidant activity of Roselle petal extract is occurred in dose dependent manner against radical DPPH (R^2 = 0.996) and ABTS (R^2 = 0.991). Anti-oxidant activity of control substance, ascorbic acid, against (B) DPPH and (C) ABTS

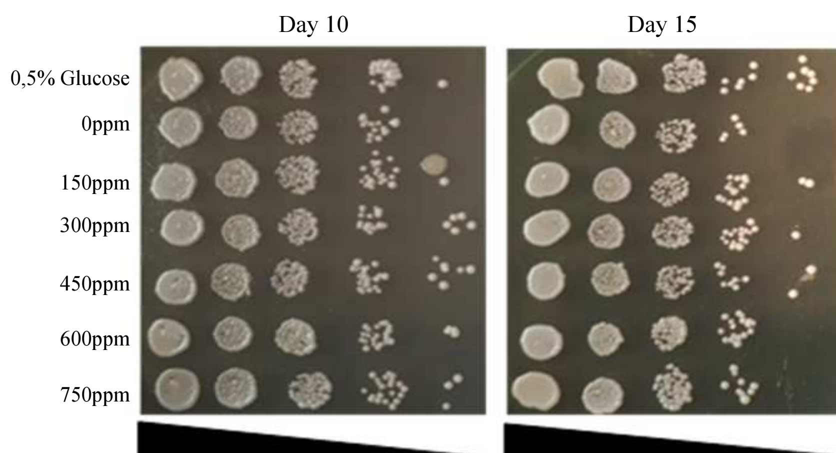


Fig. 2: Effects of Roselle petal extracts on the lifespan of *S. cerevisiae* BY4741. Yeast cells were grown in various concentrations of extract for 15 days. Spot test was performed at indicated time. Yeast cultured in YPD medium with low glucose (0.5%) was used as positive control. Spotted medium were incubated for 3 days in room temperature

Table 2: Antioxidant and antiglycation activity of roselle petal extracts *in vitro*

	IC_{50} Antioxidant activity (ppm)	
Sample	DPPH assay	ABTS assay
Ascorbic acid	2.1±0.345[a]	19.3±2.200[a]
Roselle petal extract	367.6±5.214[b]	921.4±13,400[b]

Each value with different letters are significantly different within each assay at the level of $p<0.05$

Oxidative Stress Tolerance

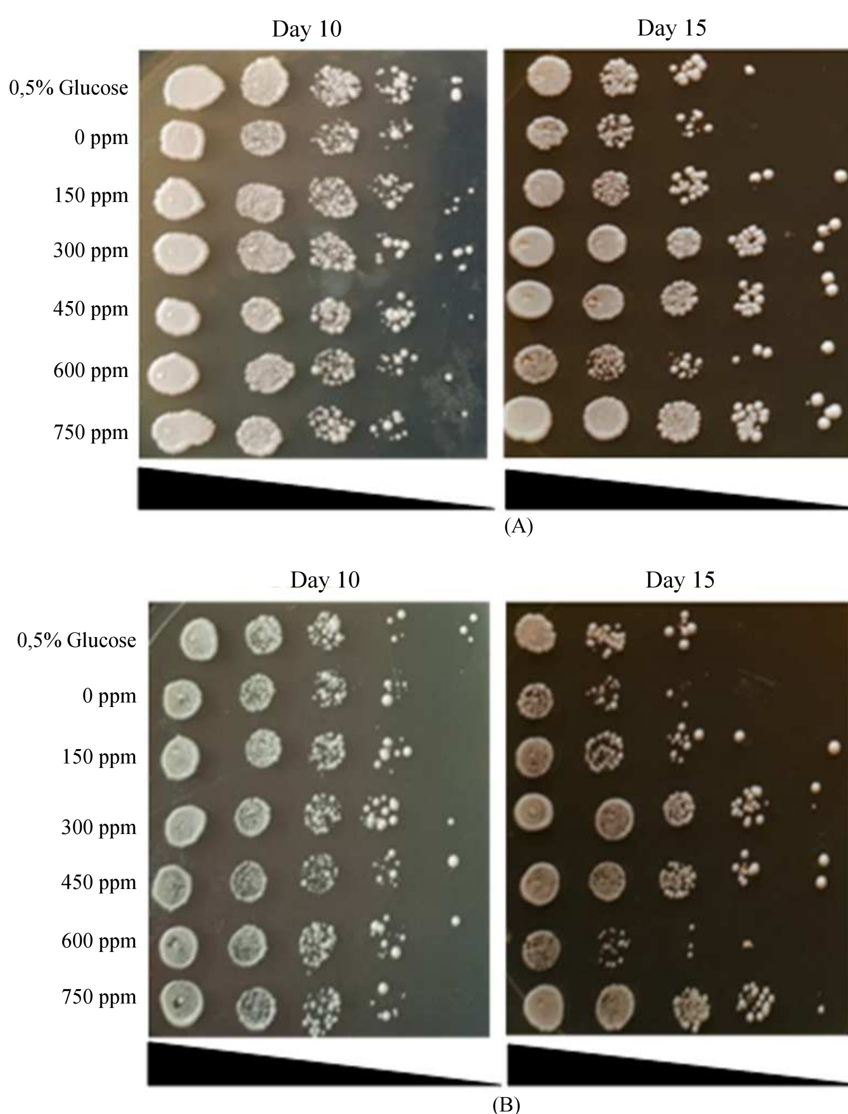
The results showed that treatment of roselle petal extract improved tolerance of yeast to H_2O_2-induced oxidative stress induced. This effect obviously occurred primarily on 15-days old yeast culture (Fig. 3). Interestingly, the yeast viability treated with the extract was higher than that positive control (CR condition). In addition, we found that the extract at 300 ppm was regarded as the best concentration in relation to provide the most desirable viability against oxidative stress with a high concentration of H_2O_2 (9 mM). The capability of the extract on inducing intracellular oxidative stress responses for yeast could indicate promising candidate as pro-oxidant.

Compound with pro-oxidant activity enables to enhance cellular oxidation that involves free radicals. Previous study successfully reported polyphenols as the pro-oxidant agent isolated from green tea, capable of inducing oxidative stress responses in yeast through producing free radicals H_2O_2 (Maeta *et al*., 2007). The

induction of intracellular response against oxidative stress is reported to be associated with mitochondrial activity. The rising mitochondrial activity allows to induce mitochondrial-ROS adaptive signaling mechanism which plays a central role in assisting yeast cells to cope with oxidative stress. The mechanism could be triggered through CR condition (Burtner *et al*., 2009). Hence, further test was performed to observe role of the extracts on mitochondrial activity.

Mitochondrial Activity Assay

The test aimed to observe the possible *in vivo* induction of yeast mitochondrial activity by roselle extract. In comparison with negative control (0 ppm), the mitochondrial activity of extract-treated yeast was much higher, as indicated by stronger and brighter red color (Fig. 4). Interestingly, the extract could induce mitochondrial activity in a dose-independent manner. As compared to positive control (0.5% glucose), mitochondrial activity of yeast treated with the extract seemed to be similar, even higher at extract concentration of 300 ppm. Potential energy in mitochondrial membrane could induce fluorescence from rhodamine, while the rate of fluorescence was comparable with potential membrane activity of mitochondria (Baracca *et al*., 2003). This suggests that brighter color means a greater mitochondrial activity. Our result showed that the addition of extract could enhance mitochondrial activity, in which the best effect was attributed to concentration of 300 ppm. Our finding confirmed that the roselle petal extract could likely promote mitochondria-ROS adaptive signalling mimicking to that intracellular stress response under CR condition. Additionally, *Gastrodia elata* was also reported to have similar effect (Lin *et al*., 2016).



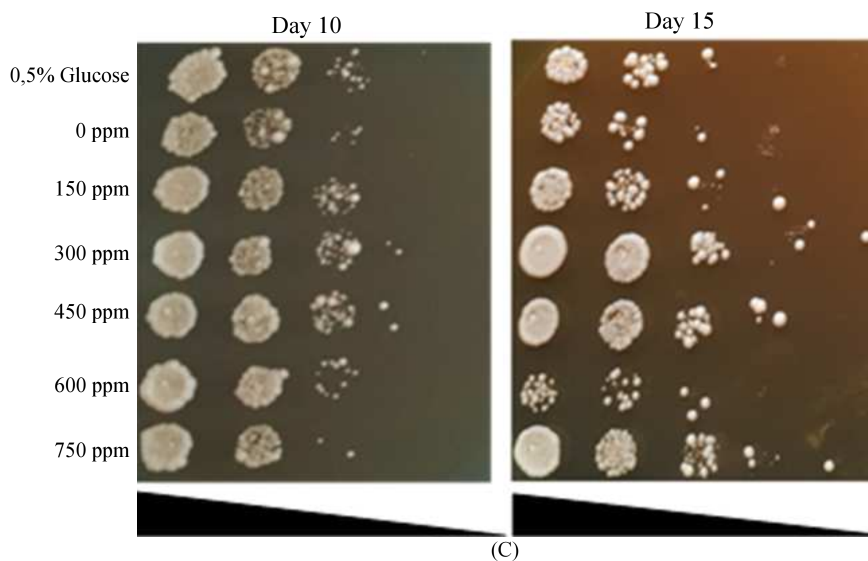


Fig. 3: Effects of Roselle petal extracts on viability of *S. cerevisiae* BY4741 against H2O2 at (A) 7 mM, (B) 8 mM and (C) 9 mM. Yeast were grown in various concentrations of extract for 15 days. Spot test was performed at indicated time. Yeast cultured in YPD medium with low glucose (0.5%) was used as positive control. Spotted medium were incubated for 3 days in room temperature

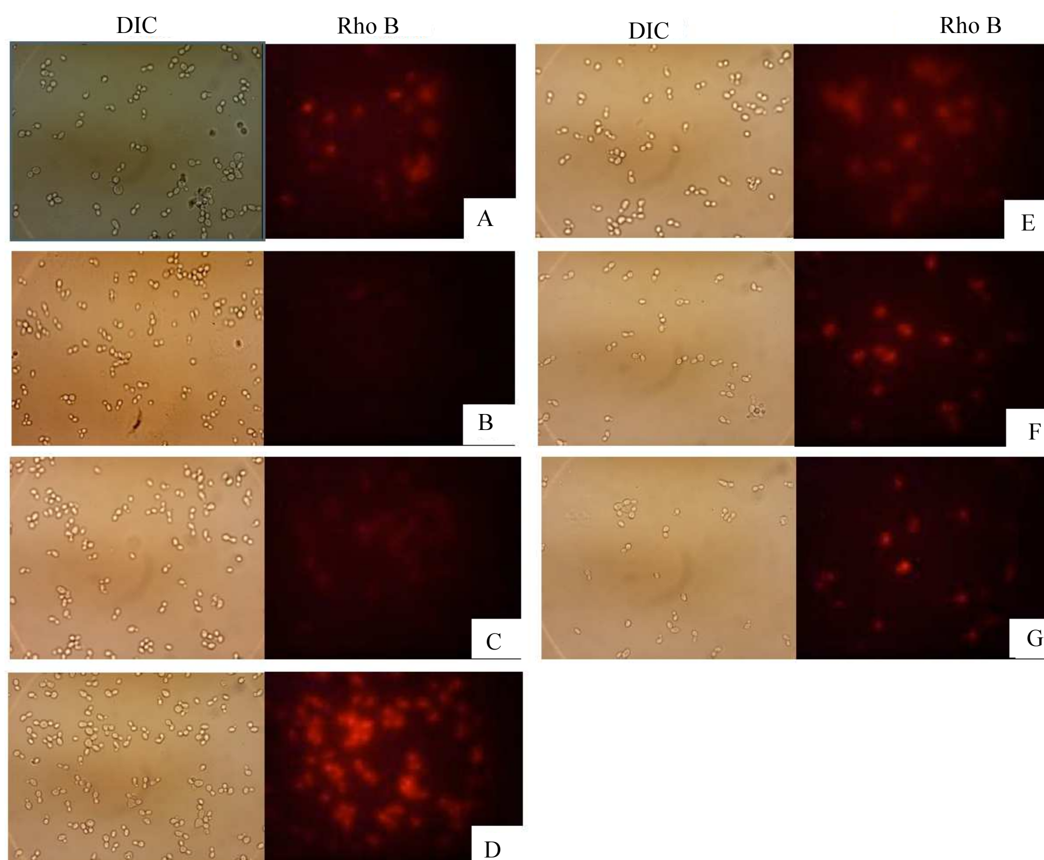


Fig. 4: The effects of Roselle petal extract on mitochondrial activity of *S. cerevisiae* BY4741 using rhodamine B staining. The treatment groups: (A) 0,5% glucose, (B) 0 ppm, (C) 150 ppm, (D) 300 ppm, (E) 450 ppm, (F) 600 ppm and (G) 750 ppm. Yeast cultured in YPD medium in low glucose (0.5%) was used as positive control

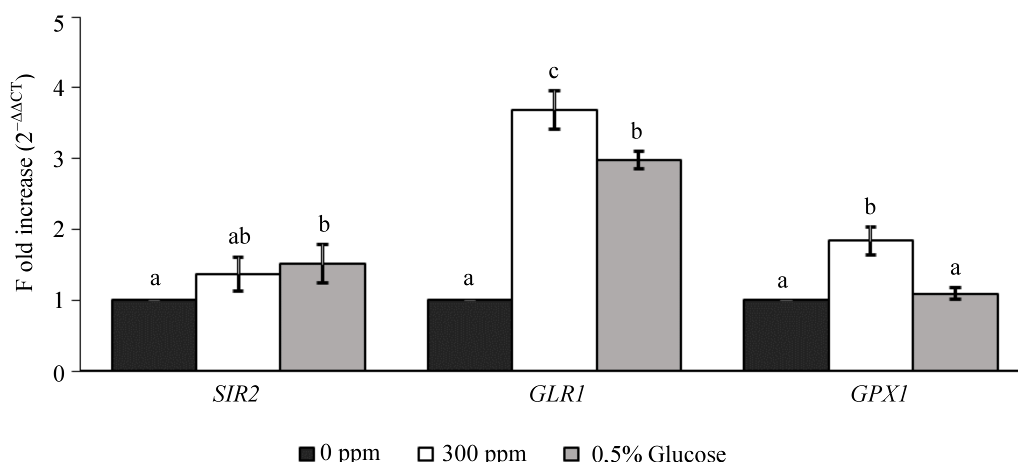


Fig. 5: The effects of Roselle petal extract on gene expression of aging pathway (*SIR2*) and tolerance against oxidative stress (*GLR1* and *GPX1*) investigated using RT-PCR experiment. Yeast cultured in 0.5% glucose was used as positive control. The level of gene expression *SIR2, GLR1* and *GPX1* was normalized using reference gene *ACT1.* Bar data which followed with the same alphabet not significant in DMRT (Duncan's Multiple Range Test) 5% test

Real-Time PCR

The RT-PCR analysis was performed to investigate the effects of roselle petal extract on gene expression that involves in response pathways against oxidative stress (*GLR1* and *GPX1*) and aging pathways (*SIR2*). Treatment of extract (300 ppm) upregulated the expression of all targeted genes, compared to that without extract treatment (Fig. 5). However, in comparison with glucose 0.5% (CR), the gene expression of extract treatment was much higher for gene expression involved in oxidative stress (GLR1 and GPX1) and lower for gene expression involved in the aging pathway (SIR2) (Fig. 5). CR condition could alter metabolic system of yeast, shifting from fermentation (2% glucose) to respiration system. Activation of respiration system promoted formation of ROS and Sty1 MAP kinase. Consequently, the activated Sty1 gene could induce essential gene expression from stress response to extend yeast's lifespan. The level of those gene expressions at concentration of 300 ppm indicated the involvement of the genes on antiaging and extract. The enhancing *SIR2* expression would promote extension of yeast's lifespan (Lin *et al*., 2016), meanwhile the expression of *GPX1* and *GLR1* genes presented a mitochondrial enzymatic defense against ROS. GPX1 is recognized as one of the antioxidant enzymes that enable to alleviate free radicals and lipid peroxide (Shirazi *et al*., 2013), while *GLR1* shows a pivotal role in protecting and avoiding cellular oxidative stress (Grant *et al*., 1996).

This present work is the first report that uncovers potential of roselle petal extract for inducing genes associated with response pathway against oxidative stress (*GLR1* and *GPX1*) and aging pathway (*SIR2*). Previous report found that polyphenolic compound in apple extract could also upregulates gene expression of aging pathway (*SIR2*) and antioxidant (*SOD1* and *SOD2*) in yeast (Xiang *et al*., 2011).

Conclusion

Antiaging and antioxidant properties of roselle petal extract were best found at 300 ppm. The extract was potential antioxidant agent since it enabled to induce intracellular yeast mechanisms against oxidative stress including mitochondrial activity and upregulation of key gene in aging pathway (SIR2) and genes involved in tolerance mechanism against oxidative stress (*GPX1* and *GLR1*). It is likely that the antiaging mechanism following extract treatments mimics to that calorie restriction conditions, in yeast cells.

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Author's Contributions

Sarima: Has contributed in a whole experiment, data analysis, paper writing and publication.

Rika Indri Astuti: Has handled data analysis, reviewed and revised the manuscript.

Anja Meryandini: Has reviewed and revised the manuscript.

Ethics

All authors read and approved the final version of this manuscript. There are not any ethical issues to declare that could arise after the publication of this manuscript.

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