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Anti-Aging Activity of *Xylocarpus Granatum* Phytoextracts and Xyloccensins K Compound

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ABSTRACT

Cellular aging is promoted by the deleterious effect of free radicals. This can be lowered by antioxidant treatments. *Xylocarpus granatum* and its compound, Xyloccensins K have been reported to have antioxidant activity but there have been no reports of antioxidant and anti-aging activities at the cellular level. Thus, this study aimed to investigate the antioxidant and anti-aging properties of *X. granatum*-derived extract and Xyloccensins K at a cellular level in yeast *Schizosaccharomyces pombe*. Four vegetative and three generative parts of *X. granatum* organs including root, stem, leaf, twig, seed, flesh of the fruit, and peel of the fruit were extracted using 70% ethanol by the maceration method. Whereas, Xyloccensins K was obtained from the seed of *X. granatum*. The samples, other than peel of the fruit, were tested for prolonged cell longevity at lower concentrations as compared to that without phytoextracts treatment. Also, our data indicate that all samples could promote oxidative stress tolerance phenotype, as yeasts were capable of dealing with H_2O_2-induced oxidative stress treatment at 1, 2, and 3 mM H_2O_2 with the best phenotypes by the administration of twig extracts. Most of the phytoextracts showed an increase in mitochondrial activity, except that of seed extract. The result showed the administration of Xyloccensins K compound did not increase the expression of transcriptional factors of oxidative stress response gene cluster, *sty1,* and *pap1*. We suggest that the Xyloccensins K compound acts as a direct Reactive Oxygen Species (ROS) scavenger. Thus, further study in elucidating the phenomenon of *X. granatum* extract-induced longevity is required.

Keywords: *X. granatum* Antioxidant, Longevity, Reactive Oxygen Species (ROS)

INTRODUCTION

Aging is characterized by a loss of physiological integrity that causes malfunction and increased susceptibility to death (Otín *et al*., 2013). Aging is induced by various factors, including oxidative stress affected by free radicals (Rahal *et al*., 2014). The cause of oxidative stress is free radical compounds that come from both extrinsic (ex. pesticides and environmental chemicals) and intrinsic (ex. metabolism process) factors. The damaging effect of free radicals can cause the loss of function and structure of healthy cells (Liochev, 2013). However, a cell has an endogenous (enzymatic) antioxidant mechanism to maintaining redox homeostasis in cells. On the other hand, there is also an exogenous (non-enzymatic) antioxidant that can be obtained from nutritional intake (Chodakowska *et al*., 2018). The antioxidant compounds have been commonly found in plant resources, including mangroves. Indonesia has the highest mangrove diversity in the world with a total of 89 species (Zamani *et al*., 2015), yet,

exploration toward antioxidant activity from mangrove plant species is very limited, including *X. granatum*.

Mangrove has unique metabolite compounds caused by environments with high abiotic and biotic stress (Simlai and Roy, 2013). Thus, this is suggesting its potential application in the pharmaceutical field (Das *et al*., 2019). One of the unique mangrove metabolite compounds is the limonoid, including Xyloccensins K-derived *X. granatum* seed (Kokpol *et al*., 1996). Based on the previous study, the antioxidant activity of the ethanol extract of *X. granatum* stem, assessed with with the *2,2-diphenyl-1-picrylhydrazyl* (DPPH) method, had 50% inhibition concentration (IC_{50}) value of 23.8 $\mu g.mL^{-1}$ (Batubara *et al*., 2010), while that of Xyloccensins I was 0.041$\mu g.mL^{-1}$(Das *et al*., 2015). The antioxidant activity of *X. granatum* belongs to the very active category (Minami *et al*., 1998). However, Xyloccensins K, another Xyloccensins compound, has not been known to have antioxidant activity.

Furthermore, *S. pombe* as a model organism of eukaryotes is commonly used to understand the mechanism of antioxidant and anti-aging activities in humans (Fontana *et al*., 2010; Roux *et al*., 2006). Yeast has several advantages, among others, including having a short lifespan, no complex genome of DNA, some information on some metabolic characters, and molecular genetics that homologous with humans (Palermo *et al*., 2012; Roux *et al*., 2006; Xiang *et al*., 2011). The homology of these cellular pathways includes the activity of the *sir2* gene, which regulates cell longevity (Roux *et al*., 2010), autophagy-a programmed cell death mechanism (Barbosa *et al*., 2019), and a mitochondria-dependent mechanism that plays a role in oxidative stress responses (Fehrmann *et al*., 2013). In yeast, mitogen-activated protein kinase (MAPK) Sty1 is essential for the regulation of transcriptional responses that promote cell survival in response to different types of environmental stimuli including oxidative stress conditions which are induced by high peroxide conditions (Berlanga *et al*., 2010). In response to mild oxidative stress, transcriptional factor, Pap1 mediates the expression of stress response element genes, which overlapped with the Sty1-modulated downstream gene (Veal *et al*., 2007). In this regard, the activity of Sty1 and Pap1 modulate the expression of antioxidant enzymes (*gpx1, ctt1, sod, tpx1, srx1*) (Calvo *et al*., 2012; Zuin *et al*., 2010), and the other cellular mechanisms in delaying cell aging (*rad52, ste11*) (Bellini *et al*., 2012). Both Sty1 and Pap1 play a key role in oxidative stress response in yeast, these two genes are often used as a marker for understanding the underlying mechanism of oxidative response mechanism in various environmental conditions (Kar *et al*., 2018; Madrid *et al*., 2004; Zuin *et al*., 2010).

To date, there have been no reports available regarding the antioxidant and anti-aging activities from phytoextracts of *X. granatum* and Xyloccensins K compound at the cellular level. Thus, in this study, we attempted to evaluate the potential of antioxidant and anti-aging activities from phytoextracts of *X. granatum* and Xyloccensins K compound by using *S. pombe* as a model organism. To the best of our knowledge, this is the first study to describe the activity of phytoextracts from *X. granatum* and Xyloccensins K compound in modulating yeast longevity.

MATERIAL AND METHODS

Cultures and medium

The fission yeast *S. pombe* ARC 039 (*h-leu1-32 ura4-294*) (Prastya *et al*., 2018) *S. pombe* was routinely maintained in Yeast Extract with Supplement (YES) medium. YES (1 L) composition is as follows: 5g yeast extract, 20g glucose, 0.128g histidine, 0.128g leucine, 0.128g adenine, 0.128g arginine, and 0.010g uracil (Prastya *et al*., 2018). The yeast was sub-cultured in a liquid YES medium for the stock and working cultures then incubated at room temperature.

Preparation of the samples

The phytoextracts of *X. granatum* were derived from both vegetative and generative organ parts of the plant including root, stem, seed, the flesh of the fruit, leaf, peel of the fruit, and twig. The phytoextracts were obtained from the previous study (Batubara *et al*., 2010). All samples were extracted using the maceration method with ethanol as a solvent (Batubara *et al*., 2010). The Xyloccensins K compound was derived from the previous study obtained from *X. granatum* seeds (Kokpol *et al*., 1996). All samples were stocked with 10000$\mu g.mL^{-1}$ using distilled water for the extracts and DMSO (*dimethylsulfide*) for the compound of Xyloccensins K.

Aging assay

The aging assay was conducted by a spot test as described previously with slight modification. The inoculum was re-inoculated on a 3mL YES treatment culture (in a 10mL test tube) with an initialOD_{600}of0.05.Phytoextractsdilutedon

Table I. Primer pairs for Real-Time PCR used in this study

Gene	Forward primer	Reverse primer
sty1	5'-TCACCGTGATCTGAAACCA-3'	5'-AGCCAAACCGAAATCGCAGA-3'
pap1	5'-TGGATGGCGATGTTAAGCCT-3'	5'-GCAGCACGGTTTTGAGCTTT-3'
act1	5'-CGGTCGTGACTTGACTGACT-3'	5'-ATTTCACGTTCGGCGGTAGT-3'

aquadest were supplemented in the treatment culture. The assay was performed at three different concentrations (250, 500, and 750µg.mL^{-1} for the phytoextracts treatment and 25, 50, and 100µg.mL^{-1} for the Xyloccensins K compound treatment). Yeast cultured in YES medium containing 3% glucose without samples was used as the negative control while yeast was cultured in Edinburgh Minimum Medium (EMM) containing 0.5% of glucose without phytoextracts was used as the positive control. Low glucose in the positive control is considered as calorie restriction (CR) conditions. Such CR condition will induce yeast cellular mechanisms including mitochondrial activity response to oxidative stress and autophagy, which contribute to the yeast lifespan extension (Roux *et al*., 2010; Sarima *et al*., 2019). Each culture was incubated for 11th-days where a spot test was conducted at days 7 and 11 of incubations. Each culture was adjusted to OD = 1 then serially diluted (up to 10^{-4}). About 2µL from each diluted suspension was then spotted on a solid YES medium. The spotted medium was incubated for 3 days at room temperature.

Oxidative stress tolerance assay

The spot test was carried out as mentioned above but the concentration used is the best concentration during the aging assay. About 2µL from each dilution suspension was spotted on solid YES medium containing H_2O_2 at various concentrations (1, 2, and 3mM).

Active mitochondrial staining

The procedure was performed according to the previous study by using Rhodamine B as a probe (Sarima *et al*., 2019). The yeast was incubated in liquid YES medium supplemented with phytoextracts of *X. granatum* and Xyloccensins K at the best concentration during the aging assay. The yeast suspension (1mL) was centrifuged and the pellet obtained was suspended using phosphate buffer and added with 100nM rhodamine B. Yeast suspensions were then incubated for 30min at 25°C. Light exposure was avoided throughout the experiment. Mitochondrial activity was observed under fluorescence microscopy using 1000x magnification. Strong fluorescence intensity correlates to strong mitochondrial membrane potential (Sarima *et al*., 2019).

Gene expression assay using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Yeast was cultured in 3mL liquid YES medium containing 25µg.mL^{-1} Xyloccensins K overnight in a shaking incubator. The yeast culture was divided into 2 groups, the oxidative stress conditions (administration of H_2O_2) and normal conditions. Furthermore, yeast-mRNA was extracted by using the *RNeasy Mini Kit* (Qiagen, USA). The total of mRNA was used as a template for cDNA synthesis using the *iScript cDNA Synthesis Kit* (Bio-Rad, USA). The qRT-PCR experiment was performed using the particular cDNA template with *Fast Evagreen® qPCR Master Mix*. PCR mix was prepared following the manufacturer's procedure with specific primers of gene *sty1* and *pap1* (Table I). The qRT-PCR conditions were pre-denaturation for 30s at 95°C, 50 amplification cycles (denaturation for 30s at 98°C, annealing for 20s at 58°C, and melting curve for 5s at 65°C). Data obtained from qRT-PCR were analyzed using relative quantification method $2^{-\Delta\Delta CT}$ and normalized towards reference gene *act1* (Livak and Schmittgen, 2001).

RESULT AND DISCUSSION

In the present study, we report the anti-aging properties of phytoextracts derived from vegetative (Figure 1) and generative (Figure 2) organ parts of *X. granatum,* and Xyloccensins K (Figure 3) were able to prolong the lifespan of *S. pombe*. Our findings showed various phenotypic responses upon administration of samples on yeast cell viability. Phytoextracts derived from root, stem, leaf, twig, seed, flesh of the fruit, and Xyloccensins K which were administrated the lowest concentration (250 µg.mL^{-1} for phytoextracts and 25µg.mL^{-1} for Xyloccensins K)

showed as the optimum concentration in prolonging the lifespan of *S. pombe* cells at the stationary phase (7th and 11th days of incubation), compared to that without phytoextracts addition. On the other hand, peel of the *X. granatum* fruit extract showed an anti-aging effect at a higher concentration (500µg.mL^{-1}) (Figure 2).

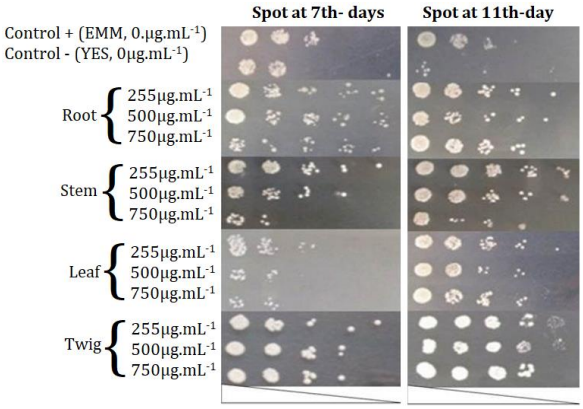

Figure 1. The effect of phytoextracts derived from the vegetative organ parts of *X. granatum* on the viability of *S. pombe* cells. All extract could promote yeast survival rate at the stationary phase (7 and 11 days of incubation). Yeast was grown in YES medium supplemented with various concentrations of phytoextracts (250, 500, and 750µg.mL^{-1}) and incubated for 7 and 11 days. Yeast cultures of each designated incubation time were then spotted on YPD agar and incubated for three days prior to observation.

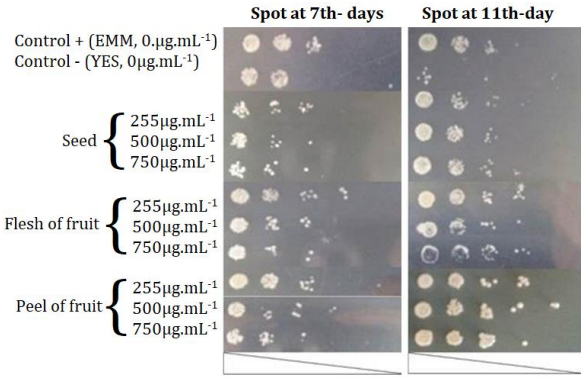

Figure 2. The effect of phytoextracts derived from the generative organ parts of *X. granatum* on the viability of *S. pombe* cells. All extract could promote yeast survival rate at stationary phase (7 and 11 days of incubation) compared to that without extract treatment. Yeast was grown in YES medium supplemented with various concentrations of phytoextracts (250, 500, and 750 µg.mL^{-1}) and incubated for 7 and 11 days. Yeast cultures of each designated incubation time were then spotted on YPD agar and incubated for three days prior to observation.

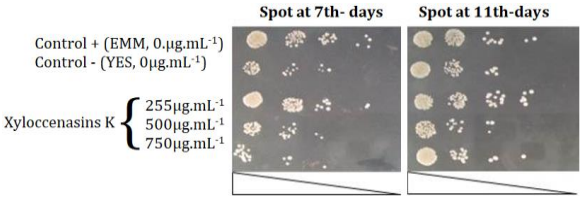

Figure 3. The effect of *Xyloccensins* K on the viability of *S. pombe* cells. Yeast was grown in YES medium supplemented with various concentrations of *Xylloccensins* K (25, 50, and 100 µg.mL^{-1}) and incubated for 7 and 11 days. Yeast cultures of each designated incubation time were then spoted on YPD agar and incubated for three days prior to observation.

Furthermore, the administration of the samples resulted in better cell viability for the 11 days culture, except that of root-derived extract treatment (Figure 1). Assessment of the viability of the cells on days 7 and 11 was based on the previous study as the viability of *S. pombe cell* grown on nutrient-rich medium decrease on the 7th day and almost none survived on the 10th day onwards.[30] It is worth noting, phytoextracts of the root, stem, leaf, twig, flesh of the fruit, and peel of the fruit could promote yeast viability better than that of yeast grown in *calorie restriction* (CR) conditions (positive control). Whereas, administration of seed extract and Xyloccensins K showed no difference to that of positive control (Figure 3). Interestingly, Xyloccensins K compounds showed better density results (10^{-3}) compared to *X. granatum* seed extracts (10^{-2}) (Figures 2 and 3).

CR condition has been known to be able to prolong the lifespan of various organisms including yeast, worms, flies, and mice (Masoro, 2005). In yeast, CR conditions could change the metabolic system from fermentation to respiration that affected various cellular aspects including the activity of mitochondrial and intracellular antioxidants mechanisms, which will ultimately inhibit the cell aging process. Whereas in non-CR conditions, including negative control and treatments, *S. pombe* was treated in nutrient-rich conditions. Commonly, the amount of nutrient concentration used for the antiaging assay is

determined by reducing 3% to 0.5-0.05% of glucose (Roux *et al*., 2010). The viability of *S. pombe* on spot medium is the basis of qualitative determination of antiaging activity.

Previous studies have also reported that some phytoextracts of *Hibiscus sabdariffa* petal (Sarima *et al*., 2019), *Cimifuga racemosa* rhizome, *Valeriana officinalis* root, *Passiflora incarnata*, *Gingko biloba* leaves, *Apium graveolens* seeds, and *Salix alba* stems could extend the lifespan of *S. cerevisiae* under conditions that mimic CR conditions with different cellular mechanisms (Lutchman *et al*., 2016). This is in line with this study, the phytoextracts administration of *X. granatum* (vegetative and generative organ parts) and Xyloccensins K were able to prolong the lifespan of yeast that mimicking CR conditions, even though yeast was grown in non-CR conditions. Thus, we suspect the phytoextracts of *X. granatum* and Xyloccensins K might induce anti-aging mechanisms like CR conditions. The spot viability appearance of *S. pombe* yeast on the solid YES medium is the basis for determining the ability of qualitative anti-aging activities. To further clarify the cellular modulation of the phytoextracts and Xyloccensins K in *S. pombe*, we assessed the capability of extract in promoting intracellular defense against exogenous oxidative stress treatments, mitochondrial activity, and expression of transcriptional factors (*sty1* and *pap1*) for oxidative stress response-related genes in *S. pombe.*

The viability assay against H_2O_2-induced oxidative stress could induce oxidative stress tolerance of yeast *S. pombe* with the administration of all phytoextracts derived from both vegetative (Figure 4) and generative organ parts (Figure 5) of *X. granatum*, and also Xyloccensins K (Figure 6) on the 11th day of incubations as compared to the positive control (CR condition). This treatment may likely interfere with yeast cellular function, thus promoting oxidative stress response in yeast.

This is in line with the previous studies using several phytoextracts in modulating the oxidative stress tolerance phenotype of different yeast isolates. For instance, phytoextracts of *Syzygium aromaticum* leaves and *Coffea* sp. seed (*caffeine* compound) could increase the longevity of *S. pombe* (Fauzya *et al*., 2019; Maeta *et al*., 2007). In addition, phytoextracts of *Hibiscus sabdariffa* and *quercetin* could promote the longevity of *S. cerevisiae* (Belinha *et al*., 2007; Sarima *et al*., 2019). Interestingly, the administration of *catechin* derived from *Camellia sinensis* leaves could also induce oxidative stress tolerance on both *S. pombe* and *S. cerevisiae* cells (Rallis *et al*., 2013). Besides, the administration of *Bacillus* SAB E-41 crude extract could also promote modulate oxidative stress tolerance phenotype of *S. pombe* cells (Prastya *et al*., 2018).

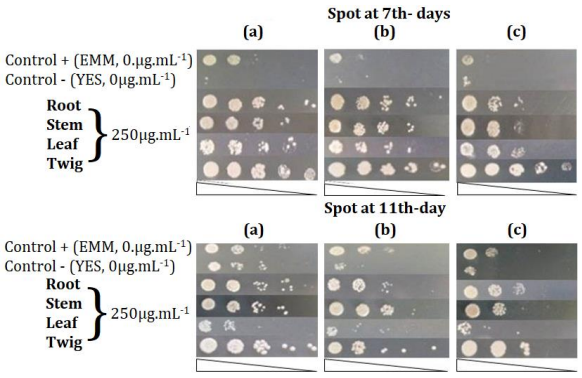

Figure 4. Effect of phytoextracts derived from the vegetative organ parts of *X. granatum* on the viability of *S.pombe* cells against oxidative stress. All extract could promote yeast survival rate against oxidative stress conditions at the stationary phase (7 and 11 days of incubation). Yeast was grown in YES medium supplemented with selected concentrations of extracts and incubated for 7 and 11 days. Yeast cultures of each designated incubation time were thenspottedd on YPD agar supplemented with various concentrations of oxidative stress using H_2O_2 ((a) 1mM, (b) 2mM, (c) 3mM) and incubated for three days prior observation.

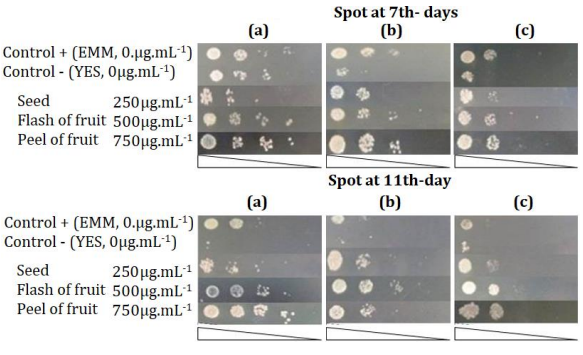

Figure 5. Effect of phytoextracts derived from the generative organ parts of *X. granatum* on the viability of *S.pombe* cells against oxidative stress. All extract could promote yeast survival rate against oxidative stress conditions at the stationary phase (7 and 11 days of incubation). Yeast was grown in YES medium supplemented with selected concentrations of extracts and incubated for 7 and

11 days. Yeast cultures of each designated incubation time were then spotted on YPD agar supplemented with various concentrations of oxidative stress using H_2O_2 ((a) 1 mM, (b) 2 mM, (c) 3mM) and incubated for three days prior to observation.

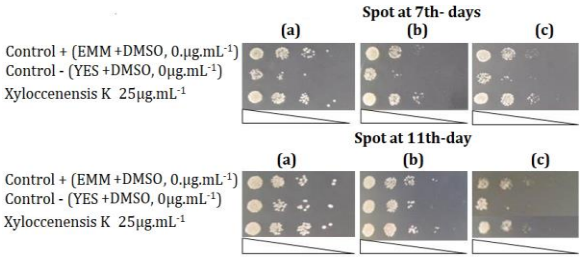

Figure 6. Effect of *Xyloccensins* K on the viability of *S.pombe* cells against oxidative stress. *Xyloccensins* K could promote yeast survival rate against oxidative stress. Yeast cultures of each designated incubation time were then spotted on YPD agar supplemented with various concentrations of oxidative stress using H_2O_2 ((a) 1mM, (b) 2mM, (c) 3mM) and incubated for three days prior to observation.

Our data showed that the administration of 250µg.mL^{-1} of twig-derived extract of *X. granatum* significantly increased cell viability against a high concentration of H_2O_2 (3 mM) on day 11 (Figure 4). Such activities have also been reported from the treatment of 100 µg.mL^{-1} of *S. aromaticum* clove extract on *S. cerevisiae* (Fauzya *et al*., 2019). On the other hand, a higher concentration of *Bacillus* SAB E-41 extract was needed to promote yeast viability against low 0.75mM H_2O_2 (Prastya *et al*., 2018).

Up to this date, this study is the first report to show the potential properties of phytoextracts from vegetative (root, stem, leaf, and twig) and generative (seed, flesh of fruit, and peel of fruit) organ parts of *X. granatum* and Xyloccensins K as an antioxidant at the cellular level. In the previous study at the acellular level, the phytoextracts compounds that play a role as antioxidants are limonoids. In the DPPH, ABTS, superoxide, and hydrogen peroxide assays, the limonoid such as Xyloccensins I was known to have IC_{50} at 0.041, 0.039, 0.096, and 0.235mg/mL, respectively (Das *et al*., 2019). Xyloccensins are limonoid group compounds found only in the Rutaceae and Meliaceae families (Bandaranayake, 2002; Cui *et al*., 2008). Limonoid structures are classified into phragmaline, mexiconalide, obacunol, and andyrobin types based on their carbon rings (Wu *et al*., 2015; Zhou *et al*., 2014). Limonoids are highly oxygenated, have moderate polarity, insoluble in water and hexane, but are soluble in hydrocarbons, alcohols, and ketones (Roy & Saraf, 2006).

The induction of intracellular oxidative stress response has been reported to be related to the mitochondrial activity (Sarima *et al*., 2019). This is supported by the theory of mitochondrias as ROS-producing organelles (Barzilai *et al*., 2012) and mitochondrial dysfunction as a signal of aging. Furthermore, phytoextracts of *X. granatum* (vegetative and generative organ parts) and Xyloccensins K were analyzed towards mitochondrial activities.

Based on our observation, all the phytoextracts of vegetative organ parts could promote mitochondrial membrane activity (Figure 7), compared to the control. In addition, most of the phytoextract of generative organ parts, except seed extract, could also promote mitochondrial membrane activity (Figure 8), compared to the control. Interestingly, the administration of Xyloccensins K (Figure 9) had a different effect to the seed extracts of *X. granatum* (Figure 8). Such difference was likely due to the presence of compounds in crude extracts of seed that act antagonistically in increasing mitochondrial activity. Thus, we suggest that the anti-aging activity of seed extract was unlikely to occur via mitochondrial signaling as likely mediated via Xyloccensins K.

The mitochondrial activity assay aimed to clarify the possible *in vivo* induction of yeast mitochondrial activity after the administration of phytoextracts. Thus, it was likely that phytoextract of *X. granatum* and Xyloccensins K may mediate the intracellular oxidative stress response of yeast *S. pombe* via the induction of mitochondrial activity. However, further, the study must be conducted to clarify this phytoextract-mode of action. This was similar to a previous study that suggests plant extracts of *H. sabdariffa* petal promote the cellular response of *S. cerevisiae* against oxidative stress (Sarima *et al*., 2019). Besides that, other studies revealed a mitochondria-independent action of the ethanol extracts clove leaves and buds in mediating oxidative stress tolerance phenotype of yeast *S. pombe* and *S. cerevisiae* (Astuti *et al*., 2019; Fauzya *et al*., 2019).

Mitochondria are known to be associated with aging because these organelles produce intracellular ROS via respiration.

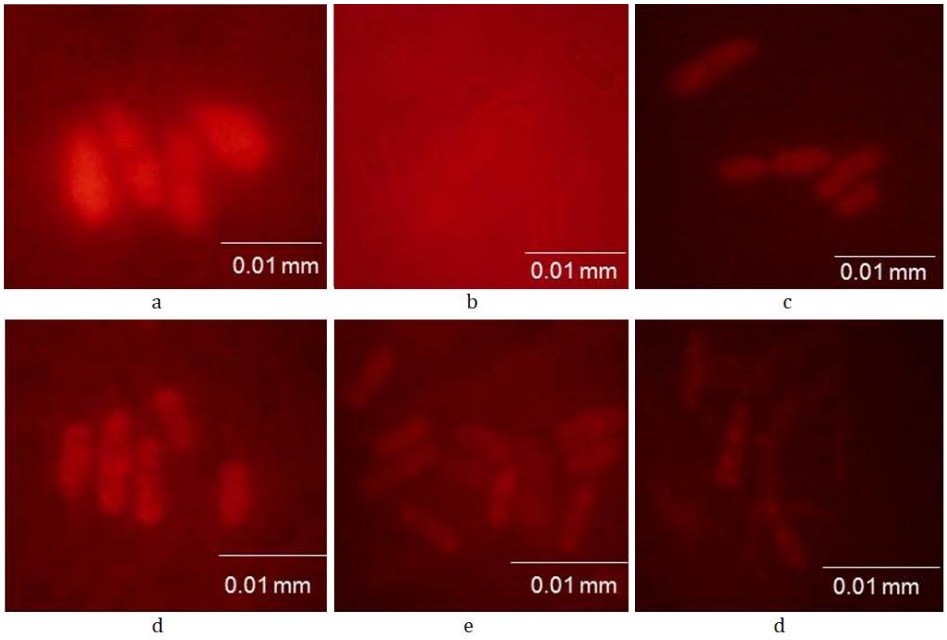

Figure 7. Effect of phytoexracts derived from the vegetative organ parts of *X. granatum* on mitochondrial activity of yeast *S. pombe*: (a) EMM 0.5% of glucose (control +, 0 µg.mL^{-1}), (b) YES 3% of glucose (control -, 0 µg.mL^{-1}), (c) YES 3% of glucose + 250 µg.mL^{-1} of root phytoextract, (d) YES 3% glucose + 250 µg.mL^{-1} of stem phytoextract, (e) YES 3% glucose + 250 µg.mL^{-1} of leaf phytoextract, and (f) YES 3% glucose + 250 µg.mL^{-1} of twig phytoextract. Bar in each picture represents 10µm scale.

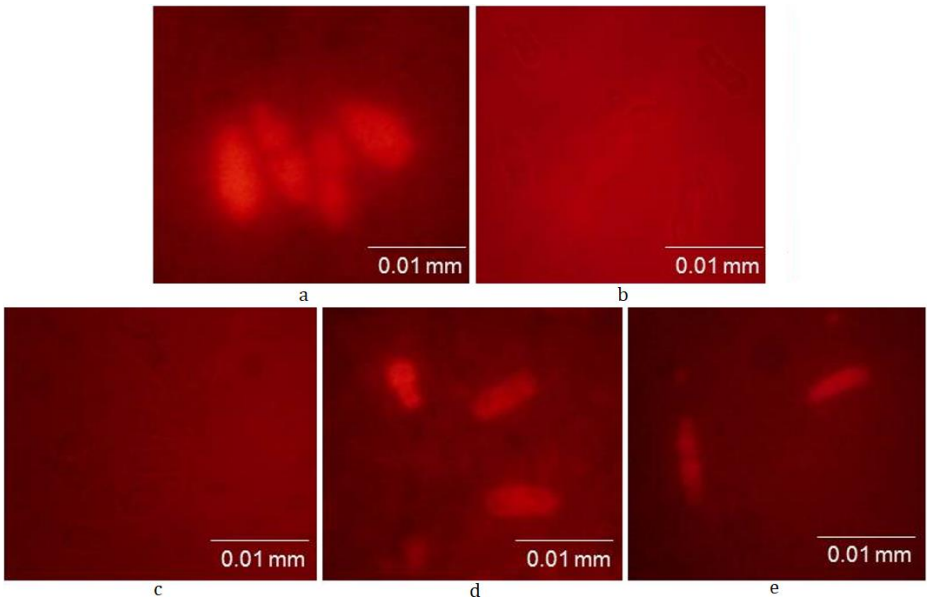

Figure 8. Effect of phytoexracts derived from the generative organ parts of *X. granatum* on mitochondrial activity of yeast *S. pombe*: (a) EMM 0.5% of glucose (control +, 0 µg.mL^{-1}), (b) YES 3% of glucose (control -, 0 µg.mL^{-1}), (c) YES 3% of glucose + 250 µg.mL^{-1} of seed phytoextract, (d) YES 3% glucose + 250 µg.mL^{-1} of flesh of fruit phytoextract, and (e) YES 3% glucose + 500 µg.mL^{-1} of peel of fruit phytoextract. Bar in each picture represents 10µm scale.

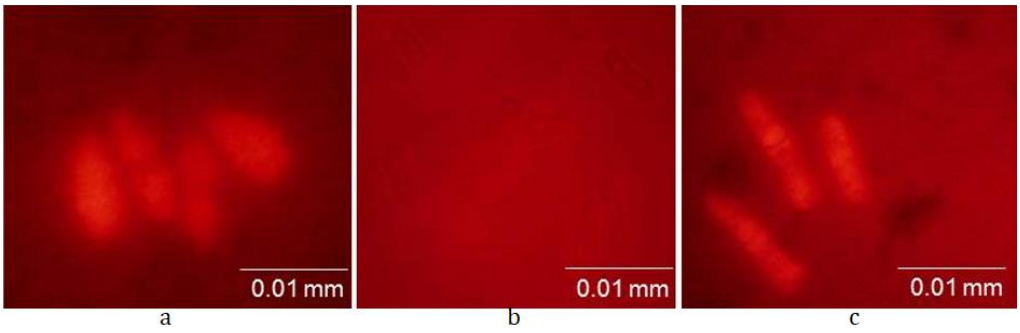

Figure 9. Effect of *Xyloccensins* K on mitochondrial activity of yeast *S. pombe*: (a) EMM 0.5% of glucose (control +, 0µg.mL^{-1}), (b) YES 3% of glucose (control -, 0µg.mL^{-1}), (c) YES 3% of glucose + 25µg.mL^{-1} of *Xyloccensins* K. Bar in each picture represents 10µm scale.

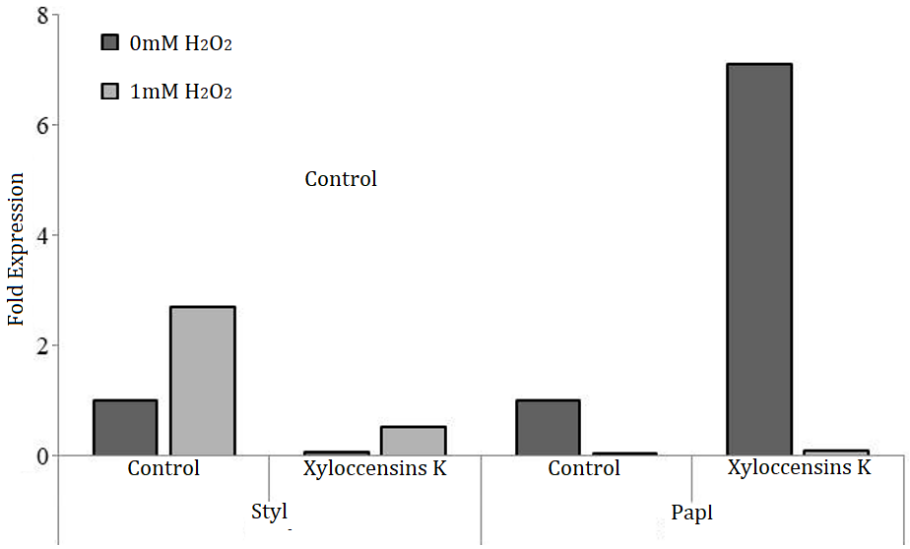

Figure 10. The effects of 25 µg, mL^{-1} *Xyloccensins* K (25 µg.mL^{-1}) on the gene expression level os transcriptional factor *sty1* and *pap1*-investigated by using qRT-PCR analysis. Yeast re-cultured in YES medium without *Xyloccensins* K addition was used as control. The level of gene expression *sty1* and *pap1* were normalized using reference gene *act1.*

Thus, mitochondrial dysfunctions could be one of the fundamental causes of cellular aging (Barzilai *et al*., 2012). Interestingly, the administration phytoextracts of *X. granatum* (vegetative and generative organs), except seed extracts and Xyloccensins K showed strong fluorescence intensity relatively similar level to that of CR conditions. The CR condition is known to be responsible for the development of adaptive mitochondrial ROS-signaling that prolong yeast chronological life span (Pan *et al*., 2012). Indeed, treatment of those phytoextract and Xyloccensins K could prolong yeast life span as revealed in this study. Further study is required to understand the effect of phytoextract and Xyloccensins K at molecular levels. It is worth noting that the development of adaptive mitochondrial-ROS signaling occurs via the reduction of TOR activity (Barzilai *et al*., 2012).

Amongst all treatments, we specifically analyzed the effect of Xyloccensins K in modulating transcriptional factors of oxidative stress response gene cluster, *sty1,* and *pap1.* The expression of both *sty1* and *pap1* genes was increased following the oxidative stress conditions, suggesting the development of intracellular oxidative stress

conditions in the *S. pombe* cells. Such results were in agreement with the previous study as reported elsewhere (Calvo *et al*., 2012; Vivancos *et al*., 2004). Indeed, both *sty1-atf1* and *pap1* pathways are reported as redox sensors that are directly activated by elevated H_2O_2 levels (Vivancos *et al*., 2004). Interestingly, it is shown that there was no increase in the expression of *sty1* and *pap1* genes following administration of 25 $\mu g.mL^{-1}$ Xyloccensins K, compared to the control treatment (Figure 10), thus suggesting that H_2O_2-induced oxidative stress conditions did not develop in Xyloccensins K-treated cells.

We suspect that the antioxidant mechanism of Xyloccensins K was mediated via a direct ROS scavenging mechanism. This was supported by the high activity of Xyloccensins in the *in vitro* assay (Das *et al*., 2015). Xyloccensins K is suspected to rapidly reduce free radicals (H_2O_2) before affecting the stress oxidative responses genes cluster in *S. pombe*. The Xyloccensins compound is a compound derived from a modified terpenoid group that is highly oxygenated with the general framework 4,4,8-trimethyl-17-furanilsteroid (Roy & Saraf, 2006). Thus, both functions as antioxidant agents. This is supported by the high activity of the *Xyloccensins* compound group in a previous *in-vitro* assay (Das *et al*., 2015).

Xyloccensins group of compounds is thought to be able to quickly reduce free radicals (H_2O_2) such that intracellular oxidative stress conditions are not formed. Such phenomenon has previously been reported as the administration of *Curcumin* can directly reduce ROS, including O_2^-, OH^- and H_2O_2 on skeletal muscle rats by penetrating cell membranes and transferring H atoms to free radical compounds in the cytoplasm (Barzegar & Movahedi, 2011). It is worth noting that, Xyloccensins K belongs to *mexaconalide* group which has a unique functional group structure that features easily oxidized properties (Wu *et al*., 2004). Thus, the Xyloccensins K may exert an excellent antioxidant agent via direct ROS scavenging activity both *in vitro* and cellular levels (Das *et al*., 2015).

CONCLUSION

Phytoextracts of both vegetative and generative organs of *X. granatum*, and Xyloccensins K could prolong the lifespan of *S. pombe* cells at different concentrations. Most of the samples showed anti-aging activities at the lowest concentrations (250 $\mu g.mL^{-1}$ for phytoextracts of root, stem, leaf, twig, seed, and flesh of fruit; 25 $\mu g.mL^{-1}$ for Xyloccensins K), except that of the peel of *X. granatum* fruit. Twig-derived extract (250 $\mu g.mL^{-1}$) induced the antioxidative stress phenotype on yeast. Most of the samples could induce mitochondrial activity, except that of the seed extract. It is worth noting that the anti-aging activity of *X. granatum* extract is not all mitochondria-dependent. Furthermore, Xyloccensins K likely induces a cellular antioxidative mechanism independent of that from *Sty1* and *Pap1* genes, thus suggesting another mechanism such as direct ROS scavenging activity. Further research is necessary to clarify the underlying mechanism of Xyloccensins K as an antiaging agent.

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