Life span extension of model yeast *Saccharomyces cerevisiae* upon ethanol derived-clover bud extract treatment

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Life span extension of model yeast *Saccharomyces cerevisiae* upon ethanol derived-clover bud extract treatment

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Abstract. Antioxidant properties of clove bud have been widely studied due to its potential use in the pharmaceuticals field. One of the chemical bioactive compounds that show antioxidant activity is flavonoid. Our study revealed that the flavonoid content of ethanol-derived extract of clove bud was approximately 93.245 mg QE/100 gram. Less is known regarding the mode of actions of antioxidant from clove bud in cellular systems. In this study, we used model organism yeast *Saccharomyces cerevisiae* to study the action of antioxidant activity in cellular systems. We found that ethanol-derived clove bud extract (100 ppm) enhanced cells viability following H_2O_2-induced oxidative stress. Interestingly, clove bud extract increased yeast-antioxidative stress tolerance phenotype in a dose-independent manner. Suggesting, prooxidant activity of clove bud extract. Mitochondria have been known to involve in oxidative stress tolerance mechanisms primarily via mitochondrial adaptive ROS-signaling. Our data revealed that yeast mitochondrial membrane potential was unchanged following 100 ppm extract treatment yet significantly increased in higher extract treatment. Our study indicated that 100 ppm extract-supplementation in yeast culture resulted in a higher survival rate of yeast after 15-days of incubation, compared to that without extract treatments. We suggest that clove bud extract (100 ppm) could enhance oxidative stress tolerance phenotype in yeast *S. cerevisiae*, which then attributed on life span extension through its ROS scavenging activity. Further study must be conducted to confirm the underlying mechanisms of clove bud extract both physiologically and genetically.

1. Introduction

The development of oxidative stress due to an accumulation of free radical, including reactive oxygen species (ROS) is believed to be one of the main factors of cellular aging [1]. Oxidative stress is occurred due to the imbalance of free radicals toward oxidative stress response systems in the cells. Severe oxidative stress conditions may then culminate to cell death [2]. Various methods have been implemented to combat the deleterious effect of free radicals. One of the promising approaches is via the treatment of antioxidant agents [3].

Antioxidant agent, such as phenolic compounds, has been reported to be an abundant presence in plants. In addition to antioxidant properties, interestingly, such phenolic compounds are attributed with anticancer, antibacterial, antiatherosceloris [4]. It is, therefore, phenolic compounds are highly demanded its pharmaceutical uses. Some tropical herbs plants have been studied as sources of phenolic compounds including, *Cinnamomum zeylanicum, Curcuma longa, Myristica fragrans,* and *Syzygium aromaticum.* In this study, we are interested in assay the antioxidant properties of *S.*

aromaticum (clove bud) and further examined its potential as an anti-aging agent. It is reported that clove bud extract has some bioactive compounds with antioxidant properties, including phenolic or polyphenolic compounds such as flavonoid, tannin dan eugenol[5]. Nevertheless, no data available regarding its potential in extending the cell`s life span. The activity of clove bud extract in inhibiting cellular aging may potentially be used to prevent or control the development of degenerative diseases.

In this study, we used yeast *Saccharomyces cerevisiae* as model organisms to study cellular aging. Aging pathway of *S. cerevisiae* is highly homolog to mammalian cells [4]. It is reported that aging pathway in both *S. cerevisiae* and mammalian cells may involve autophagy, cell cycle, mitochondria, and oxidative stress responses pathways [5]. Besides, both *S. cerevisiae* and mammalian cells show similar response in regards to molecular and cellular mechanisms toward calorie restriction treatments, which leads to cell`s longevity [6]. In this study, we investigated the anti-aging activity of ethanol-derived clove bud extract in *S. cerevisiae*. Furthermore, we clarified the possible target of cellular mechanisms, particularly mitochondria activity, affected by the extract. This study provides fundamental data in the effect of clove bud extract toward cellular aging thus could be potentially used in advance study of its pharmaceutical uses upon the prevalence of degenerative diseases.

2. Materials and Methods

2.1. Clove, Yeast, and Medium

Dried clove buds (*S. aromaticum* var Zanzibar) were used as a source of extract. *S. aromaticum* var Zanzibar was obtained from the Indonesian Spice and Medicinal Crops Research Institute (ISMCRI), Cimanggu, Bogor, Indonesia. *S. cerevisiae* BY4741 was routinely maintained in Yeast Extract Peptone Dextrose medium (Yeast Extract 1%, Peptone 2%, Dextrose 2%). *S. cerevisiae* grown in 0.5% glucose was determined as calorie restriction treatment and be used as positive control throughout the study.

2.2. Extraction

The procedure for extraction was adopted from the previous study with slight modifications [7]. Phenolic compounds were extracted using 70% ethanol as solvent. About 500 gram dried clove buds were soaked with 70% ethanol (1:5 ratio). The filtrate was filtered using filter paper. The extracted compounds were concentrated using rotary evaporator (45°C) to result in the crude extract.

2.3. Total Flavonoid Content

Flavonoid content was measured using the standard method described elsewhere [8]. Flavonoid content was quantified spectrophotometrically in 425 nm using $AlCl_3$ as chemical reagent. Quercetin was used as flavonoid standard.

2.4. Oxidative Stress Tolerance Assay

This assay was conducted to assay the effect of clove extract in modulating yeast response against H_2O_2-induced oxidative stress [9]. The assay was done using the spot test assay. *S. cerevisiae* BY4741 cells was sub-cultured in liquid YPD medium for 24 hours at room temperature, started at OD: 0.1. The particular subculture was then transferred to the YPD fresh medium containing a various concentration of clove extract (100, 200, and 500 ppm). YPD medium without extract addition was designed as control. Cultures were then incubated for 15 days. Every five days of incubations, cells were harvested and serially diluted (up to 10^{-4}) in starting OD of 1. Each serial dilution was then spotted in YPD agar medium containing a different concentration of H_2O_2 (1, 3, 5 and 7mM) and incubated at 30^{o}C for three days.

2.5. Mitochondrial membrane potential assay

To evaluate the mitochondria-dependent oxidative stress response following extract treatment, we conducted mitochondria assay using probe Rhodamine B [9]. Observation of mitochondria activity

was done qualitatively using a fluorescence microscope. *S. cerevisiae* was grown in YPD medium containing a various concentration of extract (100, 200, and 500 ppm) in starting OD: 0.1. Yeast grown without extract was used as control. Besides, yeast grown in 0.5% glucose was used as a positive control of active mitochondria treatment. After 24 hours, cells were then harvested using centrifugation (4000 rpm, 4 minutes) and washed twice with phosphate buffer pH 7. Yeast cells were then suspended in 1 ml phosphate buffer following the addition of 100nM rhodamine B solutions. Cells suspensions were kept in the dark for 30 minutes at room temperature. Mitochondrial activity was then observed under a fluorescence microscope (Olympus BX51) observations.

2.6. Aging assay

The aging assay was conducted by using the spot test assay, as described previously [10]. *S. cerevisiae* BY4741 cells was sub-cultured in liquid YPD medium for 24 hours at room temperature, started at OD: 0.1. The particular subculture was then transferred to the YPD fresh medium containing various concentration of clove extract (100, 200, and 500 ppm). YPD medium without extract addition was designed as control. Besides, *S. cerevisiae* grown in 0.5% glucose was used as a positive control of life span extension. As previously reported, S. *cerevisiae* grown in 0.5% glucose experience calorie restriction conditions [11]. Indeed, such situations result in a longer life span due to the activation of autophagy and mitochondria activities compared to that *S. cerevisiae* grown in higher glucose content (> 2%) [11-13]. Cultures were then incubated for 15 days. Every five days of incubations, cells were harvested and serially diluted (up to 10^{-4}) in starting OD of 1. Each serial dilution was then spotted in YPD agar medium and incubated at 30^0C for three days.

3. Results and Discussion

3.1. Total Flavonoid Content

By using ethanol as solvent, the total flavonoid content of clove extract was 93.25 mg QE/100 gram clove extract. Such value is considered low as compared to the previous study. It was reported that by using 80% acetone, flavonoid content of clove bud extract was higher (501 mg QE/gram). In this study, ethanol was used to support a simple solvent evaporation procedure for its future commercial use. Indeed, most of the phytoextract with commercial use were extracted by using ethanol-based flavonoid extraction such as *Ginko Biloba, Zingiber Officinale, Astragalus membranaceous* (Idunn technologies) [14].

3.2. Clove extract shows antioxidative properties

Based on the data, we found that extract treatment could enhance yeast capability in dealing with weak (1 mM) and severe (3, 5, and 7 mM H_2O_2) oxidative stress conditions (Figure 1). Amongst the concentration of extracts, 100 ppm extract strongly induced cells viability against oxidative stress conditions. Interestingly such activity was unlikely occurred in a dose-dependent manner since higher extract concentration (200 and 500 ppm) showed an opposite phenotype. It is suggested that ethanol-clove bud extract possess antioxidant properties, whereas 100 ppm is the optimum concentration of extract to regulate yeast cells in combating exogenous oxidative stress.

To our knowledge, this is the first report with respect to the potential properties of clove extract as an antioxidant agent ever conducted using yeast as model organisms. The previous study had assayed the potential of phytoextracts in inducing the capability of yeast in dealing with oxidative stress. In instance, ethanol extract from *Hibiscus sabdariffa*, *Croton caudatus,* and *Acacia nilotica* could significantly neutralize the ROS level in *Saccharomyces cerevisiae* treated with H_2O_2 induced oxidative stress conditions [15-16]. Remarkably, the later was also found as an antioxidant agent which capable of protecting macromolecules such as DNA and protein against oxidants, both *in vivo* and *in vitro* [17].

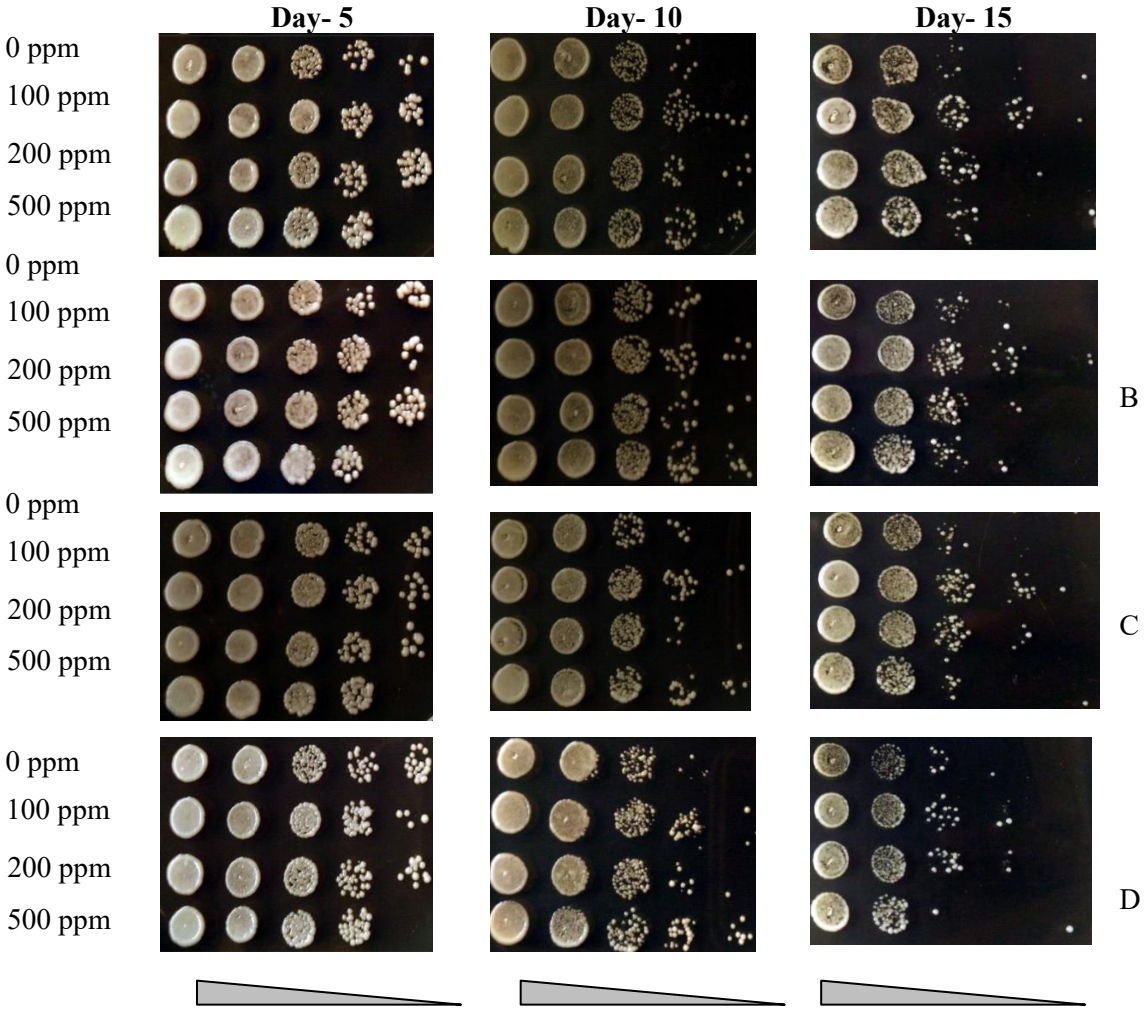

Figure 1. Oxidative stress response of *S. cerevisiae* treated with clove extract. Yeast cells were cultured in YPD liquid medium supplemented with various concentration of extract (100, 200, and 500 ppm). Cells grown in YPD without extract addition is designed as control. Following 5, 10 and 15 days of incubation, yeast was harvested (adjusted to OD =1) and serially diluted (up to 10^{-4}). Each dilution was then spotted (3µl) in YPD agar medium containing different H_2O_2 concentration (A) 1 mM, (B) 3 mM, (C) 5 mM and (D) 7 mM. Plates were then incubated in 30^0C for three days.

Based on our data, a high concentration of clove extract (500 ppm) results in a stress-sensitive phenotype of yeast cells. Such a phenomenon may occur due to the prooxidant activity of the particular clove extract. Indeed, flavonoid compounds have been reported to have dual effect as both antioxidant and prooxidant, which the later is believed to able to cause oxidative damage by reacting with various biomolecules, such as lipids, proteins and DNA [18]. Switching activity of antioxidant to prooxidant might be due to the occurrence of transition metal [19], does [20], co-oxidant such as vitamin [21] and pH [22]. In our study, it is indicated that a higher dose of clove extract leads to prooxidant activity, thus culminate in cells sensitive phenotype against H_2O_2-induced oxidative stress. Previous studies exhibited dose-dependent prooxidant activity of plant extract, including *Olea europaea*, *Morus nigra* leaves [23] and parsley [24].

The oxidative stress response in yeast has been known to involve mitochondria activity [3]. As previously reported, high mitochondria activity may lead to the development of adaptive mitochondrial ROS-signaling that stimulate cellular mechanisms in combating oxidative stress [25].

Such tools his conserved from yeast to higher mammalian cells [6]. Adaptive mitochondrial ROS signaling has been reported to induce key transcriptional factor *pap1* and *yap1* in *S. pombe* and *S. cerevisiae*, respectively, which are a key regulator of the expression of oxidative responses genes[26]. Thus, we then evaluate the potential mitochondrial-dependent regulatory mechanisms of antioxidant properties of clove extract in yeast.

3.3. Low concentration of extract treatment does note affect yeast mitochondrial membrane potential, but high concentration.

Our data indicate that 100 ppm extract treatment did not induce mitochondrial yeast activity, similar to that without extract and 200 ppm treatment (Figure 2). On the other hand, high extract concentration (500 ppm) resulted in vigorous fluorescence intensity, suggesting its effect on inducing mitochondria activity. Thus it is likely that clove extract mediates antioxidative cellular response independent to that adaptive mitochondrial ROS signaling as proposedly occurred in calorie restriction-mediated antioxidative stress response. The previous study implicated bioactive compounds such as quercetin, resveratrol, and curcumin can directly scavenge reactive oxygen species, including O_2-, OH^- and H_2O_2 [27-29]. Indeed, clove extract has been analyzed to having quite high of quercetin concentration (28.40 mg/100 g FW) [30].

Previous data (Figure 1) showed that calorie restriction could induce oxidative stress resistance phenotype of yeast cells both following H_2O_2-induced oxidative stress treatment and ROS accumulation during stationary phase. Intriguingly, our data suggest that a high concentration of extract (500 ppm) showed a similar phenotype to that calorie restriction treatment (0.5% glucose). Conversely, 500 ppm extract-treated yeast cells were sensitive toward H_2O_2-induced oxidative stress treatment (Figure 1). Thus, our data strongly suggest pro-oxidant activity of high dose of clove extract, which likely interferes the adaptive mitochondrial ROS signaling in yeast cellular systems resulting in oxidative stress-sensitive phenotype. The previous study showed that antioxidant compound quercetin had been implicated in stimulating mitochondria activity [31], yet, the particular compounds were able to inhibit thioredoxin-mediated antioxidant system in A549 cells [33].

3.4. Clove extract shows promote longevity in yeast cells

From our data (Figure 1), it is indicated that clove extract showed its significant cytoprotective effect against oxidative stress in aged cells (after 15 days of incubation), whereas 5-days old yeast cells remain unaffected. From this data, we assume that clove extract likely has anti-aging activity in addition to that antioxidant agent. Indeed, treatment of 100 ppm clove extract increased cells viability of stationary phase cells, as shown in 10 and 15 days old of yeast culture (Figure 3). Cells viability of 100 ppm-extract treated cells was weaker than cells grown under calorie restriction conditions (0.5% glucose). In agreement to that previous essay, higher clove extract, however, decreased cells viability of stationary phase yeast cells. Thus it is indicating that clove bud extract could extend yeast life span likely in a dose-independent manner.

Stationary phase cells are exposed to various conditions including nutrient starvation, ROS accumulation from OXPHOS reactions, accumulation of toxic metabolites. Stationary phase yeast cells are highly homolog to most of the life span spent by eukaryotic cells, it is, therefore, the study of aging in model yeast is primarily conducted in stationary phase cells, or known as chronological life span [4]. Yeast will develop various mechanisms in dealing with those unfortunate conditions during stationary phase. Indeed, it is reported that accumulation of glutathione is found high in stationary phase cells than that log cells, or in oxidative stress-exposed log cells. Glutathione plays a critical role in balancing redox potential during ROS exposure since this thiol-containing compound can fix radical molecules [26]. The ability of stationary phase cells to deal with stationary phase harmful conditions may lead to life span extension [5]. Our data indicate that 100 ppm extract could lead to yeast cells longevity and viability. Such phenotype may be occurred due to the antioxidant activity of clove bud extract via ROS scavenging capability thus resulting in intracellular redox homeostasis. Further analysis in ROS and glutathione intracellular level must be conducted to clarify this circumstance. The

previous study reported the anti-aging activity of phytoextract by increasing yeast oxidative stress resistance, including *Cimicifuga racemosa*, *Ginkgo biloba*, *Apium graveolens* L. [33]

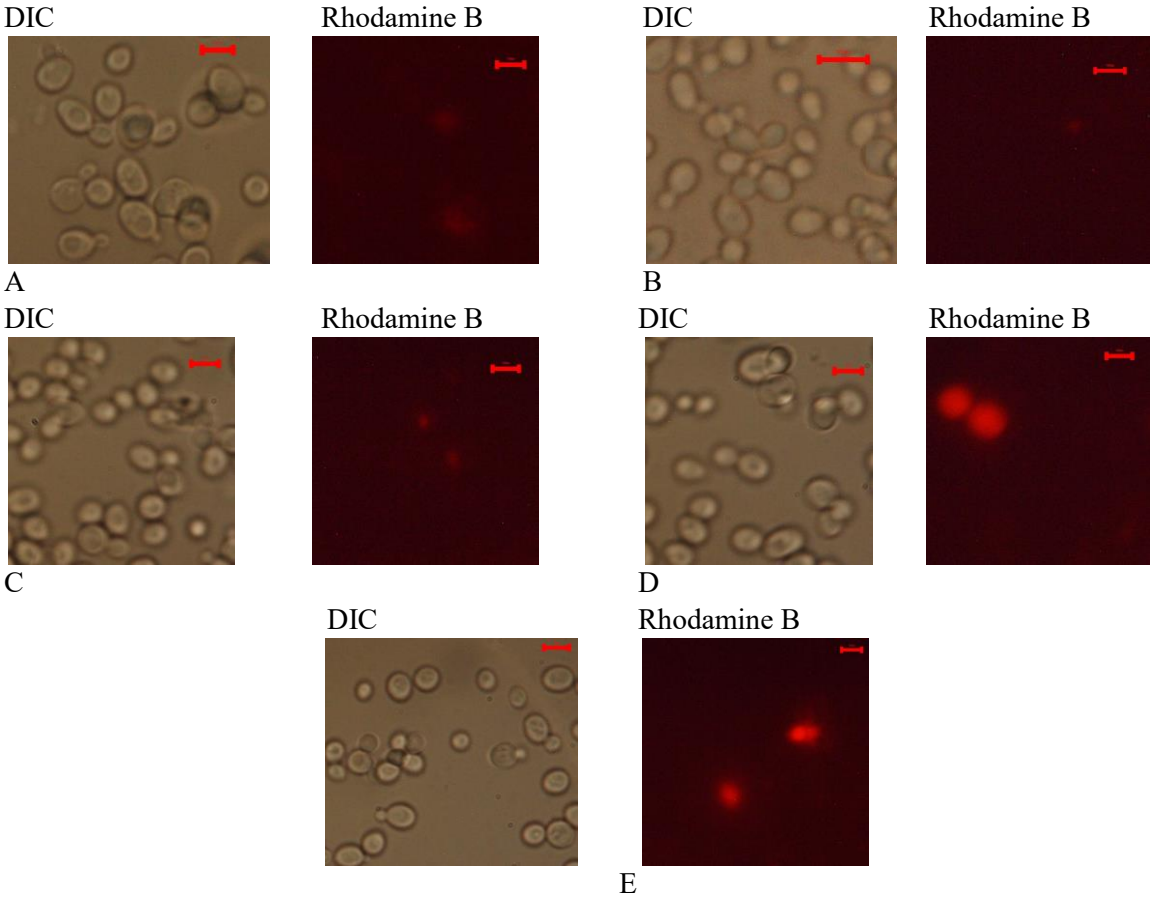

Figure 2. Effect of clove extract on mitochondria activity. Yeast cells were cultured in YPD liquid medium containing 2% of glucose with various concentration of extract (A) 0 (B) 100, (C) 200 and (D) 500 ppm for 24 hours and stained with rhodamine 123 to assay mitochondria activity. Yeast grown in YPD with 0.5% glucose was used as a positive control (E). Yeast cells were observed under fluorescence microscope observation in 1000x magnification.

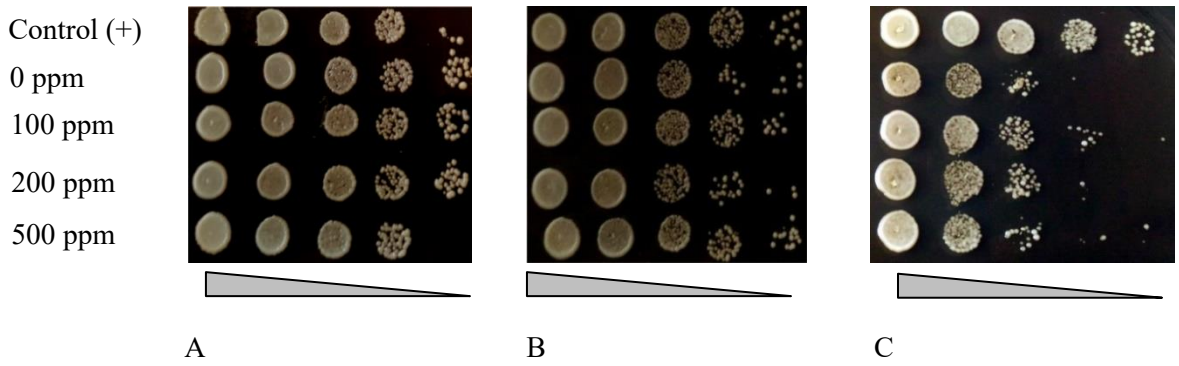

Figure 3. Effect of clove bud extract on yeast life span. Yeast cells were cultured in YPD liquid medium supplemented with various concentration of extract (100, 200, and 500 ppm). Cells grown in YPD without extract (0 ppm) addition is designed as control. Yeast grown in 0.5% glucose is designated as a positive control. Following (A) 5, (B) 10 and (C) 15 days of incubation, yeast was harvested (adjusted to OD =1) and serially diluted (up to 10^{-4}). Each dilution was then spotted (3µl) in YPD agar medium. Plates were then incubated in 30^0C for three days.

4. Conclusion

From our study, it is shown that clove bud extract (100 ppm) shows antioxidant properties, which likely lead to prolonging cells longevity and viability of yeast cells. Interestingly, the anti-aging activity of clove bud extract does not occur by intensifying mitochondrial membrane potential, as happen in calorie restriction treatment cells. Clove bud extracts likely exhibit ROS scavenging activity, instead. However, further study must be conducted to reveal the underlying mechanisms of clove bud extract, including its potential in modulating aging-related genes which involved in genome stability, stress protection, and autophagy**.**

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