

Cell Longevity of Yeast *Saccharomyces cerevisiae* by Clove Bud Extract Treatment May Occur in *Sirtuin*-Independent Pathway

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Article history

Received: 21-07-2020

Revised: 07-09-2020

Accepted: 09-10-2020

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Abstract: Accumulation of free radicals causes cell damage that leads to a phenomenon called aging. Antioxidant application is one potential strategy to avoid the damage of free radical accumulation. Many reports resume that clove, one of Indonesia's spices, contain high amount of antioxidant. Our previous research had proven that clove extract could extend longevity in yeast *Saccharomyces cerevisiae*. However, cellular mechanism on how this extract can induce cell longevity remains unknown. One of the cellular mechanisms that involve in aging pathway is the *sirtuin* pathway. Therefore, this research aimed to analyze the role of *sirtuin* (*sir2*) gene in extending the life span of yeast cell. In doing so, *S. cerevisiae sir2* gene deletion mutant (*Δsir2*) was used to clarify the *sir2*-dependent longevity mechanism mediated by clove extract. Our data revealed that clove extract could extend yeast longevity independent to *sir2* gene. In addition, clove extract could induce oxidative stress resistance phenotype of *S. cerevisiae* in *sir2*-independent manner. Interestingly, the number of normal cell and autophagy activity also increased in *Δsir2* mutant cells, thus suggesting autophagy may involve in prolonging longevity of *Δsir2* mutant cells. In conclusion, clove extract could extend longevity of yeast likely via *sir2*-independent pathway.

Keywords: Antioxidant, Longevity, Model Organism, Autophagy, H₂O₂ Scavenger

Introduction

Free radicals are unstable and harmful compound that can steal electron from other macromolecules such as lipid, protein and nucleotide in order to make itself in stable state. This compound exposure in protein and nucleotide increases susceptibility of protein to structural and functional alterations and nitrogen base damage in the DNA structure (Dizdaroglu and Jaruga, 2012). Free radicals can be produced naturally inside cell by enzymatic activity of respiration, phagocytosis and prostaglandin synthesis. Apart from enzymatic reaction in cells, free radicals also can be increased by external source such as smoke, environmental pollution, radiation, ultraviolet, ozone, several drugs, pesticides and anesthetic drug (Phaniendra *et al.*, 2015). Free radical accumulation as cell aged leads to a phenomenon called aging. Aging caused the increasing of risk to age-related diseases such

as cancer, diabetes mellitus type II, Parkinson disease, Alzheimer, heart disease and rheumatoid arthritis (Franceschi *et al.*, 2018).

Antioxidants are potential compounds that can stabilize free radicals. This compound consists of enzymatic molecules such as catalase and superoxide dismutase. In addition, non-enzymatic compounds such as vitamin C, flavonoids and phenolic compounds have also been reported to have antioxidant activity (Flora, 2009). Non-enzymatic compounds of antioxidant are highly produced by plants. Clove is one of Indonesian spices that contain high amount of antioxidants with eugenol as main bioactive compounds. Eugenol content in cloves varied between 9,281.60 to 14,650.00 mg per 100 g fresh weight (Neveu *et al.*, 2010). Phenolic compound such as gallic acid, caffeic acid, ferulic acid, ellagic acid and salicylic acid also found in cloves 783.50 to 2,375.8 mg in 100 g fresh weight (Shan *et al.*, 2005). In addition, cloves

also contain other flavonoid compounds such as caempherol, quercetin and its derivate in lower concentration. Flavonoid content of ethanol-derived extract of clove bud was reported for approximately 93.245 mg QE/100 gram (Astuti *et al.*, 2019).

High amount of antioxidant in cloves shows that cloves are potential as an anti-aging agent. Previous research had proven that antioxidant in clove extract can extend longevity in yeast *Schizosaccharomyces pombe* (Fauzya *et al.*, 2019) and *Saccharomyces cerevisiae* (Astuti *et al.*, 2019). Addition of 100 and 200 ppm of ethanol-derived clove bud and leaves extract could also increase yeast survival in H₂O₂-induced oxidative stress conditions (Astuti *et al.*, 2019; Fauzya *et al.*, 2019). However, the mechanism of how clove extract can extend yeast longevity remains unknown.

Silent information regulator 2 (*sirtuin/sir2*) is one of genes that involved in *S. cerevisiae* longevity pathway. Deletion in this gene can decrease the replicative life span of *S. cerevisiae* for up to 50% (Kaeberlein *et al.*, 1999). This gene encodes NAD-dependent histone deacetylase that acts in gene regulation, metabolism and NAD (H) synthesis (Ralser *et al.*, 2012). Because of the important function of *sirtuin*, this gene is conserved from yeast to higher eukaryotes including mice and human. This phenomenon suggests that studying the role of *sirtuin* gene in extending longevity which induced by clove extract in yeast can be a reference to study the same case in higher eukaryotes. Therefore, this study was aimed to analyze the role of *sirtuin* gene in yeast longevity that was induced by clove bud extract.

Materials and Methods

Yeast Isolates and Medium

Yeast *S. cerevisiae* strain BY7471 Wild Type (WT) and *Asir2* mutant (BY4741, MATa his3D1 leu2D0 met15D0 ura3D0 *sir2::kanMX6*) were grown in Yeast Extract Peptone Dextrose (YEPD) medium culture with 2% glucose. Hydrogen peroxide was used to induce oxidative stress environment.

Extraction of Clove Bud

The clove (*Syzygium aromaticum*) extract was prepared by using ethanol as described previously (Fauzya *et al.*, 2019). The clove buds (500 grams) were macerated using 70% ethanol at a ratio of 1:5 (sample: Solvent) at room temperature (28°C). The sample was soaked for 24 h and stirred at each 12 h. Following the harvest of the macerated substance, the process was repeated twice by using the same volume of solvent. Substance was then collected and concentrated by using the rotary evaporator (45°C) within one h. The ethanol-derived extract was obtained

in paste with dark brown color. The rendement of extract was 16.32% (w/w).

Construction of *Asir2* Mutant

Gene deletion was conducted through chromosomal integration of PCR-amplified cassettes of the targeted DNA-flanking regions as described (Janke *et al.*, 2004). The PCR cassettes for gene deletion were constructed by using template plasmid pUG6 with KanMX resistance gene (*KanMX6^r*) as marker. An upstream *sir2* gene cassette was amplified by PCR using primer S1 (5'-GAAATGATTATTAGCAGTC-3') and S2 (5'-CCAGCTTTAATGTGCCGATGtgagtcttttccttaccat-3') with *S. cerevisiae* genomic DNA as template, resulting 500bp amplicon. The downstream *sir2* gene cassette was amplified using primer S3 (5'-gtcgtatgagtttttctaGTAGAAGGCAATTTAATAAT-3') and S4 (5'-ATGTGCATTGTATTACATAG-3'), with genomic DNA as template, resulting 500bp amplicon. Primer sequence with underlined indicated the sequence of *KanMX6^r* gene. PCR reactions were prepared as follows: 5X PrimerSTAR buffer (Mg²⁺ Plus) PCR Buffer 10 µL, dNTP mixture (2.5 mM each) 4 µL, primer forward 1 µL (10-15 pmol), primer reverse 1 µL (10-15 pmol), DNA template (genomic DNA) 0.2 µL (150 ng), PrimeSTAR HS DNA Polymerase 0.5 µL (1.25 units/50 µL) and distilled water to adjust the final volume for up to 50 µL. PCR was performed at the following conditions: Pre-denaturation 98°C, 1 min; denaturation 98°C, 10 sec; annealing 55°C, 5 min; polymerization 72°C, 50 sec; post PCR 4°C. PCR was done for 30 cycles. Amplicons were then checked by using agarose gel electrophoresis and purified.

KanMX6^r -flanked *sir2* gene domain was then constructed for each upstream and downstream region using pUG6 as template. Two sets of PCR were performed for each upstream and downstream cassette. PCR was conducted by using 500 bp upstream cassette and primer S5 (5'-ctattaattcccctcgta-3') as primer resulting *KanMX6^r* -flanked *sir2* upstream region (1100 bp). PCR reactions (50 µL) were prepared as follows: PCR Buffer 10 µL, dNTP mixture (2.5 mM each) 4 µL, primer forward 4 µL (500 bp upstream cassette), primer reverse 1 µL (10-15 pmol), DNA template (genomic DNA) 0.2 µL (150 ng), Prime STAR HS DNA Polymerase 0.5 µL (1.25 units/50 µL) and distilled water to adjust the final volume for up to 50 µL. Concurrently, *KanMX6^r* -flanked *sir2* downstream region (1100 bp) was conducted by using 500 bp downstream cassette and primer S6 (5'-tgccaatgatgttacagatg-3'). PCR reactions (50 µL) were prepared as follows : PCR Buffer 10 µL, dNTP mixture (2.5 mM each) 4 µL, primer forward 1 µL (10-15 pmol), primer reverse 4 µL (500 bp downstream

cassette), DNA template (genomic DNA) 0.2 µL (150 ng), PrimeSTAR HS DNA Polymerase 0.5 µL (1.25 units/50 µL) and distilled water to adjust the final volume for up to 50 µL. PCR was performed at the following conditions : Pre-denaturation 98°C, 1 min; denaturation 98°C, 10 sec; annealing 55°C, 5 min; polymerization 72°C, 50 sec; post PCR 4°C. PCR was done for 30 cycles. Amplicons were then checked by using agarose gel electrophoresis and purified.

The resulted two amplicon cassettes were then introduced to yeast *S. cerevisiae*. A simple yeast transformation procedure was then performed to introduce the corresponding PCR cassettes in to *S. cerevisiae* BY4171 (Gietz and Schiestl, 2007). For selective growth of cell transformants, 100 µg/mL geneticin was added to YPDA medium. Transformant candidates were checked via colony PCR analysis.

Viability Assay

Spot test was used in order to analyze yeast viability following clove extract treatment within stationary phase, which correlates, to that yeast chronological life span (Fauzya *et al.*, 2019). For sub-culture, both yeast strains (WT and $\Delta sir2$ mutant) were grown in YEPD broth medium for 18-24 h at 30°C. For main culture, yeast sub-culture was transferred in YEPD broth medium containing various concentrations of clove extract (0, 100, 200 ppm) at starting OD₆₀₀ = 0.1. Main cultures were then incubated for 6 days. Main cultures were then addressed for spot assay at day 3 and 6. Cultures were harvested and serially diluted up to 10⁴. About 2 µL of each dilution were spotted in YEPD agar plates. Spot test results were analyzed after 3 days incubation at 30°C.

Oxidative Stress – Resistance Assay

The assay was conducted using spot test assay as described previously (Astuti *et al.*, 2019). Cultures were prepared as above mentioned in the viability assay. Spot was done in YEPD agar medium containing H₂O₂ in various concentrations (2, 4 and 6 mM). Yeast viability was observed after three days of incubation at 30°C.

Yeast Morphological Assay

Yeast cells (WT and $\Delta sir2$ mutant) were cultured in YEPD broth medium containing various concentration of clove extract (0, 100 and 200 ppm). At day-3 of incubation, high concentration of H₂O₂ (2 and 4 mM) was then applied in culture to induce oxidative stress condition. Morphology of yeast cells was observed under light microscope in order to determine the presence of autophagic body (Tallóczy *et al.*, 2002). To enumerate the number of autophagic bodies, about 200 cell

profiles containing the vacuole were screened. Profiles containing one or more autophagic bodies inside the vacuole were classified as positive (Tallóczy *et al.*, 2002).

Results and Discussion

Clove Extract Promotes Yeast Life Span Both in Wild Type and $\Delta sir2$ Mutant Cells.

Cell viability assay showed that the addition of clove extract could promote the viability both WT and $\Delta sir2$ cells in the stationary phase both in 3 and 6 days of incubation. In 6 days of incubation, the $\Delta sir2$ cells showed slight decrease in the cells viability compared to WT cells, in non-treated cells (Fig. 1). Such phenotype is in agreement with previous study indicating that deletion of *Sir2* resulted in decrease lifespan of *S. cerevisiae* (Smith Jr. *et al.*, 2007). Interestingly, addition of clove extract (100 and 200 ppm) could promote $\Delta sir2$ cells viability (Fig. 1). This data suggests that *sir2* is unlikely to involve in the cells viability of yeast cells in stationary phase following clove extract treatment.

Sirtuin has been known as conserved key modulator of aging in eukaryotes (Grabowska *et al.*, 2017). *Sir2* in yeast is highly homolog to that SIRT1 in mammalian cells (Frye, 2006). Activation of *Sir2* is reported to extend life span of model yeast *S. cerevisiae* (Lin *et al.*, 2002; Wierman and Smith, 2014). Previous studies reported that *sir2* gene could be activated by exogenous treatment of phytoextract primarily those containing phenolic or flavonoid compounds, such as resveratrol (Howitz *et al.*, 2003). *Sirtuin* activation by butein, fisetin and resveratrol increased lifespan by 31, 55 and 70%, respectively (Howitz *et al.*, 2003). Furthermore, extract of lemon beebrush and lycopene of tomato could activate SIRT1 activity in the rabbit's heart and liver tissues (Corbi *et al.*, 2018). On the contrary, our data indicates that sirtuin may not be involved in maintaining yeast cells viability of $\Delta sir2$ cells at the stationary phase. Thus suggesting that clove extract may modulate other pathway in promoting yeast life span. Pathway relevant for aging in yeast involves nutrient signaling via key gene *TORC1* (target of rapamycin complex 1) and *PKA* (protein kinase A) (Wei *et al.*, 2008; Deprez *et al.*, 2018), cell cycle regulation, DNA repair mechanisms, autophagy, mitochondrial homeostasis, lipostasis, protein folding and secretion, proteostasis, stress response and regulated cell death (Zimmermann *et al.*, 2018). Those mechanisms are conserved in humans as well. Thus, we further analyze the potential activity of clove extract in modulating other aging pathways of *sirtuin*, including the induction of oxidative stress response and autophagy.

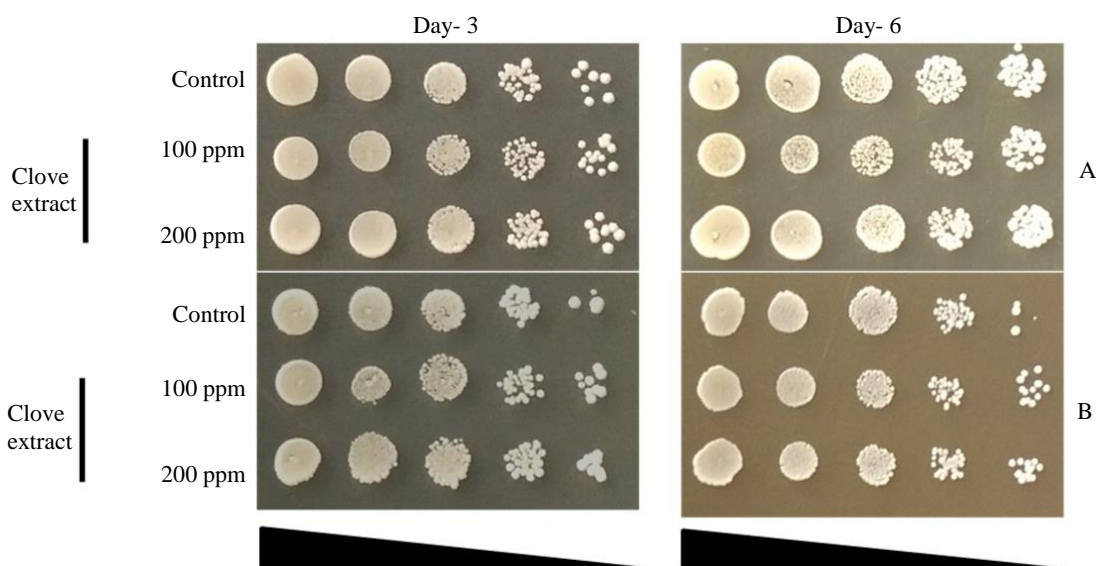


Fig. 1: Effect of clove extract treatment on the cell viability of stationary phase yeast cells of (A) *S. cerevisiae* WT BY4741 and (B) *S. cerevisiae* $\Delta sir2$. Both WT and $\Delta sir2$ were grown in YEPD broth medium containing various concentration of clove extract (100 and 200 ppm) and incubated at 300C for six days. The 3 and 6-days culture were spotted in YEPD agar plates and were incubated at 300C for three days. Yeast grown in YEPD medium without clove extract was designated as control

Clove Extract Promotes Yeast Life Span Both in Wild Type and $\Delta sir2$ Mutant Cells Under Oxidative Stress Conditions.

$\Delta sir2$ mutant was less resistant to H_2O_2 stress than WT cells (Fig. 2). Clove extract was shown to increase the tolerance phenotype of $\Delta sir2$ mutant cells against 4mM H_2O_2 treatment (Fig. 2). Such phenotype was also seen in WT cells particularly following 200 ppm treatment compared to that without extract and 100 ppm treatment. Interestingly, supplementation of 200 ppm clove extract caused cell death phenotype of WT cells following severe oxidative stress treatment (6 mM), opposite to that $\Delta sir2$ mutant cells (Fig. 2). These data suggest that *sir2* is not involved in modulating stress tolerance phenotype of yeast cells following clove extract treatment. It is reported that *sirtuin* plays critical role in anti-oxidant protective response in mammalian cells (Merksamer *et al.*, 2013). However, our data indicates that addition of clove extract could diminish the negative effect of *sir2* gene abrogation in yeast cells.

Oxidative stress is one of the causal factors of cellular aging. Thus, antioxidant compound, such as flavonoids and phenolics compounds that could combat the oxidative stress may potentially be applied as antiaging agent. Previous studies exhibit anti-aging properties of clove extract (Astuti *et al.*, 2019), roselle petal extract (Sarima and Meryandini, 2019), hesperidin-derived from *Citrus* genus (Sun *et al.*, 2012) as antiaging agent in yeast *S. cerevisiae* due to its high antioxidant activity. Oxidative stress

response pathway might be one of the targets of action of the antioxidant compounds at cellular and molecular levels. Such compounds may induce genes that involve in oxidative stress response pathway thus inducing the stress tolerance phenotype (Maeta *et al.*, 2007). It is worth noting that clove has been reported to have flavonols compounds such as quercetin (28.40 mg/100 gram) and kaempferol (23.80 mg/100 gram) (Shan *et al.*, 2005). Previous studies showed that quercetin exhibits a protective effect in *S. cerevisiae* against oxidative stress via induction of trehalose biosynthesis (Vilaça *et al.*, 2012), attenuation of lipid peroxidation and protein carbonylation (Belinha *et al.*, 2007). Thus, further study is required to gain more insight in the clove extract-dependent oxidative stress tolerance mechanism in yeast, since clove contains other bioactive compounds including phenolic compounds in which gallic acid is found in high levels (458.19 mg/100 g FW). Interestingly, gallic acid, has been reported to have antiaging activity toward yeast *S. cerevisiae* (Đorđević *et al.*, 2018). However, cellular mechanism of gallic acid in extending yeast life span remains unclear.

Clove Extract Increases Autophagy Activity

Autophagy has been implicated as one of the regulatory mechanism in prolonging yeast life span (Howitz *et al.*, 2003; Borklu-Yucel *et al.*, 2015). Our data indicate that clove extract treatment increased autophagic body of WT cells in both normal and oxidative stress conditions (Fig. 3).

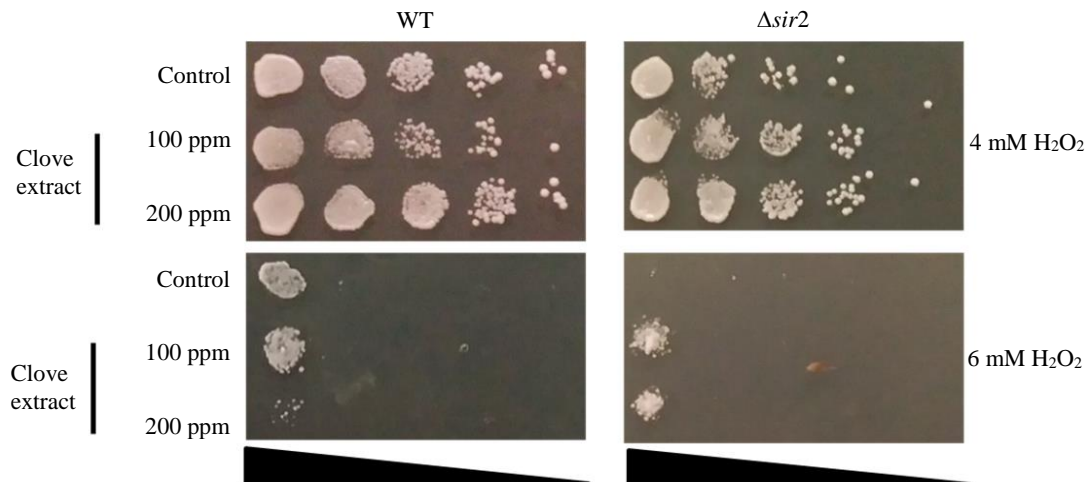
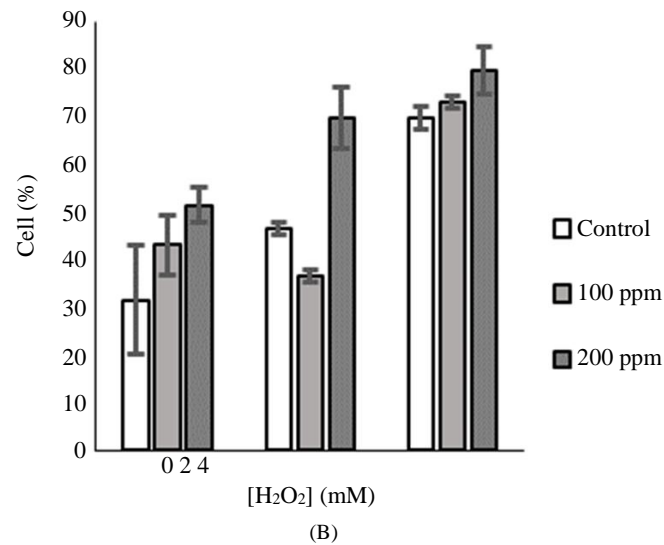
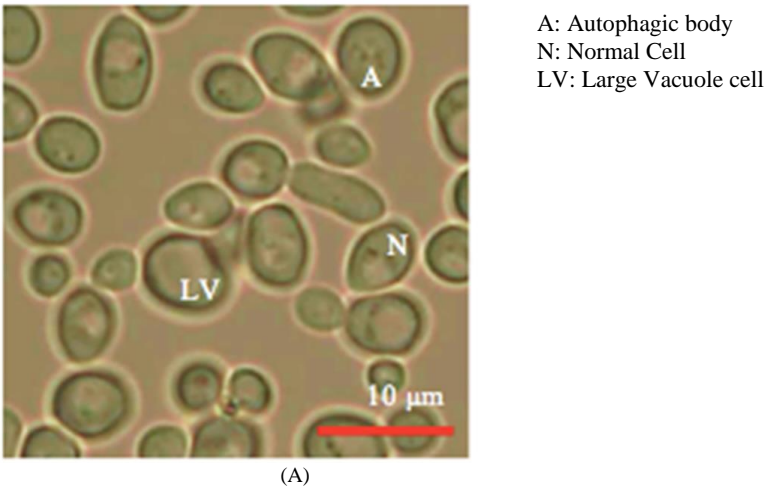


Fig. 2: Effect of clove extract supplementation on yeast viability of both *S. cerevisiae* WT and mutant $\Delta sir2$ in combating 4 and 6 mM H₂O₂-induced oxidative stress treatment. Yeast grown without clove extract addition was used as control. Yeast cells were grown in YES medium containing 100 or 200 ppm clove extract and incubated for 3 days at 30°C. Culture was then spotted in YES-agar medium containing high concentration of H₂O₂ (2 and 4 mM). Yeast grown without clove extract addition was designated as control



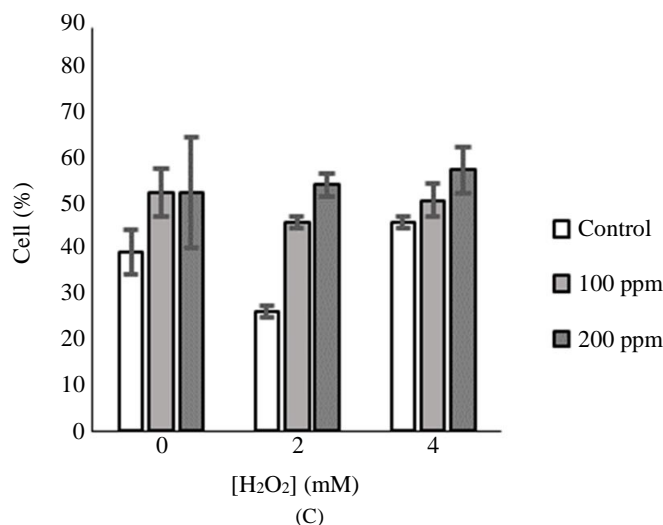


Fig. 3: Effect of clove extract on the autophagic bodies formation (A) in (B) *S. cerevisiae* WT and (C) *S. cerevisiae* $\Delta sir2$ in various hydrogen peroxide treatment. Yeast cells were grown in YES medium containing 100 or 200 ppm clove extract and incubated for 6 days at 30°C. At day-3 of incubation, high concentration of H₂O₂ (2 and 4 mM) was then applied in culture to induce oxidative stress condition. Yeast grown without clove extract addition was used as control

Following 200 mm clove extract, the number of autophagic bodies in *S. cerevisiae* and $\Delta sir2$ mutant, under 2 mm H₂O₂-induced oxidative stress conditions, was increased for 49.9 and 105.9%, respectively. The number of autophagic body in $\Delta sir2$ mutant was significantly lower than WT cells. Thus, indicating that *sir2* gene is involved in the autophagy activity (Fig. 3B). This data is consistent with previous study which revealed that during chronological aging, *Sir2* activates autophagy (Lee *et al.*, 2019). *Sir2* activation is also reported to occur as an intersection between nutrient and energy-sensing pathways, mediated by target of rapamycin (TOR)/S6 kinase homolog Sch9 through effects on autophagy (Tyler and Johnson, 2018). It is worth noting that, in WT strain, the number of autophagy body in 100 ppm clove extract treatment was found lower than that of 200 ppm extract treatment. We suggest that 100 ppm clove extract is the optimum concentration of clove extract that elicits rapid changes in the oxidative stress- response in WT yeast. Thus at 6 days of incubations, we found that the autophagy body of WT treated with 100 ppm clove extract was relatively similar to that without H₂O₂ treatment. This particular condition is considered as the WT cells were no longer experiencing stress conditions (normal conditions). However, further study is needed to clarify these dynamic intracellular conditions.

Based on the potential increasing pattern of autophagy in WT strain in without oxidative stress conditions, we suggest that *sir2* may involve in the regulation of

autophagy body via clove extract treatment. Thus the loss of *sir2* gene resulted in the similar number of autophagy body. Interestingly, in oxidative stress conditions, our data shows that treatment of clove extract could induce the development of autophagic body in both WT and $\Delta sir2$ mutant. These data indicate that clove extract likely involve in inducing autophagic body independent to that *sirtuin* activity.

Induction of oxidative stress tolerance and autophagic body phenotype in $\Delta sir2$ mutant following clove extract treatment showed that the particular extract may likely act as direct scavenger of ROS-derived H₂O₂ which in turn lowers intracellular ROS level thus avoiding the development of severe oxidative stress conditions. Previous data revealed that clove oil (22.9±2.3%) indeed had similar H₂O₂ scavenging activity to trolox (25.6±3.3%) (Gülçin *et al.*, 2012). In addition, clove extract have also been reported to have high metal chelating activity and Ferric Reducing/Antioxidant Power (FRAP) (Yadav and Bhatnagar, 2007). Thus, it is likely that clove extract may function as direct radical scavenger resulting in decreasing intracellular level of ROS against H₂O₂-induced oxidative stress conditions. However, as longevity is apparently occurred in multiple pathways, thus it is worth to further analyze the target of action of clove extract in prolonging yeast life span. Furthermore, the use of pure antioxidant compound from clove extract may give essential information regarding the modulatory effect of the particular compound toward aging-related cellular and molecular pathways in yeast.

Conclusion

To our knowledge, this is the first report of confirming the effect of clove extract at cellular level by using yeast as model organism. In addition, it is likely that clove extract may prolong yeast life span and induce oxidative stress tolerance phenotype in *Sir2*-independent pathway. Further analysis in revealing the mode of action of clove extract is needed to clarify the pharmacological effect or possible negative-side effects of this widely consumed spice, particularly at cellular and molecular levels.

Funding Information

The authors would like to thank the Ministry of Research and Higher Education of Republic of Indonesia/BRIN for the financial support of the project to RIA for fiscal year 2021 for Basic Research Scheme.

Author's Contributions

Zahrah Qolbaina Ariybah: Has contributed in whole laboratory experiments, data analysis, paper writing.

Rika Indri Astuti: Research coordinator, handled data analysis and paper writing and revision.

Sri Listiyowati: Has reviewed and revised the manuscript.

Ethics

The authors declare that there are no conflicts of interest regarding this paper publication.

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