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Activity of Ethanol-derived Fraction of Clove Leaves and Eugenol Compound as Antiaging Agent in the Yeast Model Organism *Schizosaccharomyces pombe*

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ABSTRACT
Clove is known to have high antioxidant and antivirulence activity. The antioxidant activity of clove extract presumably correlates with the extension of cellular life span (antiaging activity). Crude extract of clove leaves has been reported to prolong the life span of yeast cells, however lack of information available regarding antiaging activity of its corresponding ethanol fraction and eugenol. Eugenol is the major phenolic compound found in clove leaves. Thus, the aim of this study was to analyze the mode of action of ethanol fraction of clove leaves and eugenol at the cellular and molecular levels in prolonging life span of the yeast model organism Schizosaccharomyces pombe. The results showed that the ethanol fraction of clove leaves and eugenol compounds could prolong the yeast life span at the lowest concentration, 25 ppm and 10 ppm, respectively. The test results also showed that the ethanol fraction of clove leaves and eugenol compounds can induce yeast tolerance against oxidative stress (1mM, 2mM, and 3mM H_2O_2). Our docking molecular revealed that eugenol compounds can bind to regulator of oxidative stress response pathway, sty1 and ctt1 proteins but not on the active side. Further study is required to clarify the effect of eugenol toward the particular stress response pathway. Taken together, our study suggests the potential antiaging activity of ethanol fraction of clove leaves and eugenol compounds.

***Keywords**: antiaging, clove leaves, eugenol, Schizosaccharomyces pombe, oxidative stress*

1. INTRODUCTION

Oxidative stress occurs when there is an imbalance between the accumulation of free radicals in cells and the ability of cells to eliminate them [1]. Free radicals are compounds that have unpaired electrons in their atomic orbit. Losing electrons make molecules unstable and reactive and will try to maintain their balance by taking or releasing electrons from other molecules [2]. ROS are mostly produced by cells as a byproduct when carrying out enzymatic reactions and electron transfer processes [3]. The accumulation of ROS in cells can cause damage to important molecules such as DNA, proteins and lipids [4] thereby reducing the work of mitochondria and causing aging. The high accumulation of ROS, which cannot be overcome by the natural protection system of the cells causes the need for antioxidant compounds from outside to help overcome ROS.

Cloves are known for their benefits in the field of pharmaceuticals, especially in Asian regions such as China and Indonesia. Each part of the clove plant has a different composition of bioactive compounds. The buds and leaves of the clove plant contain eugenol, eugenol acetate, β-caryophilenol, nootkotin [5], β-sitosterol, nigrisin, glucopyranoside and olenolic acid lactone [6]. Clove buds are also high in ascorbate, tocopherol and low in riboflavin [7]. The most dominant compounds in clove buds and leaves are eugenol and eugenol acetate. Eugenol compounds from cloves are known to have benefits as antioxidants, anti-inflammatory, anti-tumor, antimicrobial [8], antidiabetic [9], and anticancer [10].

The high antioxidant activity of bioactive compounds from clove buds and leaves is thought to have a correlation with antiaging activity. The crude extract of clove leaves can extend the life span of the yeast model organism of *S. pombe* at a concentration of 100 ppm extract until the 9th day [11]. Crude clove bud extract at the same concentration is also known to prolong the life span of the *S. cerevisiae* model organisms until the 15th day [12].

Research about the antioxidant and anti-aging activities of crude extracts of clove leaves has been widely conducted. However, until now there is no information available regarding the effect of the ethanol fraction of clove leaves and eugenol compounds on cellular and molecular aspects. Thus, this study was conducted to obtain information about the antiaging activity of the ethanol fraction of clove leaves and eugenol compounds at cellular and molecular levels using the yeast model organism of *S. pombe*.

2. MATERIALS AND METHODS

2.1. Clove and Strains

Clove leaves (*Syzygium aromaticum*, var Zanzibar) were obtained from the Indonesian Spice and Medicinal Crops Research Institute (ISMCRI) Cimanggu, Bogor. Yeast *Schizosaccharomyces pombe* ARC 039 (h^-leul-32 ura4-294) was used in this study.

2.2. Cell viability assay

The anti-aging activity of ethanol fraction from clove leaves (EFCL) was tested using a modified spot test assay method [11]. The yeast isolates of *S. pombe* was inoculated into 3 mL of 3% YES liquid medium then incubated in a shaking incubator at 28 ^oC and a speed of 120 rpm for 24 hours and used as an inoculum. The turbidity of the culture was measured at a wavelength of 600 nm using a spectrophotometer. The treatment culture was prepared by inoculating the inoculum that had been made previously into 3 mL of liquid YES media with an initial OD_{600} = 0.05. EFCL (25, 50, 75, and 100 ppm) and eugenol (10, 20, 30, and 40 ppm) were supplemented in treatment culture. Yeast grown on 0.3% YES liquid medium was used as positive control and in YES 3% liquid medium without extract as negative control. The cultures were incubated for 11 days in a shaking incubator at 28 ° C at a speed of 120 rpm. The cell viability measurements were carried out on days 7 and 11. Each culture was adjusted to OD_{600} = 1 and then carried out with a serially dilution and spotted on YES 3% plates as much as 2 µL. The spotted cultures were then incubated at room temperature for 3 days.

2.3 Cell viability assay against oxidative stress

Cell viability against oxidative stress was carried out using 1, 2, and 3 mM H_2O_2 in YES 3% solid medium. The test is carried out as in the cell viability assay using the spot method.

2.4 Molecular docking

Molecular docking was performed using AutoDock 4.2 (Scripps Research Institute, La Jolla, CA) and visualized using BIOVIA Discovery Studio 2019. The target proteins used were Mitogen activated protein kinase sty1 and ctt1. Target protein modeling was carried out in The Phyre2 web portal for protein modeling, prediction and analysis, Kelley, LA. Protein was prepared by removing a water molecule and add hydrogen. Models of bioactive compounds were taken from the PubChem database (www.pubchem.ncbi.nlm.nih.gov) and converted to PDB files using MarvinView (Marvin Sketch 6.0.0).

3. RESULT AND DISCUSSION

The EFCL was obtained using the liquid-liquid method using n-hexane and ethanol compounds. The liquid-liquid method was used to separate compound based on their solubility in two different liquids (polar and non-polar solvents). The most dominant compound in clove flowers and leaves is eugenol. Eugenol is a type of compound that has an aromatic ring as a characteristic of phenol group compounds [13]. Eugenol compounds have long been known acts as antioxidants, anti-inflammatory, anti-tumor, antimicrobial [8], antidiabetic [9], and anticancer [10].

Anti-aging activity assay of EFCL and eugenol compounds was carried out using the spot test method. The spot test method was used to determine the viability of yeast cells qualitatively. If the spot culture results of *S. pombe* with EFCL and eugenol have a higher density than the *S. pombe* spot culture results without additional EFCL and eugenol (negative control), it can be assumed that the EFCL and eugenol exhibit anti-aging activity.

The viability assay results showed that EFCL (Figure 1) and eugenol (Figure 2) could increase cell viability on days 7 and 11 of incubation times compared to negative control. EFCL in low concentration (25 ppm) is known to be the best in increasing cell viability. This is evidenced by the results of *S. pombe* spot culture which added with 25 ppm EFCL can grow at 10^{-4} dilution compared to negative controls (without EFCL) which can only grow at 10^{-3} dilution on day 11th. The density of *S. pombe* cells added with 25 ppm EFCL was also

almost the same as the yeast culture treated with calorie restriction (CR) in the positive control (Figure 1).

The purer eugenol compound requires a lower concentration to increase cell viability compared to the EFCL, which is about 10 ppm. Eugenol with a concentration of 10 ppm increased cell viability better than the negative and positive controls. This is indicated by spot culture of *S. pombe* which can still grow up to day 11 at 10^{-3} dilution, compared to negative control which can only grow up to 10^{-1} dilution and positive control which can only grow at 10^{-3} dilution (Figure 2).

In this study, the positive control we used was yeast cells grown on 0.3% YES liquid medium or in a state of calorie restriction (CR). Calorie restriction is also known to be one way to overcome free radicals. Calorie restriction activates the autophagy system and activates genes that defend cells from stress [14]. Indeed, Chen and Runge (2009) also revealed that decreasing glucose levels in SD media from 3% to 0.3% can prolong the life span of *S. pombe* up to 24 days, and increase resistance of cell to oxidative stress [15]. Calorie restriction will cause Sty1 MAPK (mitogen activated protein kinase) to be activated, which affects the cellular response to stress and mitogens. The results of this cell viability test showed that the ethanol fraction of clove leaves and eugenol compounds were able to prolong the yeast life as in the CR condition even though the yeast was grown on sugar-rich media (YES 3%).

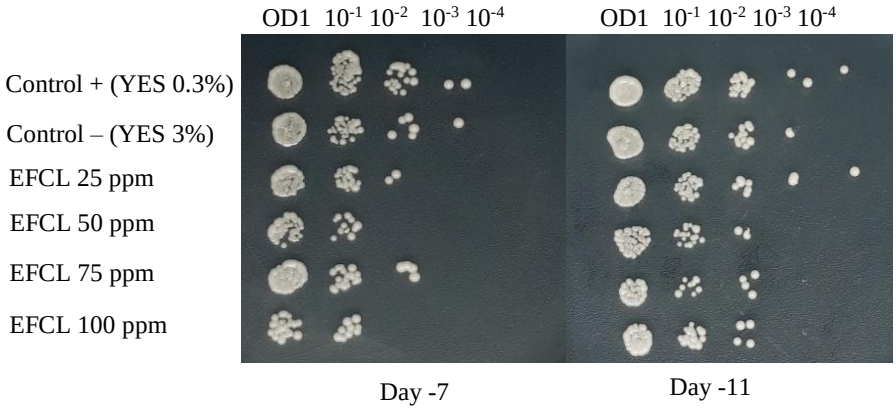

Figure 1. The effect of EFCL on the viability of *S. pombe* yeast cells grown on YES medium with 25, 50, 75 and 100 ppm of EFCL. *S. pombe* was grown on 0.3% YES media as positive control and YES medium without EFCL as negative control. Yeast culture was spotted on YES 3% solid medium on days 7 and 11

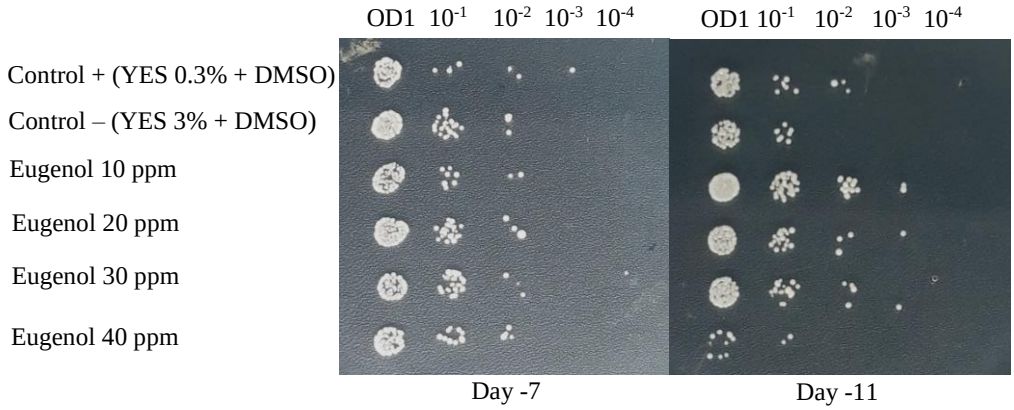

Figure 2. The Effect of eugenol compounds from cloves on the viability of *S. pombe* yeast cells grown on YES medium with eugenol concentrations of 10, 20, 30, and 40 ppm. *S. pombe* was grown on 0.3% YES + DMSO medium as positive control and on 3% YES + DMSO medium without eugenol as negative control. Yeast culture was spotted on days 7 and 11.

The EFCL and eugenol were also tested for their potential in inducing yeast resistance to oxidative stress. The test results showed that the EFCL and eugenol could induce yeast tolerance to oxidative stress (H_2O_2 1, 2, and 3 mM). The EFCL and eugenol compounds also showed a better ability than the calorie restriction treatment under 3 mM stress conditions. The EFCL at a concentration of 50 ppm can maintain yeast viability up to 10^{-1} dilution on days 7 and 11, better than the negative control and the density is better than the larger fraction concentrations (Figure 3). The concentration of EFCL needed is much smaller than previous studies using crude extract of 100 ppm [11]. Eugenol compounds are also known increase the ability of yeast to deal with oxidative stress with a smaller concentration than the ethanol fraction from clove leaves, which is at a concentration of 10 ppm (Figure 4). Eugenol with a concentration of 10 ppm can induce yeast tolerance up to 10^{-1} dilution at 3mM H_2O_2 stress. Hydrogen peroxide in the media aims to increase cellular oxidative stress. Oxidative stress can cause damage to important molecules such as DNA, proteins and lipids [4]. Generally, *S. pombe* yeasts deal with oxidative stress through three pathways, namely the MAPK (mitogen activated protein kinase) pathway, the Pap1p pathway, and the multistep phosphorelay system (Prr1p) pathway.

Hydrogen peroxide in high doses (> 1mM) is known to induce the activation of the sty1 signaling pathway [16]. Sty1 is a mitogen-activated protein kinase that plays a role in cell responses to environmental stress, such as oxidative stress, heat stress and osmotic stress [17]. The activation of the Sty1 MAPK pathway will induce the expression of genes that are important for an increased chronological life span (CLS) and overexpression of ecl1 + which increases the resistance of cells to stress. Sty1 will also inhibit Pka1 and Sck2. Loss of Sck2 and Pka1 causes a delay in aging in *S. pombe* [18].

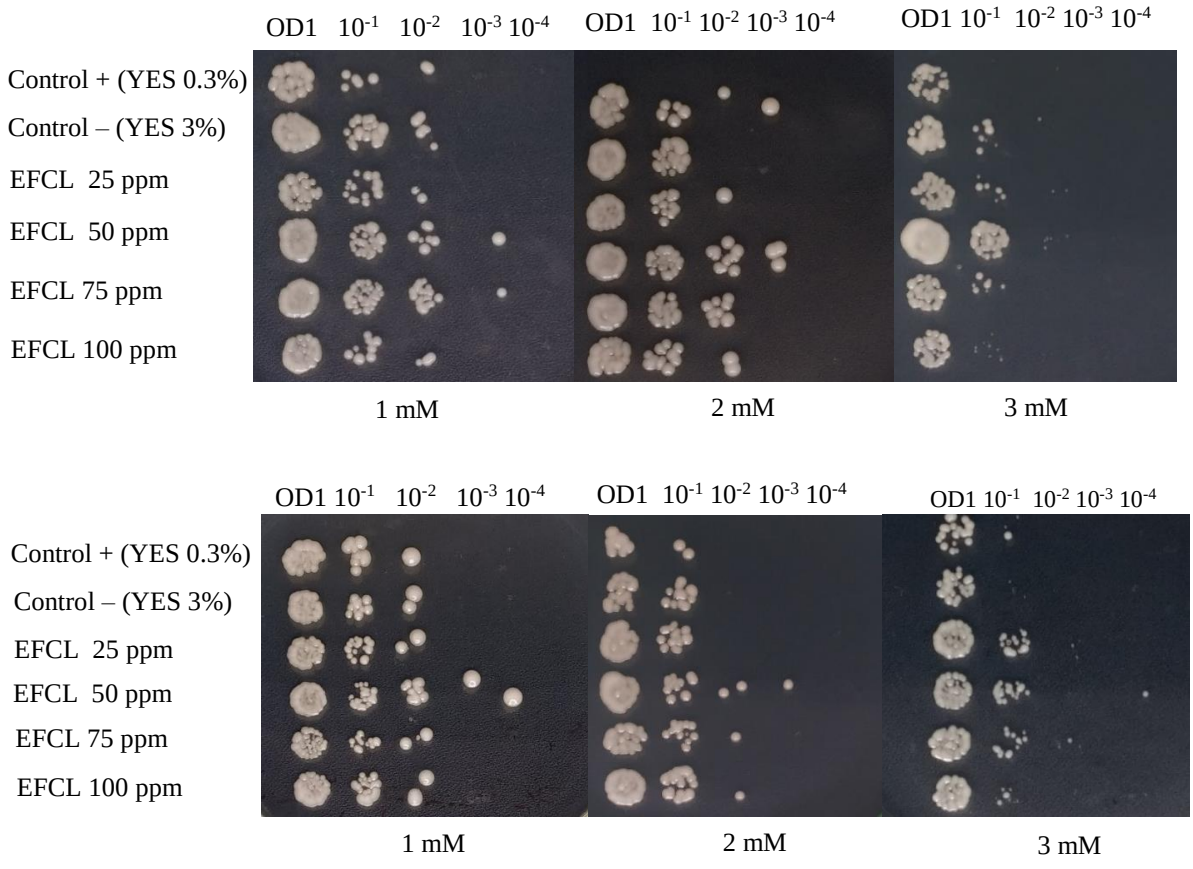

Figure 3. The effect of EFCL on the viability of *S. pombe* yeast cells grown on 3% YES medium with 1, 2 and 3 mM H_2O_2. Above: yeast cell viability on day 7 and; Below: yeast cell viability on day 11. *S. pombe* was grown on 0.3% YES medium as positive control and on 3% YES medium without EFCL as negative control. Yeast culture was spotted on days 7 and 11.

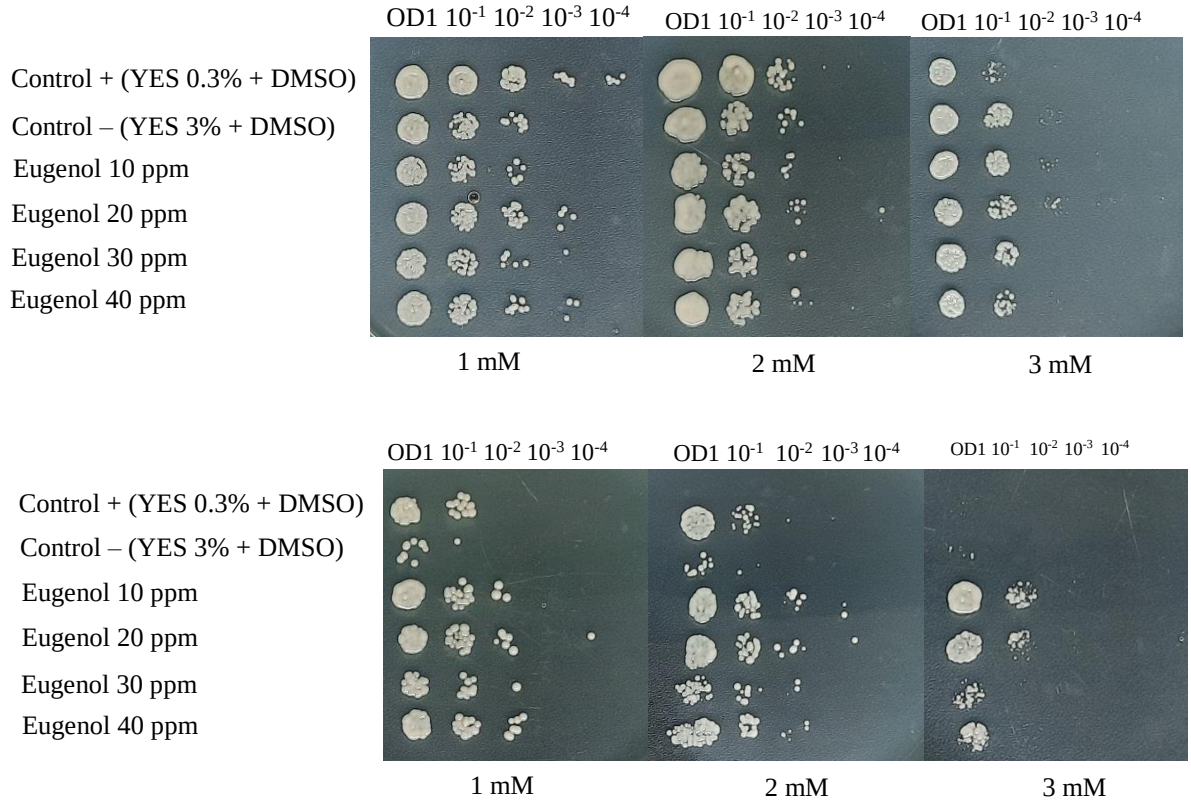

Figure 4. The effect of eugenol compounds from clove leaves on the viability of *S. pombe* yeast cells grown on 3% YES medium with 1, 2 and 3 mM H_2O_2. Above: yeast cell viability on day 7 and; Below: yeast cell viability on day 11. *S. pombe* was grown on 0.3% YES + DMSO medium as positive control and 3% YES + DMSO medium without eugenol as negative control. Yeast culture was spotted on days 7 and 11.

Molecular docking was performed on Sty1 and Ctt1 proteins to determine the effect of eugenol compounds on these proteins. The protein 3D model was built based on the amino acid sequence of the protein from NCBI and modeled using PYRE2 (Figure 5). The ligand model (eugenol) was obtained from the PubChem database and the ligand properties were checked (Figure 6). Ligands can be used in the docking process if they comply with the Lipinski's rules, including molecular weight less than 500 Da, logP value less than 5, number of hydrogen bond donors less than 5, and hydrogen bond acceptors less than 10 [19].

Lipinski's rule can determine the physicochemical properties of a ligand, for example a log P value which can determine the solubility coefficient of a compound in fat or water. Lipinski's rule describes solubility certain compounds to penetrate the cell membrane by passive diffusion. Eugenol compounds have properties that match the Lipinski rules (molecular weight = 164.2 Da, log P = 2, hydrogen bond donor = 1, and hydrogen bond acceptor = 2) so that they can be used in the docking process. Molecular weight of eugenol was 164.2 Da.

The docking process is carried out using AutoDock with a blind docking system. Blind docking was performed regardless of the grid box parameters of protein sty1 and ctt1. 20 repetitions resulted in the best pose based on the best Gibbs free energy (ΔG). The Gibbs free energy of the docking model chosen was the most negative. The results of molecular docking were visualized using the Discovery studio to see the hydrogen bonding that occurs between the target protein and the ligand. The visualization process shows that there is hydrogen bonding and amino acid interactions between Sty1 (Figure 7B) and Ctt1 (Figure 7A) proteins with eugenol compounds, but not on the active side. Data obtained from UniProt (www.uniprot.org) shows that the Sty1 protein has an active site on the amino acid ASP 141, while the Ctt1 protein has an active site on the amino acids HIS 60 and ASN 133 and metal bind on the amino acid TYR 344.

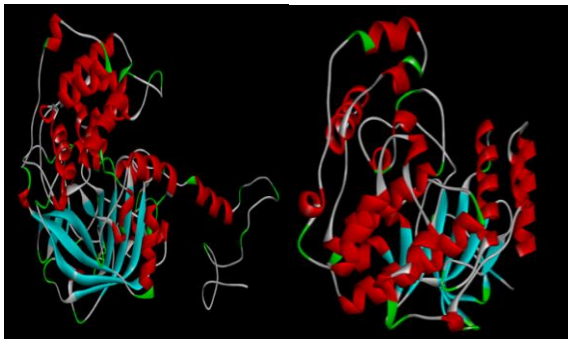

Figure 5. 3D protein model with PYRE2 (a) Ctt1 and (b) Sty1 MAPK

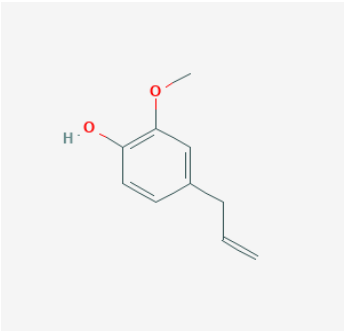

Figure 6. 2D Stucture of eugenol

Visualization results showed that the Sty1 protein binds to eugenol in the 179 and 206th amino acids with hydrogen bonds, while the Ctt1 protein binds to eugenol in the 347, 58 and 59 amino acids with conventional hydrogen bonds (Table 1). The results of the molecular docking process show that eugenol does not bind to the active sites of Sty1 and Ctt1 proteins, so it is suspected that eugenol does not directly affect the activity of Sty1 and Ctt1 proteins. Further study is required to clarify the effect of eugenol toward the particular stress response pathway.

Antioxidants in cells can work in various ways, such as donating electrons (such as ramalin), accepting electrons from free radical molecules or being immunostimulators that increase cell resistance to radical compounds [20]. Eugenol is known for its ability to reduce reactive Fe^{3+} to Fe^{2+} by donating electrons. Eugenol can also chelate Fe^{2+} to prevent the formation of superoxide, and is effective as a hydrogen peroxide scavenger [21]. The antiaging activity of the eugenol compound in the yeast *Schizosaccharomyces pombe* is thought to occur through its activity as a hydrogen peroxide scavenger and an electron donor for free radical molecules.

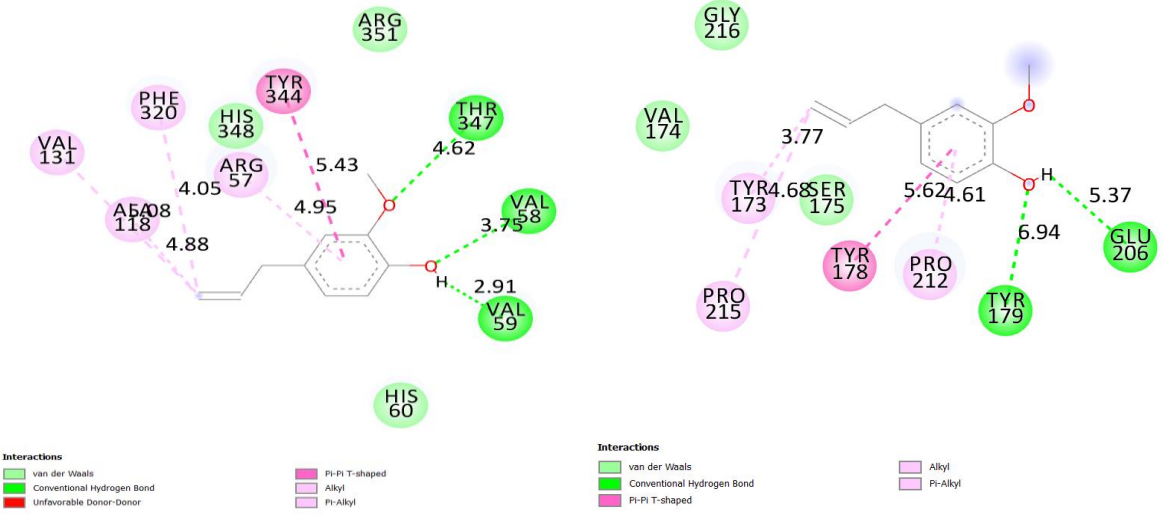

Figure 7. Visualitation the docking result for: A. Ctt1 protein; B. Sty1 protein using Discovery studio

Table 1. Hydrogen bond and Gibbs free energy

No	Protein	Docking Result		
		(ΔG)	Bond distance	Amino acid number and name
1	Catalase (Ctt1)	-6.1	4.62	Thr (347)
			3.75	Val (58)
			2.91	Val (59)
2	MAPK -Sty1	-5.7	6.94	Tyr (179)
			5.37	Glu (206)

4. CONCLUSION

The ethanol fraction of clove leaves and eugenol compounds can prolong the life of the *Schizosaccharomyces pombe* yeast cells at the lowest concentration and and induces yeast tolerance to oxidative stress. Molecular docking between eugenol compounds with Sty1 and Ctt1 proteins shows that eugenol does not prolong yeast life by affecting the action of Sty1 and Ctt1 proteins. The antiaging activity of eugenol compounds and the ethanol fraction of clove leaves is thought to occur through the activity of these compounds as a ROS scavenger or electron donors for free radical molecules.

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REFERENCES

[1] G. Pizzino, N. Irrera, M. Cucinotta, G. Pallio, F. Mannino, V. Arcoraci, F. Squadrito, D. Altavilla, Oxidative Stress: Harms and Benefits for Human Health, Oxidative Medicine and Cellular Longevity 2017 (2017). DOI: https://doi.org/10.1155/2017/8416763.

[2] V. Lobo, A. Patil, A. Phatak, N. Chandra, Free radicals, antioxidants and functional foods: Impact on human health, Pharmacognosy Reviews 4(8) (2010) 118–126. DOI: https://doi.org/10.4103/0973-7847.70902.

[3] A.P. Wickens, Ageing and the free radical theory, Respiration Physiology 128(3) (2001) 379–391. DOI: https://doi.org/10.1016/S0034-5687(01)00313-9.

[4] A. Phaniendra, D. B. Jestadi, L. Periyasamy, Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases, Indian Journal of Clinical Biochemistry 30(1) (2015) 11–26. DOI: https://doi.org/10.1007/s12291-014-0446-0.

[5] M.I. Nassar, A.H. Gaara, A.H. El-Ghorab, A.R.H. Farrag, H. Shen, E. Huq, T.J. Mabry, Chemical constituents of clove (*Syzygium aromaticum*, Fam. Myrtaceae) and their antioxidant activity, Revista Latinoamericana de Quimica 35(3) (2007) 47–57.

[6] S. Begum, Sara, B.S. Siddiqui, R. Khatoon, F. Aftab, Phytochemical studies on syzygium aromaticum linn, Journal of the Chemical Society of Pakistan 36(3) (2014) 512–516.

[7] A. Dua, A. Singh, R. Mahajan, Antioxidants of Clove (Syzygium Aromaticum) Prevent Metal Induced Oxidative Damage of Biomolecules, International Research Journal of Pharmacy 6(4) (2015) 273–278. DOI: https://doi.org/10.7897/2230-8407.06460.

[8] J.N. Barboza, C. da Silva Maia Bezerra Filho, R.O. Silva, J.V.R. Medeiros, D.P. de Sousa, An overview on the anti-inflammatory potential and antioxidant profile of eugenol, Oxidative Medicine and Cellular Longevity 2018 (2018). DOI: https://doi.org/10.1155/2018/3957262.

[9] C.H. Jung, J. Ahn, T.I. Jeon, T.W. Kim, and T.Y. Ha, Syzygium aromaticum ethanol extract reduces high-fat diet-induced obesity in mice through downregulation of adipogenic and lipogenic gene expression, Experimental and Therapeutic Medicine 4(3) (2012) 409–414. DOI: https://doi.org/10.3892/etm.2012.609.

[10] S.K. Jaganathan, E. Supriyanto, Antiproliferative and molecular mechanism of eugenol-induced apoptosis in cancer cells, Molecules. 2012. DOI: https://doi.org/10.3390/molecules17066305.

[11] A.F. Fauzya, R.I. Astuti, N.R. Mubarik, Effect of Ethanol-Derived Clove Leaf Extract on the Oxidative Stress Response in Yeast Schizosaccharomyces pombe, International Journal of Microbiology 2019 (2019). DOI: https://doi.org/10.1155/2019/2145378.

[12] R.I. Astuti, S. Listyowati, W.T. Wahyuni, Life span extension of model yeast Saccharomyces cerevisiae upon ethanol derived-clover bud extract treatment, in: IOP Conference Series: Earth and Environmental Science, vol. 299, no. 1, 2019. DOI: https://doi.org/10.1088/1755-1315/299/1/012059.

[13] A. Munisa, W. Manalu, T. Wresdiyati, N. Kusumorini, The Effect of Clove Leaf Methanol Extract on the Profiles of Superoxide Dismutase and Malondialdehyde in the Liver of Rabbits under Hypercholesterolemia Condition, Translational Biomedicine 6(2) (2015) 1–5. DOI: https://doi.org/10.21767/2172-0479.100012.

[14] N. Barzilai, D.M. Huffman, R.H. Muzumdar, A. Bartke, The critical role of metabolic

pathways in aging, Diabetes 61(6) (2012) 1315–1322. DOI: https://doi.org/10.2337/db11-1300.

[15] B.R. Chen, K.W. Runge, A new Schizosaccharomyces pombe chronological lifespan assay reveals that caloric restriction promotes efficient cell cycle exit and extends longevity, Experimental Gerontology 44(8) (2009) 493–502. DOI: https://doi.org/10.1016/j.exger.2009.04.004.

[16] A. Ikner, K. Shiozaki, Yeast signaling pathways in the oxidative stress response, Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis 569(1–2) (2005) 13–27. DOI: https://doi.org/10.1016/j.mrfmmm.2004.09.006.

[17] M.A. Papadakis, C.T. Workman, Oxidative stress response pathways: Fission yeast as archetype, Critical Reviews in Microbiology 41(4) (2015) 520–535. DOI: https://doi.org/10.3109/1040841X.2013.870968.

[18] J. Stephan, J. Franke, A.E. Ehrenhofer-Murray, Chemical genetic screen in fission yeast reveals roles for vacuolar acidification, mitochondrial fission, and cellular GMP levels in lifespan extension, Aging Cell 12(4) (2013) 574–583. DOI: https://doi.org/10.1111/acel.12077.

[19] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, Advanced Drug Delivery Reviews 64(SUPPL) (2012) 4–17. DOI: https://doi.org/10.1016/j.addr.2012.09.019.

[20] P.A.S. White, R.C.M. Olivera, A.P. Olivera, M.R. Serafini, A.A.S. Araujo, D.P. Gelain, J.C.F. Moriera, J.R.G.S. Almeida, J.S.S. Quintans, L.J. Quintans-Junior, M.R.V. Santos, Antioxidant activity and mechanisms of action of natural compounds isolated from lichens: A systematic review, Molecules 19(9) (2014) 14496–14527. DOI: https://doi.org/10.3390/molecules190914496.

[21] I. Gülçin, M. Elmastaş, H.Y. Aboul-Enein, Antioxidant activity of clove oil - A powerful antioxidant source, Arabian Journal of Chemistry 5(4) (2012) 489–499. DOI: https://doi.org/10.1016/j.arabjc.2010.09.016.