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Apoptosis in Yeast is Modulated by Clove Leaf Extract and Eugenol Potentially by Interfering Caspase YCA1

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Abstract: Apoptosis is a process of cell death important for preventing the development of diseases such as cancer, neurodegenerative disorders, autoimmune, and cardiovascular disease. Apoptosis is reported to be potentially induced by antioxidant compounds, including phytoextract. Thus in this study, we determined the potential of apoptotic modulation in yeast cells by clove extract, which was rich in eugenol. Apoptotic in yeast (*Saccharomyces cerevisiae*) was observed by forming petite colonies. Molecular docking analysis was then performed to clarify the potential binding of eugenol towards yeast caspase YCA1, the key regulator of apoptosis in yeast. Our data indicate that clove leaf extract (500 ppm) increased the frequency of petite colonies by up to 94,2% in *S. cerevisiae*. Suggesting that the particular phytoextract induced the apoptosis phenomenon in yeast. The predominant compound in clove extract, eugenol, was capable to bind YCA1 with the affinity of -4, 4 kcal/moL. Thus it is suggested that the development of apoptotic yeast cells by clove phytoextract occurred potentially due to such direct regulation of eugenol towards YCA1. However, further in vivo studies are required to elucidate the mode of action of eugenol in the regulation of YCA1 activities.

Keywords: Apoptosis, Docking, Eugenol, Petite, *Saccharomyces Cerevisiae,* YCA1

Introduction

Apoptosis is a process of cell death that occurs naturally in cells. The apoptosis program in the cells involves several signal transduction pathways that work together continuously to cause morphological changes to cell death. Naturally, apoptosis plays a vital role in controlling the number of cells by reducing cells that cannot carry out their functions, preventing cells that are deficient in nutrients, and preventing virus transmission (Noteborn, 2004). The role of apoptosis is expanding along with the development of research on apoptosis, one of which is to avoid disease through cellular mechanisms. Studies show that apoptosis is a critical determinant of the pathogenesis and progression of cancer, inflammation, neurodegenerative disorders, and autoimmune diseases (Fleischer *et al*., 2006; Abdullah *et al*., 2018).

Cloves are a native Indonesian spice that has high antioxidant activity. The antioxidant activity of cloves is based on the eugenol and eugenol acetate compounds contained therein (El Ghallab *et al*., 2020). Eugenol is a phenolic compound that has been shown to protect against the harmful effects of free radicals (Lesmana *et al*., 2021; Rahmi *et al*., 2021). This compound is reported to potentially reduce the risk of cancer, cardiovascular disease, inflammation, and other neurodegenerative diseases associated with oxidative stress (Nagababu *et al*., 2010; Dibazar *et al*., 2014; Sohilait *et al*., 2018; Parween *et al*., 2022). It is reported that clove flower extract (*Syzygium aromaticum*) has the ability as an antiaging agent (Fauzya *et al*., 2019) and anticancer agent for breast cancer therapy by modulating apoptosis in the human breast cancer cell line (MCF-7). Not only breast cancer, but clove extract also has potential as a therapeutic agent for colorectal cancer (Liu *et al*., 2014) and brain tumors (glioblastoma) (Liang *et al*., 2015). The ability of the plant extract is based on the compounds contained therein, such as alkaloids, flavonoid, saponin, tannin, and triterpenoid. These

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compounds are thought to have high antioxidant activity, which can modulate the occurrence of apoptosis, either as an inducer or an inhibitor (Cortés-Rojas *et al*., 2014; Astuti *et al*., 2021).

Apoptosis occurs not only in mammals but also in single-cell eukaryotic organisms such as yeasts. The apoptotic mechanism in yeast is similar to mammals (Zimmermann *et al*., 2018). The occurrence of apoptosis in yeast (*Saccharomyces cerevisiae*) is characterized by DNA fragmentation, plasma membrane blebbing, and cell shrinkage due to the reduction of cytoplasm and organelles (Julian and Olson, 2015). Dysfunction of cell organelles, especially mitochondria, can occur during the apoptosis process, affecting the morphology of yeast cells which causes the formation of *petite* colonies (Farrugia and Balzan, 2012; Akintade and Chaudhuri, 2020). The Yeast Caspase-1 (YCA1) protein activity induces the apoptosis mechanism in yeast. YCA1 is a protease in *S. cerevisiae* that belongs to the caspase subfamily, homologous to apoptosis-inducing proteins found in mammals (Wong *et al*., 2012). The existence of YCA1 has an essential role in regulating the apoptotic process, namely through increasing oxidative stress in cells (Madeo *et al*., 2002; Farrugia and Balzan, 2012). There is not much known about the potential of clove leaf extract in modulating apoptosis.

Molecular docking is a simulation method to determine the orientation between ligands and receptors. The principle of docking is to place the ligand into the receptor's active site, which is followed by an evaluation of the molecule based on the structural conformation. The tethering between ligands and receptors can be directed or unidirectional (Meng *et al*., 2011). This study determined the potential for apoptosis induction in yeast cells treated with clove leaf crude extract. The phenomenon of apoptosis in yeast (*S. cerevisiae*) can be observed by forming *petite* colonies. The binding potential of the compounds contained in clove leaf extract, eugenol, to the key regulator of the apoptosis pathway, YCA1 protein, was also analyzed using the molecular docking approach.

Materials and Methods

Clove Extract Preparation

Clove extract was prepared by using ethanol as a solvent. The procedure of extraction used as described earlier (Lesmana *et al*., 2021)

Induction of Yeast Apoptosis and Petite Frequency Test

The yeast cells *S. cerevisiae* BY4741 were cultured in Yeast Potato Dextrose (YPD) liquid medium and harvested after 24 h by centrifuging at 3000 rpm for 15 min and extracting the pellets. Apoptosis induction by crude Clove Leaf Extract (CLE) was carried out by adding CLE of 100, 200, and 500 ppm. The controls used were normal control, positive control (4% glucose), and 1% ethanol solvent control. All treatment conditions are listed in Table 1. The total volume of the yeast culture suspension was 1000 μL which was placed in the Eppendorf tube. Each suspension was homogenized and incubated at room temperature for 24 h (Granot *et al*., 2003).

Yeast concentrations and *petite* colony formation were analyzed using the Total Plate Count (TPC) method. The yeast suspension, induced by apoptosis, was diluted in a liquid YPD medium to a concentration of 10-6. Yeast cells (100 μL) were then spread into a petri dish containing *petite* media (YPD with 4% of glucose). The plates were then incubated at room temperature for 72 h (Syaefudin *et al*., 2014). The observed results then counted the frequency of normal colonies and *petite* colonies formed. *Petite* colonies are indicated by colonies with a diameter of ≤1.8 mm. This method was done in three replications. *Petite* colony frequencies were calculated using the equation:

$$petite\ colony\ frequencies = \frac{\sum petite\ colony}{\sum petite\ colony + \sum normal\ colony} \times 100\%$$

Preparation of Ligand Structures and Ligand Stability Analysis

The preparation of the test ligand structure was carried out using Autodock Vina software. The ligands are downloaded on the PubChem website in 3 D and saved in PDB format. The results of the preparation were then optimized by removing water molecules and stored in PDBQT format. The ligands were then analyzed for bioavailability according to the Lipinksi rule (Lipinski *et al*., 1997).

Receptor Structure Preparation

YCA1 protein structure is downloaded on the site *www.rcsb.org/pdb*. The protein structure chosen is YCA1, derived from *S. cerevisiae*. The receptors used in molecular docking must have a crystallological resolution of less or equal to 2.5 Å and are stable in the form of a 3 D structure (Cole *et al*., 2005). The receptor protein is then cleaned from water molecules and ligands. Hydrogen ions were then added using the Discovery Studio Visualizer software. The structure is then saved in PDB format. The preparation results were then converted into PDBQT format using AutoDock Vina Tools software.

Molecular Docking

The ligands attached to the YCA1 protein structure are separated and prepared first. After that, molecular docking was carried out with the dimensions of x = 40, y = 25, z = 25 and center x = 31,148, y = center 33.79 and z = center 33.34 and spacing = 0.375 Å. Molecular docking was done 20 times until a Root Mean Square Deviation (RMSD) value <2.5 Å was obtained at least three times.

Table 1: Preparation of the conditions for yeast cell apoptosis induction with the addition of Glucose (Glu) and crude Clove Leaf Extract (CLE)

Sample	Glu (μL)	EtOH (%)	Cells (μL)	YPD (μL)	dH_2O (μL)	CLE (μL)
Normal	-	-	100	500	400	-
Ethanol 1%	-	10	100	500	390	-
Glu 4%	200	-	100	500	200	-
Glu 4% CLE 100 ppm	200	-	100	500	190	10
Glu 4% CLE 200 ppm	200	-	100	500	180	20
Glu 4% CLE 500 ppm	200	-	100	500	150	50

The ligands and receptor proteins that have been prepared and stored in PDBQT format are then put in the Vina folder. The vina config file is compiled using notepad software and saved as conf. The molecular docking process is carried out according to the parameters in validating the molecular docking method. Molecular docking is performed using the command prompt program "cmd". The "cmd" program is opened, then programming commands are carried out until they are in the Vina folder. The programming command to run the molecular docking program is "C:\vina --config conf.txt --log log.txt". The molecular docking results can be seen in the document in PDBQT format and log files that can be opened using notepad software. The out document is opened using the Discovery Studio Visualizer application. The log file is a document that contains data on the value of Gibbs free energy (ΔG^o/binding affinity) in kcal/mol units.

Analysis of molecular docking results can be determined by looking at the Gibbs free energy (ΔG^o/binding affinity). The Gibbs free energy value analyzes the bond affinity between the ligand and the receptor. In the ligand-receptor molecular docking analysis, a 3 D visualization of the test-receptor ligand complex that is closest to the control-receptor ligand interaction model using the Discovery Studio Visualizer was also determined. The test ligand is combined with the receptor by copying the selected model on the "ligand" tab screen and pasting it on the "macromolecule" tab screen. The ligands are pulled (dragged) towards the receptors until they are fused. The Gibbs free energy (ΔG^o) value obtained is then recorded.

Results and Discussion

Clove Leaf Crude Extract as a Potential Apoptosis Modulator

Based on the literature search, it is known that clove extract has the potential as an apoptosis modulator. To understand the apoptotic mechanism of clove extract, we used yeast as a model. The phenomenon of apoptosis in yeast was observed by forming small colonies (petite) as shown in Fig. 1.

Apoptosis in yeast can be indicated by cell shrinkage (Granot *et al*., 2003). The cell shrinkage affects colony morphology so that it is smaller (*petite*) compared to normal cells. The formation of *petite* colonies in yeast is influenced by the addition of glucose or CLE, which induces oxidative stress as the beginning of apoptosis signaling. The oxidative stress experienced by cells causes the mitochondria to lose their respiration capacity (mitochondrial dysfunction) (Kitagaki and Takagi, 2014; Vowinckel *et al*., 2021). Yeasts with an imperfect mitochondrial genome (rho^-) or no mitochondria (rho^0) are called *petite* cells because they form small colonies when grown in glucose-containing media. Cells with a normal mitochondrial function (rho^+) form a grande colony (Dimitrov *et al*., 2009).

Apoptosis induction was performed using *petite* media, namely YPD 0.5%, with the addition of 2 mL of absolute ethanol. Absolute ethanol acts as a second source of carbon to be used by cells after the glucose content in the medium runs out. However, *petite* cells cannot use ethanol due to mitochondrial dysfunction, so these cells will remain small in size (Vowinckel *et al*., 2021). The results showed that treatment of Glu 4% with CLE at either 100, 200, or 500 ppm could increase the formation of *petite* colonies the same as the positive control for Glu 4% (Table 2).

Petite frequency calculation is done by calculating the number of *petite* colonies divided by the total colonies formed. Apoptosis modulation can occur with the addition of glucose concentrations and the addition of Clove Leaf crude Extract (CLE) (Table 2). The test results showed that 4% of glucose could induce apoptosis better than normal controls. The frequency of *petite* with 4% glucose was 37.5%, while in normal control, the frequency of *petite* was only 11.5%. These results are consistent with those reported previously, in which 4% glucose can induce apoptosis in yeast so that it can be used as a positive control (Syaefudin *et al*., 2014).

The highest concentration of CLE (500 ppm) was able to increase the number of *petite* colonies with a frequency of 94.2%. Meanwhile, the treatment of CLE (100 ppm) was not significantly different from that of CLE (200 ppm). Thus it is suggested that CLE with a concentration of 500 ppm has the potential to induce apoptosis well.

Eugenol's Affinity for YCA1 Metacaspase Protein

Eugenol is an active compound that is the main component of clove leaves and has antioxidant activity (Bezerra *et al*., 2017). Eugenol is a phenolic compound that acts as a protector of the harmful effects of free radicals. This compound's potential biological activity includes reducing the risk of cancer, coronary heart disease, stroke, atherosclerosis, osteoporosis, inflammation, and other neurodegenerative diseases associated with oxidative stress (Jaganathan and Supriyanto, 2012; Zari *et al*., 2021).

Fig. 1: Apoptosis induction of *S. cerevisiae* grown on petite media with different treatments. (a) Normal, (b) Ethanol 1%, (c) Glucose (Glu) 4%, (d) Glu 4% + crude Clove Leaf Extract (CLE) 100 ppm, (e) Glu 4% + CLE 200 ppm, (f) Glu 4% + CLE 500.

Fig. 2: 2D visualization of contact of amino acid residues at ligand-bound YCA1 receptors. (a) 1.1 diphenylethanol (DFH), (b) eugenol

Table 2: Effect of Clove Leaf Extract (CLE) on the frequency of petite yeast cells

Sample	Normal colony	Petite colony	Petite frequency
Normal	$5,4 \times 10^7$	$6,2 \times 10^6$	11,5%
Ethanol 1%	$1,1 \times 10^7$	$1,5 \times 10^6$	13,9%
Glu 4%	$4,1 \times 10^7$	$1,6 \times 10^7$	37,5%
Glu 4% CLE 100 ppm	$3,2 \times 10^7$	$2,0 \times 10^7$	62,0%
Glu 4% CLE 200 ppm	$2,7 \times 10^7$	$1,4 \times 10^7$	53,0%
Glu 4% CLE 500 ppm	$2,9 \times 10^7$	$2,7 \times 10^7$	94,2%

Table 3: Properties and stability of the eugenol ligand according to the Lipinski rule (Lipinski *et al*., 1997)

Parameter	Eugenol properties
Molecular weight	164.2 g/moL
Log P	2
Number of hydrogen bond donors	1
Number of hydrogen bond acceptors	2

Table 4: Gibbs free energy (ΔG) is the result of docking between the ligands and the YCA1 protein

Compound Name	Gibbs free energy (ΔG) (kcal/mol)
1,1-diphenylethanol	-5.1
Eugenol	-4.4

Table 5: Hydrogen bonds and amino acid interactions between ligands and YCA1 protein

	Docking simulation results	
Compound	Bond distance (Å)	Amino acids and functional groups
1,1-diphenylethanol	4.65	Gln (184) O-N
Eugenol	4.78	Gln (184) OH-N

Table 6: The ligand contacts the amino acid residues of the YCA1 protein

Compound	Amino acid residue
1,1-diphenylethanol	Asn (185), Val (190), Val (242), Asp (182), Gln (184), Leu (187)
Eugenol	Leu (187), Asp (186), Asn (185), Gln (184), Asp (183), Asp (182), Val (190), Val (242), Thr (192), Asp (243)

The results of the apoptosis induction test showed the effect of crude extract of clove leaves in inducing apoptosis in yeast. It suggests the presence of eugenol binding as an antioxidant in the crude extract of clove leaves against the YCA1 protein in yeast. YCA1 is a receptor protein on the yeast mitochondrial membrane that modulates apoptosis (Wong *et al.,* 2012).

Eugenol is used as a ligand in this molecular docking analysis. The eugenol ligand was analyzed for bioavailability using the Lipinski rule. Lipinski's rule describes the solubility of a ligand to predict whether the ligands can be absorbed by the body or not (Lipinski *et al*., 1997). Lipinski's rule states that a drug or compound can only be absorbed by the body orally if it meets the five rules, namely relative atomic mass <500 Da, hydrogen bond donor <5, hydrogen bond acceptor <10, Log P value <5, and molar refractivity between 40. -130. The data show that the structure and stability of the eugenol ligand used have met the Lipinski solution (Table 3).

The docking simulation results show the value of Gibbs free energy (ΔG), which is used as a parameter of ligand stability. Free energy describes the binding affinity of the ligands to the receptor. The more negative (ΔG) value indicates the best level of stability between the ligand and the receptor so that the bonds formed will be very strong (Meng *et al*., 2011). The data showed that the (ΔG) eugenol value was higher than the (ΔG) value of 1, 1-diphenylethanol which is a natural ligand in the YCA1 receptor (Table 4). It shows that the binding affinity of eugenol to YCA1 is lower than that of 1,1-diphenylethanol.

Docking simulations are carried out under flexible ligand conditions. It causes the interaction between ligands and receptors. The interactions that occur can be covalent or non-covalent interactions which can increase the affinity between ligands and receptors. This interaction is observed through chemical bonds such as hydrogen bonds, electrostatic bonds, and van der Waals forces. The docking results showed a hydrogen bond between eugenol and the amino acid Gln (184) at the YCA1 receptor with a distance of 4.78 Å (Table 5).

In addition to hydrogen bonding, the interaction can be in the form of contact with amino acid residues, which can also increase the binding affinity between the ligand and the receptor. The results of docking analysis showed that there was contact between eugenol and amino acids Leu (187), Asp (186), Asn (185), Gln (184), Asp (183), Asp (182), Val (190), Val (242), Thr (192), Asp (243) at the YCA1 receptor (Table 6). Six of ten amino acids are the same as the active amino acid in YCA1, which interacts with 1,1-diphenylethanol. It indicates that eugenol can be a competitive ligand for 1,1-diphenylethanol against YCA1. The docking results were observed to prove that eugenol from clove extract can potentially interact with YCA1 protein, allowing apoptosis induction. Further research at the in vivo level is needed to prove this mechanism. The docking results are visualized in the form of a two-dimensional structure, as shown in Fig. 2.

Conclusion

Crude clove leaf extract can modulate apoptosis. Crude clove leaf extract with a concentration of 500 ppm increased the frequency of petite colonies up to 94.2% in *Saccharomyces cerevisiae*. Eugenol from clove extract was able to bind YCA1 with an affinity of -4.4 kcal/moL, likely to trigger apoptosis. However, further studies to determine this phenomenon at the cellular level are required.

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Author's Contributions

Septiyani Amini: Research experiment, data collection, writing and manuscript draft.

Rika Indri Astuti: Research coordinator, design, data analysis, and manuscript revision.

Irmanida Batubara: Data analysis, research design, manuscript revision.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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