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Antioxidant Activity of *p*-Hidroxy-*m*-Methoxy Chalcone and its Combination with Doxorubicin: *In Vitro* and *In Silico* Study

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Abstract. Chalcone has a variety of interesting biological activities, including as an antioxidant and anticancer. Antioxidants are molecules that can retard or prevent the oxidation process or inhibit the formation of free radicals. *p*-Hydroxy-*m*-Methoxy Chalcone (*p*H*m*MC) is a chalcone derivative reported has anticancer activity both in used single and in combination treatment with Doxorubicin (DOX) in breast cancer cell lines. DOX is one of the chemotherapy agents widely used in cancer treatment but the medicine has side effects of cardiotoxicity. This effect is generally associated with free radical formation. This study aims to find out the activity of *p*H*m*MC as an antioxidant both in used single and in combination treatment with DOX by *in vitro* and to explore the potential of *p*H*m*MC as an antioxidant by *in silico*. Activity tests as antioxidants were measured by 1,1-diphenyl-2- picrylhydrazyl (DPPH) test. Ascorbic acid (Vitamin C) was used as a positive control. Antioxidant activity was calculated as the value of 50% Inhibition Concentration (IC_{50}). *In silico* study was carried out by molecular docking using Protein Ligand ANT System (PLANTS) software with peroxiredoxin 5 [1HD2] as the target. The results showed that the IC_{50} values of *p*H*m*MC, DOX, and Vitamin C were 11.9; 21.6 and 3.3 μ/mL respectively. The combination of *p*H*m*MC-DOX has a higher antioxidant activity than single *p*H*m*MC or single DOX. The docking molecular showed that there were similarities amino acids involved in the interaction between PRDX5[1HD2]-*p*H*m*MC and PRDX5[1HD2]-DOX. This research indicated that *p*H*m*MC and *p*H*m*MC-DOX are potentially developed as an antioxidant.

Introduction

Chalcone (1,3-diphenylpropen-1-on) is an intermediate compound in the flavonoid biosynthesis in nature. This compound has an unsaturated keto group which causes a lot of interesting biological activity. Chalcone and its derivat have been widely studied as a therapeutic compound, especially as an antitumor drug. Because of the structure and many biological activities, at the time chalcone referred to the new era of medicines in its capacity as antitumor, antibacterial, and anti-inflammatory [1]. Chalcones and their derivatives also have been reported to show attractive activities as antioxidants [2-5].

3-(4'-hydroxy-3'-methoxyphenyl)-1-phenyl-2-propen-1-on or *para*-hydroxy-*meta*-methoxy chalcone (*p*H*m*MC) is a derivat chalcone with hydroxyl groups in the *para* position and methoxy group in the *meta* position. This compound has been reported to show potent inhibition of lipid peroxidation [6]. This activity showed that *p*H*m*MC is potential as an antioxidant. The compound also showed cytotoxic effect on HeLa cervix cancer cells, Raji lymphoblastoid cancer cells [7], and T47D breast cancer cells [8]. The attractive thing was *p*H*m*MC not have cytotoxic effect against Vero normal cells [9]. In silico study showed that *p*H*m*MC interfered the interaction of some tyrosine kinase receptors with adenosine triphosphate (ATP) [10]. The study shows that *p*H*m*MC is potentially developed as an anticancer medicine.

Doxorubicin (DOX) is an anthracycline class of chemotherapy agent that widely used in the treatment of various epithelial cancers such as breast cancer and leukemia. One mechanism of DOX is to react with cytochrome P450 reductase in the presence of Nicotinamide adenine dinucleotide phosphate reduced (NADPH) to form an intermediate substance. The intermediate substance will react with oxygen to produce free radicals that can destroy cells [11,12]. However the resulting free radicals of DOX also cause side effects such as nausea, myelosuppression, arrhythmia, and cardiomyopathy followed by heart failure [13]. The potential of treatment methods that have been developed to reduce the side effects was by reducing DOX doses or using combination therapy [14].

Combination treatment of *p*H*m*MC and DOX on T47D cancer cells showed a synergy to strong synergy effect [15]. However, the potential of *p*H*m*MC as an antioxidant has not been thoroughly studied, as its antioxidant contribution if combined with DOX. The antioxidant activity of *p*H*m*MC is expected to reduce the side effects of DOX on the use of the *p*H*m*MC-DOX combination. The study provides an overview of how the antioxidant effect of *p*H*m*MC affects the antioxidant activity of DOX on the use of the combination of *p*H*m*MC-DOX. The antioxidant activity of *p*H*m*MC-DOX combination is expected to increase compared to the activity of a single *p*H*m*MC and DOX, to reduce the side effects of DOX single that cause free radicals when used in cancer treatment.

Materials and Methods

Materials and Tools. The research used *p*H*m*MC obtained from Prof. Dr. Indyah Sulistyo Arty, 1,1-diphenyl-2- picrylhydrazyl (DPPH) from Sigma-Aldrich, and doxorubicin (DOX) from Kalbe. Whereas ascorbic acid (vitamin C) and methanol were obtained from Merck. Measurement of absorbance of DPPH test was carried out using a spectrophotometer Hitachi U-1800. The tools used for *in silico* test were a set of computers with Windows 7 32 bit specifications and Linux co-PenDrive program for Linux simulation on Windows, YASARA version 10.1.8 (C) 1993-2010 by Elmar Kreiger (licenced to Hari Purnomo Universitas Gadjah Mada) for visualization and preparation protein, Protein-Ligand ANT-System (PLANTS) software downloaded from http://www.tcd.uni-konstanz.de/research/plants.php for molecular docking, Marvin Sketch 5.2.5.1 for ligand preparation (*p*H*m*MC), and Molecular Operating Environment (MOE) program for visualizing amino acids

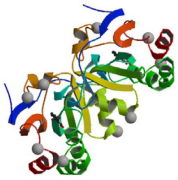
Preparation of DPPH and Samples. DPPH was diluted in methanol to get a concentration of 1 mM. The concentration of *p*H*m*MC samples were prepared as follow: 0.3125; 0.625; 1.25; 2.5 and 5 μg/mL, while DOX were prepared as as follow: 2.5; 5; 10; 20; and 40 μg/mL. The free radical scavenger reference compound or positive control was used ascorbic acid (vitamin C) in concentration 0.3125; 0.625; 1.25; 2.5; and 5 μg/mL. The concentration of combination *p*H*m*MC-DOX in this study was used under the 50% Inhibition Concentration (IC_{50}) value. All samples and control were diluted in methanol.

Antioxidant Test. Prepared samples such as procedure above were taken 1 ml and put into an Eppendorf tube. Each sample was added 0.1 mL DPPH 1 mM then mixed using a vortex mixer. The solution was incubated at 37°C for 30 minutes then its absorption was measured at 515 nm wavelength (maximum wavelength) using spectrophotometer [16]. DPPH diluted in methanol was used as blank. The percentage of inhibition (% I) was calculated based on {(blank absorption-test sample absorption)/blank absorption} x 100. The value of Inhibition Concentration (IC_{50}) was calculated from the linear regression curve between %I versus various concentration.

In silico Test. *In silico* tests are carried out in the order of protein preparation, structure preparation of test compounds, validation, and molecular docking [17]. Peroxiredoxin 5 (1HD2), an antioxidant enzyme was identified as the target receptor for antioxidant compounds. Proteins Data Bank PDB of 1HD2 was obtained from https://www.rcsb.org. [18, 19]. The protein was selected in active form that binds to native ligand (Table 1). The binding of 1HD2 and native ligand was validated using Root Mean Square Distances (RMSD) calculation. If the RMSD value is less than 2.0 angstroms (A), the protocol was received and docking of the test compound on the target protein can be carried out [17, 20] Bermen. The native ligand, all bound water, ligands and cofactors were then removed by the YASARA program to provide space (pocket/cavity). This space was used to analyze the interaction

of ligand (*p*H*m*MC, DOX, and ascorbic acid) and 1HD2. Visualization of amino acid residues that interact with the ligand was processed using the MOE program [17].

Table 1. The structure of peroxiredoxin 5 as a target protein and its native ligand

Protein	PDB	Structure	Native
peroxiredoxin 5 (PRDX5)	1HD2		BEZ

Results and Discussion

In vitro antioxidant testing or inhibition of free radicals from *p*H*m*MC, DOX and *p*H*m*MC -DOX combinations were carried out on DPPH using vitamin C (ascorbic acid) as a positive control. DPPH is an easy and fast method for testing antioxidant activity. DPPH (1,1-difenil-2-pikrilhidrazil) is a free radical that has stable properties at room temperature. DPPH solution in methanol produces violet colors whose intensity will be reduced in the presence of antioxidant molecules. The DPPH method is based on the ability of an antioxidant compound to inhibit free radicals by donating hydrogen atoms. DPPH will capture hydrogen from antioxidant compounds and convert it to 1,1-diphenyl-2-picrylhydrazine [21]. Antioxidant activity is expressed by the IC_{50} value, which is the concentration of the sample solution needed to inhibit 50% of DPPH free radicals.

The test results of antioxidant activity using DPPH (Fig1) showed that *p*H*m*MC (IC_{50} = 11.9 µg/mL) had higher antioxidant activity than DOX (IC_{50} = 21.6 µg/mL). However, the antioxidant activity of these two compounds was lower than ascorbic acid (IC_{50} = 3.3 µg / mL) which is a strong antioxidant. Antioxidant activity of *p*H*m*MC-DOX (Fig 2) was done at a concentration under IC_{50}. The variation of concentrations used are 2.5; 5 and 7.5 µg/mL of *p*H*m*MC and 5; 10 and 15 of DOX. The result showed that antioxidant activity *p*H*m*MC-DOX higher than single *p*H*m*MC and single DOX. The antioxidant activity of *p*H*m*MC-DOX is getting stronger in line with the increasing concentration of the combination.

The antioxidant activity of *p*H*m*MC was contributed from the presence of free hydroxyl group (OH) and double bonds (C = C) in its structure. Several studies have shown that the antioxidant activity of chalcone and their derivatives is affected by hydroxyl substituents and double bonds (-C = C). These groups can give 1 hydrogen molecule to free radicals such as reactive oxygen species (ROS) so that ROS becomes stable and new free radicals are formed which are less reactive [5,22].

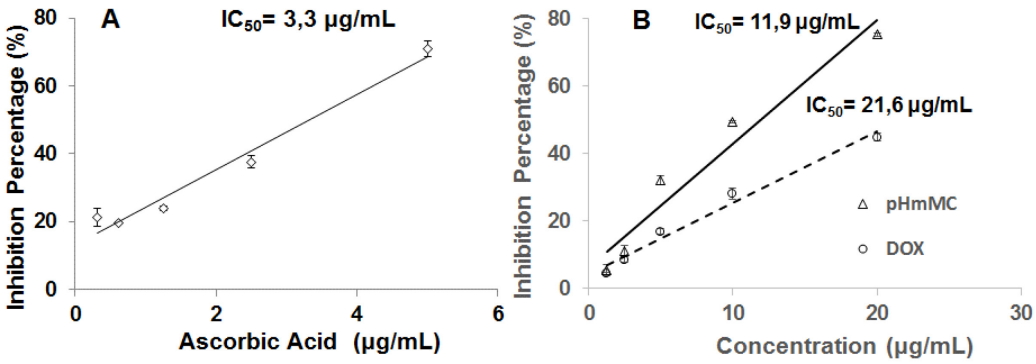


Fig. 1. The profile of antioxidant activity of ascorbic acid (A), *p*H*m*MC and DOX (B) againts DPPH. Antioxidant activity of *p*H*m*MC is higher than DOX but is lower than ascorbic acid.

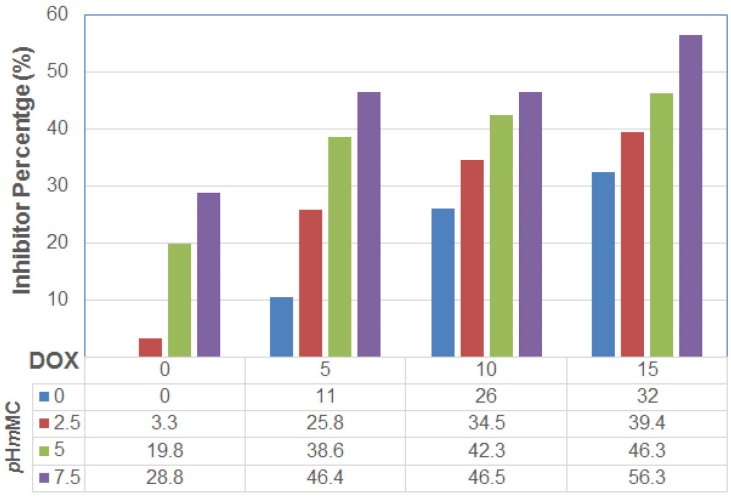


Fig. 2.Antioxidant activity of *p*H*m*MC, DOX and *p*H*m*MC-DOX. Antioxidant activity of combination *p*H*m*MC and DOX is higher compared to the antioxidant activity of single *p*H*m*MC and single DOX.

The in silico test of antioxidant activity of *p*H*m*MC, DOX, and ascorbic acid were carried out using peroxiredoxin 5 (PRDX5) as a target receptor. This target is a peroxidase enzyme that has been identified in prokaryotes and eukaryotes. PRDX5 is a new type of thioredoxin peroxidase from mammals. This enzyme widely is expressed on tissues and found in mitochondria, peroxisomes and cytosols. The enzyme has been involved in antioxidant protective mechanisms and signals transduction in cells [18]. PRDX5, PDB ID 1HD2, binds to antioxidant ligands. In this test benzoic acid [BEZ] is used as native ligand. Benzoate ions, a hydroxyl radical scavenger, are recorded close to the active site of the enzyme, so the role of benzoate in antioxidant activity is used as a comparison [17,18]. The results of validation using RMSD between 1HD2 and BEZ show that the RMSD value is 1.9252 A or less than 2. Thus BEZ can be used as a native ligand for the docking test and molecular testing can be continued used for analysis.

The results of docking scores of *p*H*m*MC, DOX and ascorbic acid with PRDX5[1HD2] are shown in Table 2 and a visualization of the interactions is shown in Fig 2. The study showed that the docking score between PRDX5[1HD2]-*p*H*m*MC and PRDX5[1HD2]-DOX have almost the same value, which is respectively -61.8134 and -61.1652. The scores are lower than PRDX5[1HD2]-Ascorbic acid (-57.0686) dan PRDX5[1HD2]-BEZ (-57.2906). This shows that the PRDX5 [1HD2]-*p*H*m*MC and PRDX5 [1HD2]-DOX bonds are more stable than PRDX5 [1HD2]-Ascorbic acid or native ligand. The difference in results between the in vitro test and in silico test can be understood considering that ascorbic acid as the highest inhibitor is caused by its nature as a radical catcher, whereas *p*H*m*MC and DOX are possible not only as radical capture but also needed in other cell work mechanisms. Polyphenol secondary metabolite compounds such as flavonoids, polyenes and compounds containing many -OH groups are multifunctional and can act with free radicals as reducing, free radical scavenger, metal chelating, and silencer formation of oxygen singlets [23].

Table 2. Docking Score of PRDX5[1HD2] with *p*H*m*MC, DOX, ascorbic acid and BEZ.

Compound	Docking Score
*p*H*m*MC	-61.8134
DOX	-61.1652
Ascorbic acid	-57.0686
BEZ (native ligand)	-57.2906

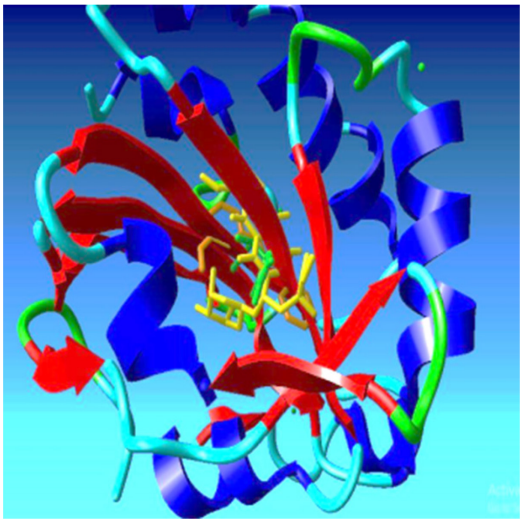


Fig. 2. The visualization of interaction between PRDX5[1HD2] with *p*H*m*MC, DOX and ascorbic acid. The *p*H*m*MC compound is shown in brown, DOX in yellow and ascorbic acid in green.

In addition to docking scores, using the in silico test can observe the amino acids involved in interactions between test compounds and target receptors. Amino acids involved in the interaction between PRDX5 [1HD2] with *p*H*m*MC, DOX and ascorbic acid are shown in Table 3. The results of the study showed that there were similarities between amino acids involved in interactions between PRDX5 [1HD2]-*p*H*m*MC, PRDX5 [1HD2]-DOX and PRDX5 [1HD2]-Ascorbic acid.

Table 3. Amino acids involved in the interaction between PRDX5[1HD2] with *p*H*m*MC, DOX and ascorbic acid.

Target receptor	Ligand	The amino acids involved in the interaction
PRDX5[1HD2]	*p*H*m*MC	**Cys 72***, **Gly 38***, **Leu 73***, **Phe 37***, Val 39
	DOX	**Gly 38***, **Leu 36****, **Leu 73***, **Leu 125****, **Phe 37***, **Phe 104****, Phe 128
	Ascorbic acid	**Cys 72***, **Gly 38***, **Leu 36****, **Leu 73***, **Leu 125****, **Phe 37***, **Phe 104****, Phe 128, Val 75

In PRDX5 [1HD2]-*p*H*m*MC there were four amino acids (Cys 72, Gly 38, Leu 73, Phe 37) that the same as those involved in PRDX5 [1HD2]-ascorbic acid. Whereas in PRDX5 [1HD2]-DOX there were three amino acids (Gly 38, Leu 73, Phe 37) that the same as those involved in PRDX5[1HD2]-*p*H*m*MC and three amino acids (Leu 36, Leu 125, Phe 104) as PRDX5 [1HD2]-ascorbic acid. The similarity of amino acids involved in the interaction between ligands (*p*H*m*MC and DOX) and receptors (PRDX5[1HD2]) will affect their activity as antioxidants. When associated with the test results of *p*H*m*MC-DOX by in vitro shows that antioxidant activity of combination is higher than the single. This shows that the activity as a free radical scavenger is increased in combination use. But it is possible that in other mechanisms competition can occur. This needs to be supported by other studies given the many pathways of antioxidant mechanisms [23]. *p*H*m*MC itself has been shown to inhibit the activity of the lipoxygenase enzyme which also shows that this compound is a potential antioxidant [6]. In relation to anticancer activity, the antioxidant activity of the *p*H*m*MC-DOX combination is expected to reduce the side effects of DOX.

Summary

*p*H*m*MC has the potential as an antioxidant and increase the antioxidant activity of DOX in combination use. There are similarities in the amino acids involved in the interaction between 5PRDX5 [1HD2] with *p*H*m*MC, DOX and ascorbic acid. The antioxidant activity of the *p*H*m*MC-DOX combination is expected to reduce the side effects of DOX.

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