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Biotransformation of eugenol to dehydroeugenol catalyzed by *Brassica juncea Peroxidase and its* cytotoxicity activities

Yulia Anita[a], Andini Sundowo[a], Puspa Dewi N. L[a], Euis Filailla[a], Hani Mulyani[a] , Chandra Risdian[a] Sofna Banjarnahor[a],Muhammad Hanafi[a] and Enade P Istyastono[b]

[a]*Research Centre of Chemistry Indonesian Institute of Science, Kawasan Puspiptek Serpong, 15314, Indonesia*

[b]*Pharmaceutical Technology Laboratory, Faculty of Pharmacy, Sanata Dharma University, Yogyakarta, Indonesia*

Abstract

A crude peroxidase biocatalyst extracted from sawi hijau (*Brassica juncea*) was used to produce dimerization phenolic synthesized from eugenol. The result from MS and NMR analysis showed that the reaction between eugenol substrate and H_2O_2 with crude extract enzyme produced a new chemical compound. The chemical structure of new compound showed coupled result in *ortho-ortho* position with C-C coupled derivative, dehydrodieugenol. It was obtained as brownish white crystal needles with yield 10 %. This compound displayed higher cytotoxicity effect on T47D breast cancer cell compared to the eugenol monomer.

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*Keywords:*Peroxidase; Eugenol; Dehydioeugenol; Dimerization; Oxidative coupling

1. Introduction

Phenolic oxidative coupling reaction plays an important role in nature and is classified as biogenetic type synthesis[1,2,3]. Alkaloids, antibiotics, lignins, melanins, and tannins are a few examples of the result in coupling reaction form[2]**.** Most of the natural product chemists have focused in the subject of oxidative coupling reactions. Iron compunds, that are employed for the last few decades,is known as oxidizing agents such as $FeCl_3$, and $K_3Fe(CN)_6$. They are effective coupling agents and the most widely used phenol oxidants[1]. Oxidative coupling of phenolic compound is successfully dimerized or polymerized by several chemical reagents as well as by enzymatic activity[2].

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* Corresponding author. Tel.:+62-21-7560090; fax:+62-21-7560545.
*E-mail address:*yuli002@lipi.go.id

Nomenclature

POD	Peroxidase
PBS	Phosphate – buffered saline
MTT	3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PI	Propidium Iodide
V-FITC	Fluorescein isothiocyanate V

Phenolate oxidation becoming a phenoxy radical using chemical catalyst is the most common way in recent years, enzyme is usually used as biocatalyst for the reaction. Peroxidases (PODs, EC1.11.1.7) are quite abundant enzymes in nature that catalyze a variety of oxidative transformations[4]. In addition, they have been successfully conducted to catalyze oxidative coupling of phenols and aromatic amines which are particularly applied for technological applications such as the removal of phenols from industial wastewater[5]. These compounds can be oxidized by peroxidase in the assistance of hydrogen peroxide to form dimeric, oligomeric, or polimeric products under mild conditions[5]. Hydrogen peroxide or other peroxides can act as scavenger[4]. Recently, peroxidase catalyzed oxidative coupling has ever been perfomed successfully to synthezise dimer derivatives such as naphthol, and tyrosine with satisfactory yields[5].

The exploitation of vegetable residuals as sources of crude enzymes can be investigated. In our research investigation will be focused on dimerization of natural methoxyphenols with using the potential biocatalytic activity of crude peroxidase (POD). Here, we used crude enzymes from stem of sawi hijau. Sawi hijau (*Brassica juncea*) as source of peroxidase was easily found and obatained since it widely distributed in nature belongs to the family of Horseradish and also from the Indonesian native plants.

Natural methoxyphenols, such as eugenol (2-allyl-4-methoxyphenol), is a mainly volatile constituent of clove essential oil resulted through hydrodistillation of *Eugenia caryophyllata* (*Syzygium aromaticum*) buds and leaves. Eugenol is an outstandingly versatile molecule used as an important ingredient in various products. It has been applied in agricultural, pharmaceutical, flavour, fragrance, cosmetic, and other several industries. Its pharmacological activities have been researched and have a potency as antimicrobial, anti-oxidants, analgesic, antiinflammatory, and anticancer activities[6,7,8].

The present study reports peroxidase from Sawi hijau (*Brassica juncea*) to catalyze phenolic coupling for the synthesis of eugenol. Eugenol was used as the starting material. Since there is no ortho- substituent next to phenolic hydroxyl group, Dimerization due to ortho-ortho C-C coupling or ortho C-O coupling can be expected. We designed dimerized eugenol in order to improve their biological activity for anti cancer activities.

2. Materials and Methods

2.1 General Experimental Procedurs.

The fresh vegetables of *Brassica juncea* were purchased before use from local supermarket. Eugenol was purchased from merck. NMR spectra were acquired on a JEOL 500 NMR spectrometer (^{1}H 500 MHz and ^{13}C 125 MHz). Eugenol dimer was dissolved in $CDCl_3$ containing tetramethylsilane as a chemical shift standard. LC-MS was done using Mariner instrument

2.2 Extraction of peroxidase from Brassica juncea (sawi hijau)

Stems of *B. juncea* were washed, cut into small size and then mechanically homogenized with sodium phospate buffer pH 7.0 in blender stirred for 10 minutes. The mixtures were filtered and put in the refrigator until used. The supernatant obtained contains *Brassica juncea* peroxidase as the main constituent. The peroxidase activity of this enzyme was determined using Bergmeyer and Lowry methods[9].

2.3 Oxidative coupling of eugenol catalyzed by Brassica juncea peroxidase

Crude *Brassica juncea* peroxidase in posphate buffer 200 mL was reacted with eugenol 10 mL in beaker glass, then added 5% H_2O_2. The mixture was vigorously stirred at room temperature for 15 minutes. After 15 minutes, the mixture was extracted with ethyl acetate for 3 times. Organic layers combined and dried over Na_2SO_4. Evaporation of the solvent yielded the brown oil which was purified by silica gel column chromatography (hexane:ethyl acetate = 8 : 2). The polymerization product was identified by LC-MS dan NMR spectrometer.

2.4 Cytotoxicity assay on cancer cell lines[10]

In order to evaluate cytotoxic effects of the tested compounds, three cancer cell lines were used. Leukemia cancer cell line (P388) and two types of breast cancer cell lines (MCF-7 and T47D) were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% PBS and antibiotics. Cells were plated in 96-well microplates at an initial cell density of approximately $3x10^4$ cells/mL and cultured in a CO_2 incubator (5% CO_2) and 37^{o}C. After 24 h of incubation for cell attachment and growth, varying concentrations of tested samples were added and the cells were incubated for another 24 hour. The compounds added were first dissolved in DMSO at the required concentration. Control wells received only DMSO. The cytotoxic effects of the test compounds were evaluated using MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; also named as thiazol blue] assay. After 24 h, 10 µL of MTT stock solution (5 mg/mL) were added to each well and incubated for 3 h at 37 °C. The formazan crystals were solubilized then by adding 100 µL DMSO per well. Absorbance of converted dye was measured at a wavelength of 540 nm by ELISA reader (Camoutsis et al. 1999). IC_{50} values were taken from the plotted graph of percentage live cells compared to control (%), receiving only DMSO, versus the tested concentration of compounds (µg/mL). The IC_{50} value is the concentration required for 50% growth inhibition. Each assay and analysis was run in triplicate and averaged.

2.5 Apoptosis Evaluation by Propidium Iodide (PI)/Annexin V double staining[11]

T47D cells were seeded into 24-well plate with 10^5 cells/mL. The cells were then incubated at 37^{o}C and 5% CO_2 for 24 h. The cells were treated with Dehidyrodieugenol at concentration 12.5 µg/mL and 25 µg/mL for another 24 h. After 24 h incubation, cells were treated with 0.5 µg/mL PI and 0.5 µg/mL Annexin V-FITC conjugate for 5 minutes in the dark at room temperature. The fluorescences of annexin V-FITC and PI were detected by using flow cytometer with excitation wavelength of 488 nm and emission wavelength of 530 nm (FL1) and 625 nm (FL2), respectively.

3. Results and discussion

The exploitation of vegetable residuals as source of crude enzymes can participate not only to reduce the pollutant waste of vegetable, but also to increased the benefit value of its vegetable. This research aimed to investigate the potential biocatalytic activity of crude peroxidase enzyme (POD) from stems of sawi hijau waste.

POD from stems of sawi hijau waste was obtained after homogenized processing using a mixer grinder. The crude enzyme in buffer phospate at pH 6-7 was produced. Then, it was filtered through filter paper to remove cell debris, and the clear supernatant was used as the crude enzyme source. The spesific activity was determined using Lowry assay and Bovin Serum Albumin (BSA) which were universally used as a standard protein. The specific activity of the enzyme has value of 14.577 U/mg. Aderson reported that oxidative coupling for phenolic compound is successfully dimerized or polymerized by several chemical reagents such as $FeCl_3$, and $K_3Fe(CN)_6$[2]. However, most of the work described here was done with crude peroxidase enzyme (POD) which is called crude *B. juncea* POD extract. In a typical experiment (Figure 1), the crude *B. juncea* POD catalyzed phenolic coupling for the biomimetic synthesis of eugenol in the presence of H_2O_2 to form C-C linked dimer of eugenol. The mixture reaction was stirred at room temperature for 15 minutes and monitored by TLC plate. The reaction was stopped after 15 minutes and then supernatant was extracted with ethyl acetate. The micture products were purified by silica flash chromatography (hexane : AcOEt, 8:2). Only major product obtained was further isolated and investigated to give dehydrodieugenol. It was obtained as brownish white crystal needles with yield 10 %.

The major product was fully characterized by mass spectrometry and 1D NMR.
LCMS spectra of dehydroeugenol showed the presence of molecular ion signal $[M+H]^+$ at m/z 327.17. The pure product was clearly identified by mass spectrometry m/z at 326 Dalton, whereas eugenol as parent compound has m/z 164. An LCMS study exhibited dimerization resulting from oxidative coupling of the starting eugenol.

In the spectrum of ^{1}H-NMR (500 MHz, $CDCl_3$, ppm): showed the presence of two methylene (CH_2) at δ_H 3.36 (4H, d, , J = 6.9 Hz, C-7,7'); two methoxyl group at δ_H 3.92 (6H, s, 2x-OMe); 5.05 (4H, m, C-9,9'); methine of allyl at δ_H 6.02 (2H, m, C-8,8'). In the presence 4H signas as singlet is sp[ecific signal for dyhydroeugenol was obtained. It was also supported by 6.73- (4H, s, C-4,4', C-6,6'); ^{13}C NMR spectra (125 MHz, $CDCl_3$, ppm): indficated the presence of two CH2 at δ_C 40.17 (C-7, C-7'); two –OMe at 56.26 ; and aromatyic and double bond of allyl at δ_C 110.85 (C-4, C-4'); 115.91 (C-9, C-9'); 123.28 (C-6, C-6'); 124.57 (C-1, C-1'); 132.11 (C-5, C-5'); 137.84 (C-8, C-8'); 141.07 (C-2, C-2'); and 147.39 (C-3, C-3'). Based on 1D NMR data, the structure of the reaction product was assigned obviously as dehydrodimer which is dehydroeugenol (**2**). Because there is no ortho- substituent next to phenolic hydroxyl group. Dimerization due to ortho-ortho C-C coupling was detected based on 1D NMR data (figure 1)[12]. Dehydrodimer (**1**) was not successfull to be isolated under above synthesis conditions.

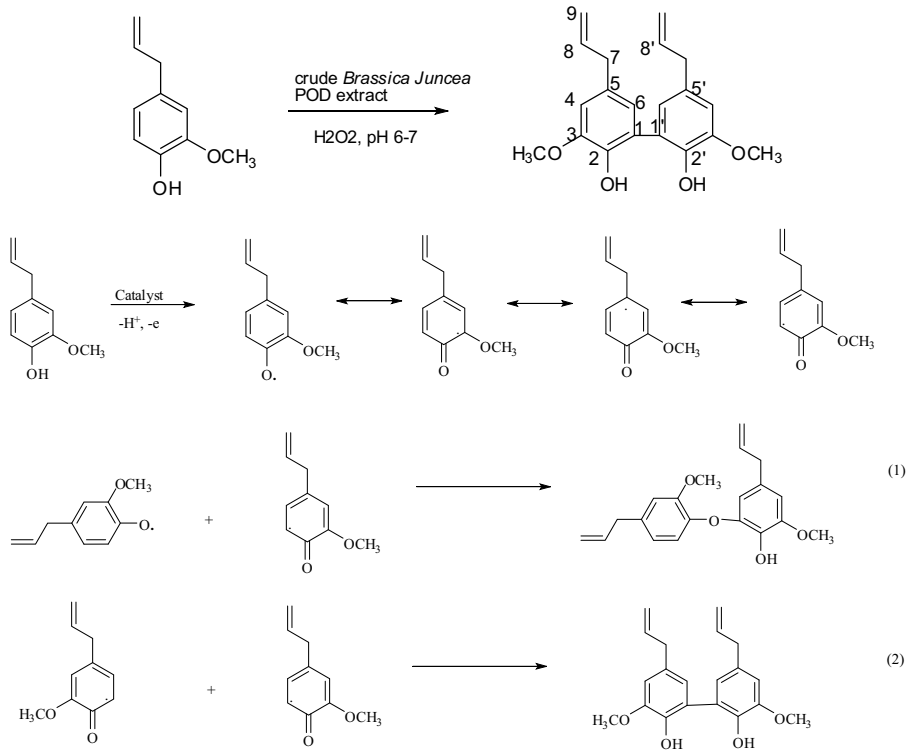


Figure 1. Oxidative coupling of eugenol catalyzed by crude *Brassica juncea POD* and some possible coupling reactions between free radicals generated during the crude *Brassica juncea* POD extract-mediated transformation of eugenol.

We have designed dimerized eugenol in order to improve their biological activity including anticancer activities. Dimerization of an active compound usually results in increased and improved in binding and pharmacological properties[13]. The IC_{50} values (concentration required to inhibit tumor cell proliferation by 50%) for the obtained

compound against cancer cell lines including leukemia cancer cell line (P388) and two types of breast cancer cell lines (MCF-7 and T47D) were determined using MTT assay. The results are showed in Table 1.

Table 1. Preliminary cytotoxic activities of dehydrodieugenol (**2**) and eugenol against three cancer cell lines

Compounds	IC_{50} (µg/mL)		
	P-388	MCF-7	T47D
Dehidyrodieugenol (**2**)	60	37.684	3.989
Eugenol	120	129.544	90.246

As shown in Table 1, dehydrodieugenol (**2**) showed improvement in cytotoxic activities against P-388, MCF-7 and T47D, compared with eugenol (monomer). Dehydrodieugenol (**2**) represented excellent cytotoxic activity with the IC_{50} value of 3.989 µg/mL againsts human breast cancer cell line T47D.

In order to discriminate between apoptosis and necrosis cells, dual staining with Annexin V and PI (Propidium Iodide) were conducted in this study by using flow cytometry. Annexin V-positive/PI-negative cells are regarded as apoptotic cells or early apoptotic cells, Annexin V-positive/PI-positive cells as secondary necrotic cells or late apoptotic cells, and Annexin V-negative/PI-positive cells as necrotic cells [14].

Apoptosis evaluation on T47D cells indicated that untreated cells were viable at 94.33% and have amount of early apoptotic cells for about 2.18% (Figure 2). Mean while the treated cells with dehydrodieugenol at 12.5 µg/mL and 25 µg/mL were viable at 83.80% and 11.87% (Figure 3 and 4), respectively. The percentage of apoptotic cells when treated with dehydrodieugenol at 12.5 µg/mL and 25 µg/mL were 12.40% and 74.45%, respectively (Figure 3 and 4). This study then indicated that dehydrodieugenol could inhibit the proliferation of T47D cells in the mechanism of apoptosis.

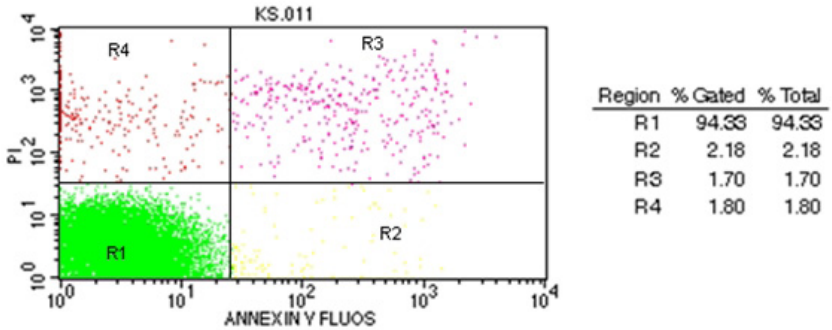
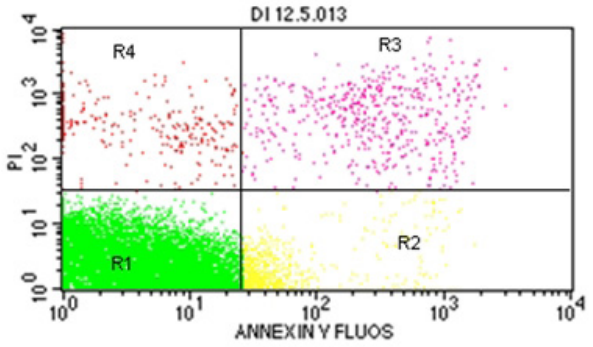
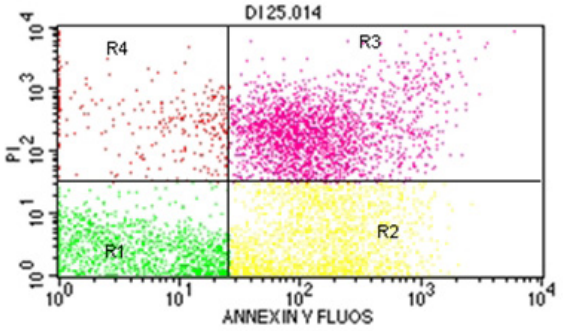


Figure 2. Flow cytometry analysis quadrant of untreated T47D cells. R1: viable cells, R2: early apoptotic cells, R3: late apoptotic cells, R4: Necrosis cells.



Region	% Gated	% Total
R1	83.80	83.80
R2	12.40	12.40
R3	2.58	2.58
R4	1.36	1.36

Figure 3. Flow cytometry analysis quadrant of treated T47D cells with 12.5 µg/mL Dehydrodieugenol . R1: viable cells, R2: early apoptotic cells, R3: late apoptotic cells, R4: Necrosis cells.



Region	% Gated	% Total
R1	11.87	11.87
R2	74.45	74.45
R3	12.21	12.21
R4	1.55	1.55

Figure 4. Flow cytometry analysis quadrant of treated T47D cells with 25 µg/mL Dehydrodieugenol . R1: viable cells, R2: early apoptotic cells, R3: late apoptotic cells, R4: Necrosis cells.

4. Conclusion

In summary, we have represented the beneficial of using crude *Brassica juncea* POD extract as a biocatalyst for the oxidative coupling-dimerization of eugenol leading to ortho-ortho C-C coupling obtained dehydrodieugenol (**2**). The cytotoxic activity of this dimer exhibited excellent cytotoxic activity with the IC_{50} value of 3.989 µg/mL againsts human breast cancer cell line T47D. It is promising as molecular scaffold for more further synthesized thus is obtained more potent cytotoxic activity on human breast cancer cell line T47D.

5. Acknowledgement

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