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Dimerization of xanthorrhizol using peroxidase enzyme extracted from broccoli (*Brasicca oleacea* L) and its influence to the antioxidant and antimicrobial activity

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Abstract
Xanthorrhizol, one of phytochemical compound in the extract of *Curcuma xanthorrhiza* Roxb, is a phenolic type compound which has abundant hydroxyl groups in its structure, thus, it is highly potential to be a hydrogen donor. Several studies showed that the hydrogen donor substances are also have antioxidative and antimicrobial capacity. The hydrogen donor substance from xanthorrhizol can be synthesized with the aid of peroxidase enzyme, via an oxidation mechanism. The as-formed radical product of xanthorrhizol will combine with the other radicals to produce dimer compound of xanthorrhizol.
This study was aimed to modify xanthorrhizol from *Curcuma xanthorrhiza* Roxb via dimerization process using peroxidase enzyme from raw extract of broccoli as catalyst. This experiment was carried out via several steps, as follows: extraction of xanthorrhizol from curcuma, extraction of peroxidase enzyme from broccoli, dimerization process of xanthorrhizol, characterization of products.
The results showed that the extraction of curcuma using ethyl acetate produce viscose liquid with Rf 0.4 which was predicted as xanthorrhizol. In the further step, this extract was dimerized using peroxidase enzyme extracted from broccoli and produced a substance which expected to be a new compound since there was a moderate shift in wavelength at UV region. FTIR spectrum confirmed the breaking of the benzene ring and the formation of ketone group. The antioxidant assay using DPPH method revealed the increase of IC_{50} from 467.5 ppm to 578.9 ppm (without Tween-80) and 1056.6 ppm (with Tween-80). The result of antimicrobial assay showed that that dimerization product have higher inhibition power than the unmodified xanthorrhizol extract, against the growth of *Bacillus cereus* at concentration of 0.2-0.8% with inhibition power of 8-14 mm (moderate to strong), but could not inhibit the growth of E.coli and some fungies (*Pestalutiopsis, Penicilium* and *Aspergillus niger*) in the concentration range of 0.2 – 1%.

Keywords: *Xanthorrhizol, dimerization, peroxidase enzyme, antioxidant capacity, antimicrobial capacity*

1. Introduction
Curcuma (*Curcuma xanthorrhiza* Roxb) is a plant that originally grows in Indonesia, especially in Java Island. Its rhizome part has been known as a medicinal herb to cure various illnesses for a long time, including stomach diseases, liver disorders, constipation, dysentery, bloody diarrhea, and children's fever[1]. Many researchers have demonstrated that xanthorrhizol from *Curcuma xanthorrhiza* has antibacterial [1-2] and estrogenic activities [3].
Phytochemical assay from literatures showed that xanthorrhizol is a volatile compound which can be classified as phenolic groups that contains hydroxyl groups[4], thus can act as hydrogen donor. Several researches has proved that the hydrogen donor compounds also act as antioxidant and antimicrobial agents [5-6].
Various modification techniques were commonly employed to enhance bioactivity of the compounds, such as dimerization reaction. The dimerization process can be performed by reducing H_2O_2 to H_2O using peroxidase enzyme as catalyst. The enzyme was oxidized in this process, and it can be changed back into the normal state by a further reduction which includes a hydrogen donor agent such as xanthorrhizol. This step can be predicted to cause oxidative coupling to form radical of xanthorrhizol which further formed dimer compounds of xanthorrhizol. The dimerization products were expected to have higher contents of bioactive compounds and can be separated to be applied as antioxidative and antimicrobial agents.
One of the source of peroxidase enzyme is broccoli, which is one of highly available vegetables in the markets. The previous research revealed that the peroxidase enzyme from broccoli has higher activity than that of green mustard [5]. In this study, broccoli was used as peroxidase enzyme source to catalyze dimerization process of xanthorrhizol. This study was aimed to modify xanthorrhizol from curcuma (*Curcuma xanthorrhiza* Roxb) rhizome extract

via dimerization process using peroxidase enzyme from broccoli as catalyst, in order to enhance their antioxidative and antimicrobial activities.

2 Materials and Methods

2.1 Materials

Curcuma rhizome and broccoli was harvested from local markets in Bogor, Indonesia. Methanol, ethyl acetate, silica gel (70-230 mesh), n-hexane, phosphate buffer pH 7.0 and 6.0, ammonium sulphate 50% (b/v), pyrogalol 5%, H_2O_2 0.5% and 30%, H_2SO_4, petroleum eter, selen catalyst, NaOH, HCl 0.5 N, mengsel indicator, buffer pH 3, Na_2SO_4 anhidrat, KBr, Tween-80, DPPH (1,1-diphenil picrylhydrazyl) reagent were purchased from Merck. Nutrien Agar medium, PDA medium, bacteria and fungi suspension, and paper disc were obtained from Sigma-Aldrich.

2.2 Methods

2.2.1 *Xanthorrhizol extraction* [3]

Curcuma rhizome was rinsed with water and cut into small size, and then dried in room temperature for 3 days. 100 g of dried curcuma rhizome was macerated using 400 mL methanol 75% for 3 x 24 hours. This mixture was filtrated, and the filtrate was evaporated to yield extract of methanolic fraction. The evaporation product was further extracted using ethyl acetate and evaporated until it yield ethyl acetate fraction.

2.2.2 Peroxidase enzyme extraction from Broccoli [7]

Fresh broccoli was crushed by blender, mixed with phosphate buffer (pH 7.0) at ratio of 1:2 at room temperature. Thi mixture was filtrated by cotton fabric, and the filtrate was centrifuged at 1700 rpm for 10 minutes. The supernatant was separated from the sediment, added by ammonium sulphate 50% (w/v), stirred for 2 hours, and the solution was precipitated overnight. The precipitate was separated by centrifugation at 1700 rpm for 20 minutes at room temperature. The precipitate was resuspended in phosphate buffer (pH 6.0), and then characterized using activity assay based on method [8], and protein determination method [9].

2.2.4 Activity Assay of Peroxidase enzyme from Broccoli

2 mL of 5% pyrogalol was added by 1 mL of 0.5% H_2O_2, 2 mL of phosphate buffer and 14 mL of water. The solution temperature was kept at 20^0 C, and then added by 1 mL of enzyme from the broccoli extract, and after ± 20 detik was added by 1 mL H_2SO_4 2 M. The as-obtained purpurogalin extracted with ether for 3 times 30 mL each. The extract was removed and collected. The remaining solution in the separation funnel was again extracted using 10 mL of ether. Purpurogalin solution was then measured by UV-Vis spectrophotometer at wavelength of 420 nm. Control solution was prepared by the same steps, but using only substrate and reagent in the absence of enzyme. The specific activity of the peroxidase enzyme was calculated from the equation:

Unit/mL = A_{420} (sample-control) x 8.5/mL of solution

2.2.5 Dimerization reaction using peroxidase enzyme from broccoli

1,0 g of sample was added by 0.3 mL of 30% H_2O_2 in closed two neck-round flask, and then reacted with 15 mg of peroxidase enzyme, 20 mL of buffer pH 3 and 20 mL of 10% methanol (the dimerization process was also done with the presence of Tween-80 surfactant). The mixture was stirred for 1 hour and the temperature was kept at 35 ºC. After 1 hour, the product reaction was extracted using ethyl acetate. Ethyl acetate fraction was separated and the remaining water in organic phase was removed by adding 5 g of Na_2SO_4 anhydrous. The extract was concentrated by evaporating the solvent.

2.2.6 Characterization of dimerization product

The dimerization product was characterized by TLC using *n*-hexane and ethyl acetate at ratio of 2: 5. Spots which predicted as the main product were separated and dissolved in ethyl acetate.

In the characterization using UV-Vis spectrophotometry method, the sample preparation was done by dissolving 1 mg sample in 10 mL ethyl acetate until homogeneus. The solution was analyzed its maximum absorption in the wavelength range of 300 – 500 nm.

The sample preparation for characterization using FTIR spectrometry method was done by crushing 1 mg sample with 50 mg KBr using mortar until homogeneus. The sample mixture was placed in the mold pellet and measured in the wavenumber range of 4000-600 cm^{-1}.

Antioxidant assay of the sample was done using DPPH method [10]. Briefly, 3 mL of 200 µM DPPH reagent in methanol was pippetted to reaction tube, then added by 1 mL of each samples at 100, 200, 300, 400 and 500 ppm. The mixtures were vortexed and measured their absorbance at 0, 5, 10, 15, 20, 25 and 30 minutes using UV-Vis spectrophotometer at wavelength of 517 nm. Percentage of inhibition was calculated as follows.

% inhibition = $((Absorbance_{initial} – Absorbansi_{final}) / Absorbance_{initial}) \times 100\%$

The linier curve was plotted with sample concentration as X axis and % inhibition as Y axis, thus the equation of Y = aX + b was obtained. The IC-50 was calculated using this equation : IC-50 = (50 – b)/a

Antimicrobial assay was performed by measuring inhibition zone of paper disc, according to previous reference [11]. The assay was done for 2 types of bacteria : *E.coli, B.cereus* and 3 types of fungi : *Pestalutiopsis, Penicilium*, and *A. niger*. Incubation was done at 37 ºC for 24 hours. Inhibition zone which formed around paper disc was calculated as follows.

Inhibition zone = diameter of (clear area + paper disc) – diameter of paper disc

3. Result and Discussion

3.1 Extraction of Xanthorrhizol from curcuma rhizome

Xanthorrhizol extraction was conducted by maceration method and solvent extraction using methanol and ethyl acetate. A viscose solution of extraction product was characterized by TLC using *n*-hexane: ethyl acetate at ratio of 5 : 1 as eluen.

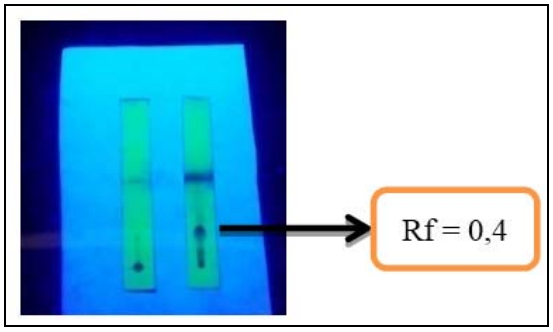

Fig 1: TLC spot of extraction product (predicted as xanthorrhizol) at methanolic (left) and ethyl acetate (right) phase

Figure 1 showed that methanolic fraction did not formed good TLC profile, since it cannot be separated into spots or has Rf of about 0. It indicated that methanol was not suitable for separation compounds in the extract, which may due to the less polarity of the substances. When the ethyl acetate has been employed as solvent in the extraction, the extraction product showed a clear separated spot at TLC profile with Rf of 0.4, which is close to the Rf of pure xanthorrhizol (0.42 with hexane:ethyl acetate at ratio of 10:1, as eluen), although the complete separation was still not achieved.

3.2 Extraction of Peroxidase enzyme from Broccoli

The peroxidase enzyme from broccoli in this experiment was crude extract, since the extraction was conducted for only two steps, extraction and centrifugation, without fractionation step. The enzyme has ability to catalyze oxidation reaction by hydrogen peroxide of many substrate which act as hydrogen donor, such as phenolic compounds [12].

Table 1: Protein content and activity of peroxidase enzyme extracted from broccoli

	Protein content (%)	Enzyme activity
broccoli	6.03	-
peroxidase enzyme extracted from broccoli	47.83	8.05 Unit/mL

Table 1 confirmed that peroxidase enzyme was successfully extracted from broccoli, as shown from increasing protein content after extraction from 6.03% to 47.83%. The activity of peroxidase enzyme from broccoli was 8,05 Unit/mL, higher than that of green mustard leaves extract, 0,179 U/mg [12]. Generally, the specific activity of peroxidase enzyme tend to drop at temperature of 30, 40 and 50 ºC but increases at temperature of 60 ºC[13] (Anis, 2003). The as-obtained peroxidase enzyme was expected to become a biocatalyst for enhancing the activity of antioxidant from curcuma rhizome extract.

3.3 Dimerization process of extract from curcuma rhizome

Extract of curcuma was reacted with H_2O_2 and catalyzed by peroxidase enzyme from Broccoli in acid condition (pH 3). According to [14], the dimer formation which occurred via coupling reaction of phenolic radicals took place in acid condition since it will be more stable. Tween-80 was also added to study the effect of the presence of surfactant in homogenization process of the reaction mixture. The experiment showed dimerization process at temperature below 35 ºC for 1 h yielded a change of solution color from clear brownish yellow to the darker turbid brownish yellow (Figure 2).

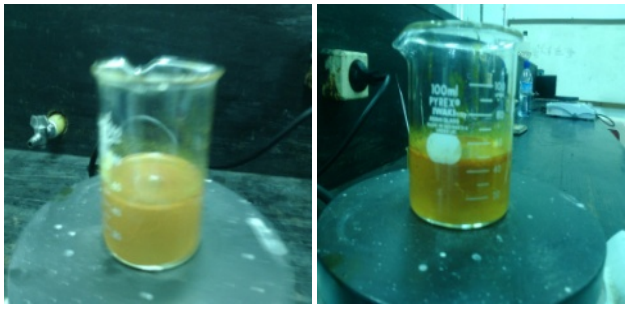

Fig 2: The extract of curcuma before (left) and after (right) dimerization process

UV-Vis spectrum of the extract showed a peak at 416 nm dan shoulder at 397 nm dan 443 nm (Figure 3). Although at UV region the peak was not well defined and completely separated due to the heterogeneous mixture of the crude extract, there was a trend of increasing absorbance at low wavelength (< 300 nm). This result was in agreement with the study, that xanthorrhizol absorbed radiation at wavelength of 276 nm, 217.5 nm and 245,5 nm which indicated phenolic groups [15]. Xanthorrhizol extracted from *Curcuma xanthorrhiza* Roxb. revealed UV peak at 275.20 nm [11], associated with electron transition of phenolic groups. Silverstein *et al*. [16] explained that maximum absorption of phenolic groups was in the range of 210 – 280 nm, which corresponds to $\pi \rightarrow \pi^*$ electron transition of aromatic rings.

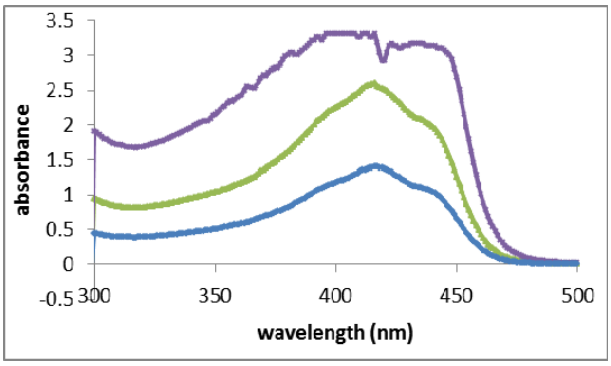

Fig 3: UV-Vis spectra of xanthorrhizol extract (purple), dimerization product in the absence of Tween-80 (green) and in the presence of Tween-80 (blue)

Figure 3 showed that there was no wavelength shift in the UV-Vis spectrum, which may be contributed from reaction between extract and enzyme. It indicated that the chromophore groups in the extract were not drastically changed in structure and chemical environment. This trend was also shown by reaction product in the presence of Tween-80, despite that the reaction mixture was physically changes in color intensity. To confirm whether the main structure was the same or any changes occurred as a result of the reaction, characterization by FTIR spectroscopy was conducted.

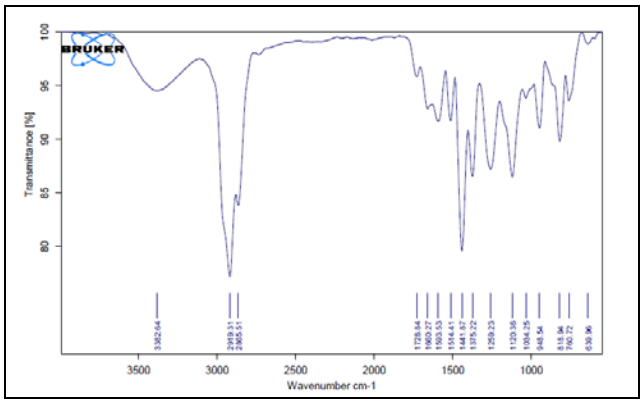

Fig 4: FTIR spectrum of extract before dimerization process

Figure 4 showed that xanthorrhizol contains -OH groups, benzene rings, CH-, CH_2-, and -CH_3 alkane, and C=C bonds, which associated with peaks at 3400 cm^{-1} (-OH fenol stretching), 2900 cm^{-1} and 2800 cm^{-1} (assym. and sym. stretching C-H alkane), 1660 cm-1 (stretching of C=C bonds). Benzene rings were shown by peak at 1514 cm^{-1} and 1728 cm^{-1}. Bending vibration of -OH was shown by peak at 1440 cm^{-1}.

Peak at about 760 cm^{-1} corresponds to the substituted benzene.

This result was in agreement with that obtained by Musfiroh *et al*. [17] who reported that xanthorrhizol extracted from *Curcuma xanthorrhiza* rhizome showed main peaks of FTIR spectrum at wavenumber of 3402, 2915, 1708, 1620, 1599 cm^{-1}.

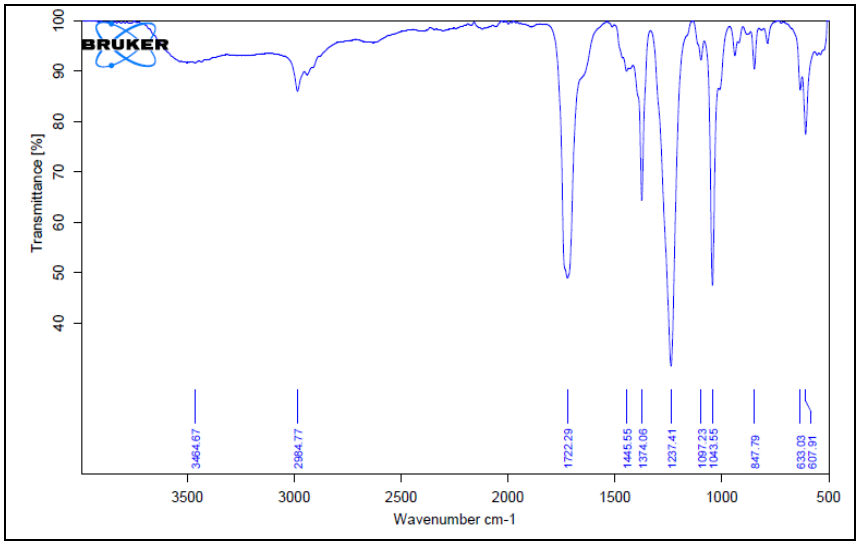

Fig 5a: FTIR spectrum of the dimerization product in the absence of Tween 80

FTIR spectrum of dimerization product in the absence of Tween-80 (Figure 5a), revealed several wavenumber shift and increase in intensity, indicated some changes of structure and chemical environment, compared to that of extract before dimerization. Peak at wavenumber of 3400 cm^{-1} and 2900 cm^{-1} region were not significantly changed, indicated that the –OH groups in benzene were not changed. In the other hand, peak at about 1660 cm^{-1} was disappeared, but the broad peak at the region of 3100-3300 cm-1 was emerged, at weak intensity. Double bonds of alkenes in xanthorrhizol were predicted to interact during the reaction, thus =C-H peak was unobserved, but C=C peak was more visible since it separated from –OH peak. Peak at 1725-1735 cm^{-1} region revealed only a slight change from 1728 cm^{-1} to 1722 cm^{-1}, indicated that the substituted benzene rings experience no significant change. The disappearance of peak at 1514 cm^{-1}, indicated the change of the benzene structure. This is contrary to the previous statement. An explanation can be drawn that may be the formation of ketone groups was dominant, as observed by the increase of intensity of the peak at about 1700 cm^{-1}. As a consequence, there was a shift in double bond position in benzene structure.

The dimerization product in the presence of Tween-80 (Figure 5b), revealed a significant profile in the –OH peak region, which tend to be flat, indicated that most of the –OH groups reacted to form its derivates. Besides that, peak at 1728 cm^{-1} shifts to 1737 cm^{-1}, with an significant increase in intensity, indicated that there was a change in chemical environment in benzene rings. The broading peak at 3100-3300 cm^{-1} with very low intensity mau due to the =C-H bond which almost all have been reacted, thus the amount was reduced significantly. Similar to the dimerization product in the absence of Tween-80, peak at 1514 cm^{-1} was also disappeared, which also can be expected as a result of ketone formation. Based on the explanation, it can be predicted that reaction between extract and enzyme occurred at both –OH of phenolic groups and at double bonds in benzene rings.

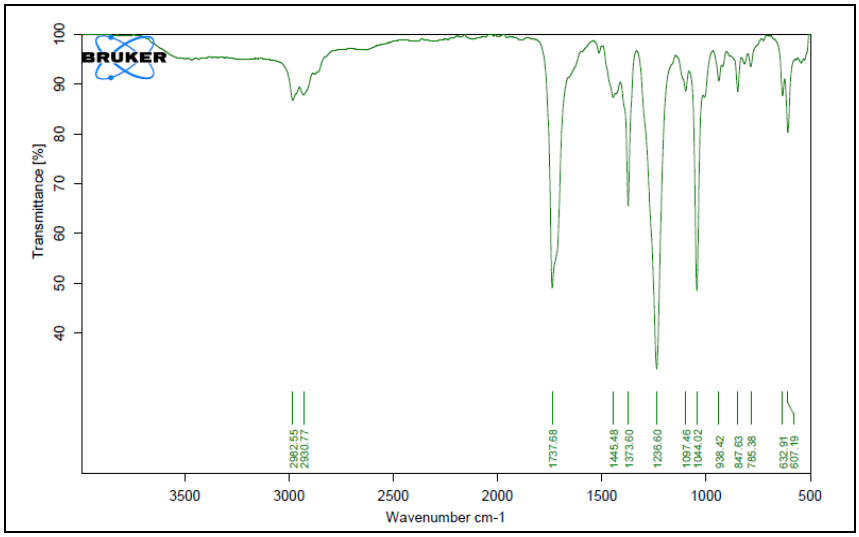

Fig 5b: FTIR spectrum of the dimerization product in the presence of Tween 80

3.4 Antioxidant Activity

Figure 6 showed that xanthorrhizol extract before and after dimerization process (both in the absence and presence of Tween-80) have capability to quench free radicals of DPPH 200 µM, as shown from the decrease in DPPH absorbance, compared to absorbance of control solution. According to [18], the decrease of DPPH absorbance related with quenching of DPPH radicals after it reacted with hydrogen atom from active compound of the samples. This reaction caused the number of diazo double bond in DPPH was reduced.

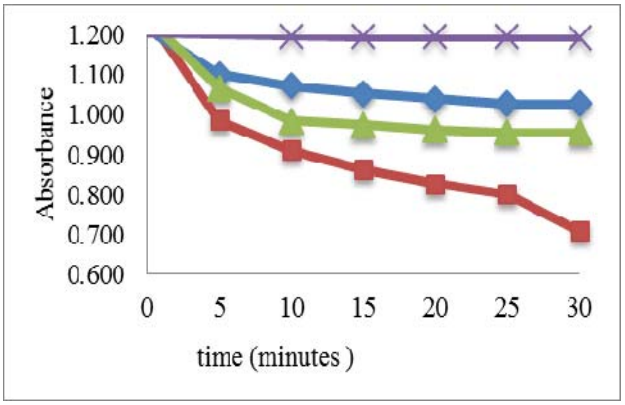

Fig 6: DPPH absorbance of control solution, 500 ppm extract before dimerization, after dimerization, in the presence and absence of Tween-80 (from top to bottom)

Dimerization process of the xanthorrhizol extract using peroxidase enzyme from broccoli reduce the antioxidant activity, as shown from the lower absorbance of DPPH. The presence of Tween-80 as surfactant in the dimerizaton process caused the decrease in DPPH absorbance was more pronounce. The IC-50 value of dimerization product was significantly lower than that of unmodified extract (Table 2), especially when the dimerization process was conducted in the presence of Tween-80. Several references stated that the strength of antioxidant activity was classified (based on IC-50 value) as strong when the value < 100 ppm, moderate when the value is in the range of 100-500 ppm and weak when the value is in the range of 500-1000 ppm[19]. Thus, the antioxidant activity of the extract was categorized as moderate and that of the dimerization product as weak.

Tabel 2: IC-50 of xanthorrhizol extract and the dimerization products

No	Sample	Concentration (ppm)	% Inhibition	IC-50 (ppm)
1	Extract before Dimerization	100 200 300 400 500	2.69 4.29 8.60 10.50 15.54	467.3
2	Dimerization product (without Tween-80)	100 200 300 400 500	10.63 21.13 27.67 37.12 54.14	578.9
3	Dimerization product (with Tween-80)	100 200 300 400 500	15.66 31.64 45.78 59.89 74.59	1056.6

The decrease in antioxidant activity for the dimerization product was predicted as a result of significant change in functional groups during the reaction, as confirmed by FTIR spectrum, which explained the change or degradation of –OH groups, especially in the dimerization in the presence of Tween-80. The ketone formation in this reaction may due to the incomplete dimerization. According to [20], one of the steps in dimerization of phenolic compound is the formation of phenoxy radicals, followed by formation of quinon intermediate and combination between phenoxy radicals. In this experiment, it can be predicted that the reaction yielded quinon intermediate at low intensity, but the next step, which should be dimerization of phenoxy radicals could not occur.

In previous research [14] explained that enzyme activity greatly influenced the intermediate formation, whereas the type and the amount of co-solvent significantly affected the dimer formation step. According to this theory, it can be explained that the low activity of the peroxidase enzyme (8.05 Unit/mL) employed in this experiment may be the main cause of the unsuccessful of dimerization process. As a comparison, the study conducted by Muryeti [21] involved larger activity of peroxidase enzyme (100 Unit/mg) to dimerize eugenol.

3.5 Antimicrobial Activity

Xanthorrhizol as a main active compound in the curcuma rhizome (*Curcuma xanthorrhiz*a Roxb) was known to possess antimicrobial activity [22, 23]. In this experiment, antimicrobial activity was assessed against 2 types of bacteria (*E.coli dan Bacillus cereus*) and 3 types of fungi (*Pestalutiopsis, Penicilium and Aspergillus niger).*

Table 3: Inhibition power against some bacteria by xanthorrhizol extract and its dimerization product

Concentration (%)	Inhibition power (mm) xanthorrhizol extract		Inhibition power (mm) dimerization product	
	E. coli	*Bacillus cereus*	*E. coli*	*Bacillus cereus*
0,2	-	-	-	8
0,4	-	4	-	8
0,5	-	6	-	10
0,8	-	4	-	12
1,0	-	14	-	14
Standard (Amoxicilin)	8	4	8	4

(-) = do not have clear zone

Table 4: Inhibition power against some fungi by xanthorrhizol extract and its dimerization product

Concentration (%)	Inhibition power (mm) xanthorrhizol extract			Inhibition power (mm) dimerization product		
	Pestalutiopsis	***Penicilium***	***A. niger***	***Pestalutiopsis***	***Penicilium***	***A. niger***
0,2	-	-	-	-	-	-
0,4	-	-	-	-	-	-
0,5		-	-		-	-
0,8	-	-	-	-	-	-
1	-	-	-	-	-	-
Standard Ketokonazole	14	-	-	16	-	-

From Table 3, it can be observed that xanthorrhizol extract have ability to inhibit the growth of gram(+) bacteria *Bacillus cereus*, with inhibition power of 4-14 mm in the concentration range of 0.2-1%. Antimicrobial activity was classified as weak (<5mm), moderate (5-10 mm), strong (10-20 mm) and very strong (>20mm) [24]. Thus, in the concentration range of 0.2-0.8% the activity was low to moderate, but at concentration of 1% was categorized as strong.

The dimerization product showed an increase in activity at concentration of 0.2% - 0.8% which yield value of 8-12 mm (moderate - strong), but showed no effect at concentration of 1%. This phenomenon revealed that the structural change of the compounds during dimerization did not greatly change active functional groups which possess antibacterial activity. The phenolic groups can inhibit the growth of bacteria through formation of protein-phenol bond mechanism, between hydroxyl groups and protein of cell membrane caused the disruption in cell membrane permeability and subsequently, the death of bacteria.

Table 3 and 4 also revealed that xanthorrhizol extract and its dimerization product cannot inhibit the growth of gram (-) *E.coli* bacteria and fungi (*Pestalutiopsis, Penicilium dan Aspergillus niger).* The results was in agreement with the study which showed that ethanolic and dichloromethane extract of curcuma cannot inhibit the growth of gram (-) *E.coli, P.aeroginosa* and fungi albicans [25]. Different from gram (+) bacteria which contains 60-80% peptidoglycan, gram (-) bacteria and fungi have complex cell wall besides peptidoglycan, in the form of external layer cell wall consists of liposaccharide, lipoprotein and periplasma which bound to peptide. The porin type lipoprotein which is hydrophilic cannot interact with hydrofobic antibacteria compound.

The difficulty of xanthorrhizol extract and its dimerization product to penetrate the sterol membran in the cell wall of fungi caused the growth of fungi *Pestalutiopsis, Penicilium dan Aspergillus niger* cannot be inhibited. According to [26] the growth of fungi was difficult to be inhibited by antimicrobial agent since it has rigid cell wall consists of chitin, glucan and mannan, and the extract cannot inhibit chitin synthesis in the cell wall.

4. Conclusion

Based on the results, it can be concluded that curcuma extraction using ethyl acetate yielded a compound with Rf of 0.4which predicted as xanthorizol. Dimerization of this compound using peroxidase enzyme from broccoli produced new substance, which characterized by wavelength shift of UV-Vis spectrum. FTIR spectrum confirmed that this new substance was classified as ketone (quinon intermediate). Antioxidant assay showed a slight decrease of antioxidant activity after dimerization with IC-50 value from 467.5 ppm to 578.9 ppm (without Tween-80) but a significant decrease of that with IC-50 value of 1056.6 ppm when Tween-80 was employed. Antimicrobial assay showed that dimerization product have higher inhibition power than the unmodified xanthrrhizol extract, against the growth of gram (+) bacteria *Bacillus cereus* at concentration of 0.2-0.8% with inhibition power of 8-14 mm (moderate to strong), but cannot inhibit the growth of gram (-) bacteria *E.coli* and *Pestalutiopsis, Penicilium* and *Aspergillus niger* fungi in the concentration range of 0.2 – 1%.

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