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Literature Review

# THE PRELIMINARY STUDY OF ANTIOXIDANT ACTIVITY FROM XYLO-OLIGOSACCHARIDE OF CORNCOB (*ZEA MAYS*) HYDROLYSIS PRODUCT WITH ENDO-$\beta$-XYLANASE ENZYME

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***ABSTRACT***

*Xylo-oligosaccharide derived from corncob hemicellulose has been reported to possess antioxidant activity. In order to assess the effective scavenging of xylo-oligosaccharide, we conducted in vitro studies based on self-made xylo-oligosaccharide with DPPH (2,2-diphenyl-1-picrilhydrazil) method. Xylo-oligosaccharide was prepared with enzymatic hydrolysis. The enzyme used for hemicellulose hydrolysis was endo-β-xylanase enzyme from PC-01 isolated bactrerium. PC-01 isolated bacterium used in this study was Pacet hot spring which was isolated from East Java. Endo-β-xylanase enzyme is an extracelluler enzyme. There was about 0.199 U/mL after purification and dialysis process. Hydrolisis product of hemicellulose A and B from corncob were analyzed with TLC (Thin Layer Chromatography) and HPLC (High Performance Liquid Chromatography). This analysis showed that hydrolysis product of hemicellulose B had a lot of xylo-oligosaccharide hydrolysis product of hemicellulose than Xylo-oligosaccharide hydrolysis product of hemicelluloses A. Xylo-olygosaccharide was analyzed as on antioxidant activity. Xylo-oligosaccharide hydrolysis product of hemicellulose B ($IC_{50}$ = 48.96) has higher antioxidant activity than Xylo-oligosaccharide hydrolysis product of hemicellulose A ($IC_{50}$ = 92.302). The toxicity of xylo-oligosaccharide can be calculated by the value of $LC_{50}$ (Lethality concentration). $LC_{50}$ of xylo-oligosaccharide derived from corncob hemicellulose was 400 ppm so that xylo-oligosaccharide has anti tumor activity because xylo-oligosaccharide has $LC_{50}$ < 1000 ppm.*

***Keywords:*** *Hemicellulose, corncob, endo-β-xylanase, xylo-oligosaccharide, antioxidant activity, toxicity*

***ABSTRAK***

**Latar Belakang***: Xilo-oligosakarida hasil hidrolisis hemiselulosa tongkol jagung dilakukan studi awal uji aktivitas antioksidan.* **Tujuan***: Uji perendaman radikal bebas oleh xilo-oligosakarida, uji antioksidan dari xilo-oligosakarida ini dilakukan secara in-vitro dengan metode DPPH (2,2-diphenyl-1- picrilhydrazil).* **Metode***: Xilo-oligosakarida diperoleh dari hasil hidrolisis secara enzimatis. Enzim yang digunakan untuk proses hidrolisis ini adalah enzim endo-β-xilanase dari isolat bakteri PC-01. Isolat bakteri PC-01 yang digunakan dalam penelitian ini adalah isolat dari sumber air panas Pacet. Enzim Endo-β-xilanase adalah enzim ekstraseluler yang memiliki aktivitas 0,199 U/ml setelah proses pemurnian dan dialisis. Produk hidrolisis hemiselulosa A dan B dari tongkol jagung dianalisis dengan KLT (Kromatografi Lapis Tipis) dan HPLC (High Performance Liquid Chromatography). Analisis tersebut menunjukkan bahwa produk hidrolisis Hemiselulosa B memiliki kandungan xilo-oligosakarida yang lebih banyak dibandingkan dengan produk hidrolisis hemiselulosa A dari tongkol jagung.* **Hasil***: Xilo-oligosakarida hasil hidrolisis hemiselulosa tongkol jagung diuji aktivitas antioksidan. Xilo-oligosakarida hasil hidrolisis Hemi B (IC50 = 48,96) memiliki aktivitas antioksidan yang lebih tinggi dibandingkan xilo-oligosakarida hasil hidrolisis Hemi A dari tongkol jagung (IC50 = 92,302). Toksisitas xilo-oligosakarida dapat dihitung dari harga LC50 (Lethality concentration). Nilai LC50 dari xilo-oligosakarida hasil hidrolisis hemiselulosa B tongkol jagung adalah 400 ppm sehingga xilo-oligosakarida ini memiliki aktivitas antitumor karena nilai LC50 < 1000 ppm.*

***Kata kunci:*** *Hemiselulosa, tongkol jagung, endo-β-xilanase, xilo-oligosakarida, aktivitas antioksidan, toksisitas*

## INTRODUCTION

Lately the medical world has been discussing free radicals that give bad effects to human health. These free radicals are physiologically produced by the cells due to the metabolic processes in the body. In addition, free radicals are also produced by other processes outside the body such as ionizing radiation, environmental pollutants (vehicle emission and industrial emissions, asbestos, cigarette smoke, etc.), alcohol, smoke and foods which contain high fat. Free radicals can be easily formed by a compound that is ready to deliver a single electron, such as fatty acids. Free radicals or oxidants in the body can be controlled by the body itself by forming endogenous antioxidants. On the situation of endogenous antioxidants that are not able to suppress free radicals that arise, it needs antioxidants from outside. Antioxidants can be obtained from the synthesis or from natural compounds in plants.[1] Recently, it has been reported that the oligosaccharide compounds also have antioxidant activities.[19] Oligosaccharide is an oligomer of hemicellulose which can be found in many agricultural waste. Oligosaccharide is one example among other xilo-oligosaccharides (XOS), galakto-oligosaccharides, and frukto-oligosaccharides (FOS).Based on this background, the research was conducted as a preliminary study testing for oligosaccharides especially xylooligosaccharides obtained from enzymatic hydrolysis of corn cob as an anti-oxidant with several stages. These stages were hemicellulose isolation of corn cob and enzymatic hydrolisis of hemicellulose into oligimer which was xylooligosaccharides. The enzyme that was used for the hydrolysis of hemicellulose subtrate was endo-$\beta$-xylanase from Bacillus subtilis PC-01.[3] Xilo-oligosaccharides derived was used to fix antioxidant tests and toxicity tests using shrimp fry.

## METHODS

### Xilanolitik enzymes production

Inoculum of *Bacillus subtilis* PC 01 was grown in 1 liter of media production and incubated for 8 hours at 60° C. Cells were harvested after ± 8 hours at 4° C and centrifuged 10000 rpm for 10 minutes. Cell pellet was discarded, while the supernatant (enzyme) was used for the enzyme presipitation process.

### Xylanase Enzyme Precipitation Using Amonium Sulfate (enzyme precipitation)

To 100 ml of crude extract enzyme that had been soaked in an ice bath, some ammonium sulfate was added slowly, stirred frequently until the levels of ammonium sulfate saturation percentage reached 60%. Ammonium sulfate saturation percentage was used based on ammonium sulfate saturation table.[4] The enzymes were centrifuged at 6000 rpm for 10 minutes. Precipitated enzyme was again precipitated and dissolved in 100 mM citrate phosphate buffer at pH 5, and then dialyzed. Dialysis was performed, until the ammonium sulfate fraction-free enzyme was marked by the formation of a white precipitate when some buffer was poured into $BaCl_2$ solution.

### Xilanolitik Enzyme Assay

The standard reaction mixture, contained 100 µl of substrate and 100 µl of enzyme which was incubated at 70° C for 1 hour and finished by adding 600 µl of DNS, after that, heated for 15 minutes together with the controls, and immediately cooled in ice water for 20 minutes. Absorbance readings were analyzed at a wavelength of λ550 nm. The controls used were 100 µl of enzyme, 100 µl of substrate and 600 of µl DNS. They were treated the same as those, above but without any incubation.

### Isolation of Corn cobs Hemicellulose

Agricultural waste of corn cob powder weighed 5 grams. We put the two neck round bottom flask containing 2.0 M NaOH solution up to 100 ml within a magnetic stirrer for heating for 4 hours. After the process was complete, cooled, and then filtered using Buchner funnel, the filtrate was acidified with 4 N acetic acid to a pH of 5.5–6.0 for precipitating hemicellulose A and continued with dicentrifuging (10000 rpm, 20 min) to separate the sediment. The precipitate was freeze-dried to obtain hemicellulose that was of free water. The filtrate obtained was mixed with 96% ethanol to precipitate hemicellulose B. The precipitate obtained was washed with 96% ethanol and then powdered and freeze-dried to obtain a free of water hemicellulose B.[5] The hemicellulose obtained could be used for further testing.

### Hemicellulose Enzymatic Hydrolysis

Every 1% of Hemicellulose A and B samples was taken as much as 100 µl and added with 300 µl of xylanase enzyme, incubated at 70° C for 24 hours. After centrifuging, the filtrate obtained was xilo-oligosaccharides and other sugar monomers were dried using a freeze drier.[6]

### Analysis of Hydrolysis Products Thin Layer Chromatography (TLC)

A number of oligosaccharide compounds contained in the hydrolysis products were analyzed by TLC with various comparison eluent. The eluent used n-propanol: $CH_3CN$: water = 5: 3: 2; n-propanol: water: ammonia (70: 29: 1) and n-butanol: acetic acid: water = 2: 1: 1. The three systems of eluent were used to obtain the best separation and as a monitor in the subsequent separation process.[7] The apparition stain used was sulfuric acid in methanol.

### High Performance Liquid Chromatography (HPLC)

The HPLC analysis used 2 different columns: the carbohydrates column (mikrobondapak, Waters 2487) and a $NH_2Si$ column, and 2 different detectors refractory index detector and ELSD (Evaporative Light Scaterring Detector), as well as solvent methanol 80% in water and 83% acetonitrile in water, flow rate of 1 µl/min, injection volume of 20 µl.

**Table 1.** The results of HPLC analysis for the hydrolysis products of corn cobs

| Sample | Glucose (%) | Xylose (%) | Arabinose (%) | Xylo-oligosaccaride (%) |
|---|---|---|---|---|
| CA ( xylo-oligosaccharides from Hemi A of corncob) | 5,0 | 0 | 0 | 4,6 |
| CB (xylo-oligosaccharides from Hemi A of corncob) | 6,0 | 0 | 0,1 | 4,9 |

**Anti - Free Radical Activity Test**

The antioxidant activity xylo-oligosaccharide standards and the product of hydrolysis spectrometry were determined by measuring absorbance at a wavelength of 497 nm, 517 nm, and 537 nm. Each sample of xylo-oligosaccharide standards and the product of hydrolysis was dissolved in water with various concentrations: 100, 80, 60, 40 and 20 ppm, taken as much as 1 ml, added with 1 ml of 0.4 M acetic acid buffer at pH 5.5 and 0.5 ml 10–4 M in ethanol and then incubated for 5 min at 20° C. After that, each solution absorbance was measured with UV-VIS spectrophotometer at a wavelength of 497 nm, 517 nm, and 537 nm. Observation of free radical activity of compounds against DPPH reagent absorbance can be calculated as follows.

$$A_{hit} = \frac{A_{517}nm - A_{497}nm\ A_{537}nm}{2}$$

Anti - Free Radical activity as % Scavenging DPPH was as follows.

$$\%\ \text{Scavenging DPPH} = \frac{[1 - (\text{A count of test material})] \times 100\%}{\text{A count DPPH (comparator)}}$$

Determination of the inhibition $IC_{50}$ (Inhibitor Concentration 50%) was based on the linear regression analysis of the concentration of % Scavenging DPPH. If $IC_{50}$ is less than 100 ppm, the compound has the activity as an anti-free radical.[8]

**Toxicity Test Using BSLT Method**

BSLT test was performed on the isolated pure compound or separation. Sample weighed as much as 10 mg, and then was dissolved in 1 ml of water. After that, it was added with 99 mL of sea water and stirred until homogeneous to obtain a solution with a concentration of 100 ppm. From 100 ppm solution with concentrations of 100, 50, 25, and 12,5 ppm respectively, replication until 3 times was made.

Further into the sample solution and control, each shrimp fry 8–15 was added, thereafter left to stand for 24 hours. The number of dead shrimp fry was counted and recorded for each concentration of the sample solution and the control solution. Good control data were obtamed when there was no dead shrimp fry. Shrimp fry mortality data at each concentration was used for the analysis of $LC_{50}$.[9] Observations were made after *Artemia salina* contact with the test solution for 24 hours. If the mortality in the control was more than 10 %, the test was canceled and re-tested.

Toxicity of xylo-oligosaccharides was determined by calculating the $LC_{50}$. To determine $LC_{50}$, data obtained from the test result bioactivity were processed using SPSS computer program to determine the $LC_{50}$ value. The test result obtained, provided information about the toxicity of the hydrolisis product.

## RESULTS

**Hemicellulose hydrolysis enzymatically**

The enzyme activity of crude endo-$\beta$-xylanase was as high as 0.119 U/mL. Crude extract of endo-$\beta$-xylanase precipitated by ammonium sulfate showed that the enzyme activity of endo-$\beta$-xylanase had on optimum activity at 60% saturation of ammonium sulfate. It was based on the previous research.[10] After the dialysis process was complete, the volume of endo-$\beta$-xylanase obtained was 15 mL from 1 liter of media production and the activity of endo-$\beta$-xylanase in total after ammonium sulfate precipitation and dialysis was 0.199 U / mL.

In this study, hemicellulose A (Hemi A) and hemicellulose B (Hemi B) were produced. Hemi A was a major hemicellulose whereas Hemi B was hemi residual hemicellulose product. A Hemi obtained was 7.6 grams, while the Hemi B obtained was 6.4 grams. From the TLC results obtained, xylo-oligosaccharide from Hemi B hydrolysis product had a retention factor (Rf) of 0.36, while xylo-oligosaccharide from Hemi A hydrolysis product had a value of Rf as high as 0.41. Based on these two Rf. It was expected that xylo-oligosaccharide from Hemi B hydrolysis product had a Degree of Polymerization (DP)which was higher than that from the Hemi A. This can be seen from

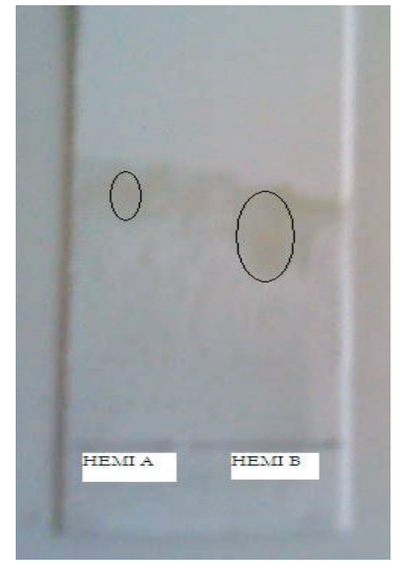


**Figure 1.** TLC result of xylo-oligosaccharide compounds from Hemi A and B.

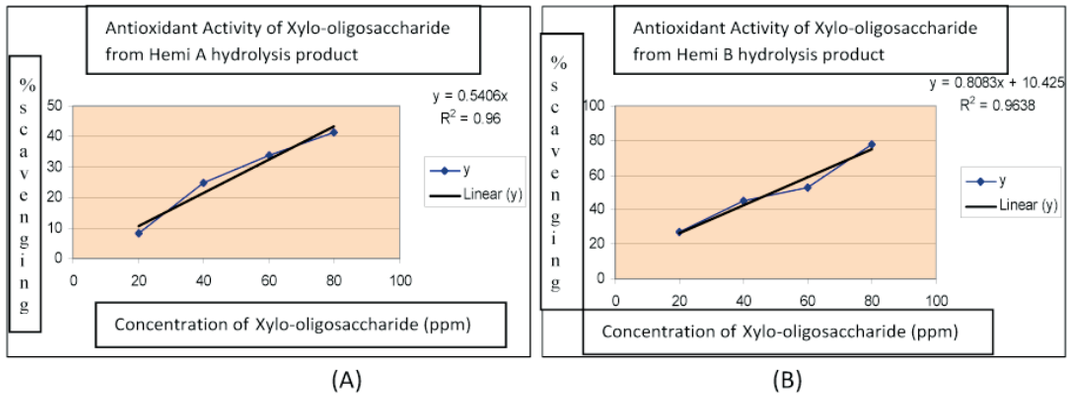


**Figure 2.** Percentage of Scavenging DPPH curve vs. DPPH concentration xylo-oligosaccharides. (A) HemiA, (B) Hemi B.

the spots on the TLC plates that the highest Rf is the spot for the monomer-monomer sugars/monosaccharides (located around the upper limit of the plate).

From Table 1, we know that Hemi B contains more xylo-oligosaccharides than Hemi A. Xylo-oligosaccharide from Hemi A was 4.6% while from Hemi B was 4.9%.

**Anti Free Radical Activity Test**

Free radical activity of each xylo-oligosaccharides can be determined based on regression equations derived from these curves of xylo-oligosaccharides (Hemi A), y = 0.5406 x and xylo-oligosaccharides (Hemi B), y = 0.8083x + 10.425. Once the calculation was done by substituting the a value y with 50, it means that the ability was reduced to 50%, and the obtained x as $IC_{50}$ values are as follows.

Based on the data in Figure 2. $IC_{50}$ value of xylo-oligosaccharides (Hemi A) equaled 92.302 ppm, whereas the $IC_{50}$ value of xylo-oligosaccharides (Hemi B) was 48.96 ppm. The antioxidant activity of compounds oligosaccharides could be affected by DP of the compound. In this study, the antioxidant activity in xylo-oligosaccharide hydrolysis results Hemi B with variations hydrolysis time were also tasted.

**Table 2.** The results of measurements and calculations % DPPH Scavenging by xylo-oligosaccharide from Hemi B hydrolysis product with hydrolysis time variations

| Xylo-oligosakarida (Hemi B) | Xylo-oligosakarida concentration (Hemi B) | Absorbance | | | Antioxidant Activity | |
|---|---|---|---|---|---|---|
| | | $A_{497}$ | $A_{517}$ | $A_{537}$ | % Scavenging | $IC_{50}$ (ppm) |
| Hemi B of corncob | 80 ppm | 0,081 | 0,082 | 0,081 | 87,5 % | 24 |
| Hydrolyzed for 6 hours | 60 ppm | 0,080 | 0,085 | 0,087 | 81,25 % | |
| | 40 ppm | 0,090 | 0,099 | 0,104 | 75 % | |
| | 20 ppm | 0,083 | 0,104 | 0,115 | 37,5 % | |
| | control | 0,081 | 0,100 | 0,103 | - | |
| Hemi B of corncob | 80 ppm | 0,269 | 0,281 | 0,288 | 68,75 % | 47,61 |
| Hydrolyzed for 12 hours | 60 ppm | 0,175 | 0,192 | 0,203 | 62,5 % | |
| | 40 ppm | 0,148 | 0,172 | 0,187 | 43,75 % | |
| | 20 ppm | 0,127 | 0,152 | 0,166 | 31,25 % | |
| | control | 0,081 | 0,100 | 0,103 | - | |
| Hemi B of corncob | 80 ppm | 0,482 | 0,541 | 0,565 | 78,125 % | 48,96 |
| Hydrolyzed for 24 hours | 60 ppm | 0,398 | 0,474 | 0,475 | 53,125 % | |
| | 40 ppm | 0,508 | 0,607 | 0,619 | 43,225 % | |
| | 20 ppm | 0,477 | 0,567 | 0,540 | 26,875 % | |
| | control | 0,516 | 0,635 | 0,594 | - | |

**Table 3.** Observational data xylo-oligosaccharide toxicity tests with *Artemia salina* L

| Concentration of test solution (ppm) | Number of *Artemia salina* larvae tested | | Number of *Artemia salina* larvae dead after treatment | | Number of *Artemia salina* larvae dead in control | |
|---|---|---|---|---|---|---|
| | Replication I | Replication II | Replication I | Replication II | Replication I | Replication II |
| 80 | 10 | 10 | 6 | 8 | 0 | 1 |
| 60 | 10 | 10 | 4 | 2 | 0 | 0 |
| 40 | 10 | 10 | 1 | 2 | 0 | 0 |
| 20 | 10 | 10 | 0 | 0 | 0 | 0 |

From Table 2, the antioxidant activity of xylo-oligosaccharide was affected by the hydrolysis time. Xylo-oligosaccharide from Hemi B hydrolysis product was incubated for 6 hours and had a high antioxidant activity when compared with the incubations for 12 hours and 24 hours, while Hemi B without hydrolysis had the lowest antioxidant activity. The antioxidant activity of xylo-oligosaccharide from Hemi B hydrolysis product incubated for 12 hours and 24 hours were almost the same.

**Toxicity test of *Brine Shrimp Lethality Test* (BSLT)**

BSLT method was performed by counting the number of dead larvae in each test solution.

Larvae mortality data were obtained and analyzed using a SPSS program to determine the relationship between the number of larvae mortality with the concentration of the test solution. Test result was obtained in $LC_{50}$ value. The calculation of $LC_{50}$ value with SPSS obtained an average $LC_{50}$ for xylo-oligosaccharide of 400 ppm.

## DISCUSSION

The development and the advancement of agriculture and agricultural industry in Indonesia have led to an increase in the agricultural waste that are largely a lignocellulosic biomass. Lignocellulosic biomass has not been optimally utilized. Most of biomass will only be destroyed by burning. Continuous combustion process can lead to the accumulation of $CO_2$ in the air that will give an impact as global warming. When examined more deeply, lignocellulosic biomass is composed of organic materials such as hemicellulose, cellulose and lignin, and has a great potential as raw material for various industries. In addition, fractionation of this waste into its constituent components will increase its utilization in various industries. Among lignocellulosic biomass, corncob is not optimally used. Corncob fibers have a composition comprising starch (10–25% (b/b)), hemicellulose (40–50% (b/b)), cellulose (15–25% (b/b)) and phenolic acid (3–5% (b/b)), while the residual consists of protein and oil.

Natural antioxidant is an antioxidant that comes from nature or synthesized through a chemical reaction, and its structure is derived also from nature. The examples of natural antioxidants are poliphenol, flavonoid (flavonon, flavonol, katekin), vitamin E (tokoferol), vitamin C (asam askorbat), and $\beta$-karoten. Synthetic antioxidants are antioxidants that are synthesized through a chemical reaction and their structure is derived from nature such as propyl galat, octyl galat, BHA, BHT and askorbil palmitat.[11] Research have recently observed that oligosaccharide compounds also have antioxidant activity.[2]

Hemicellulose hydrolysis enzymatically has specific properties. The endo-$\beta$-xylanase can hydrolyze xylan as a constituent hemicellulose. The endo-$\beta$-xylanase (1,4 - $\beta$-D-xylanxylanohidrolase, EC.3.2.1.8) can hydrolyze xylan basic structure randomly into xylo-oligosaccharides. The antioxidant activity of a compound can be determined by various methods, such as by measuring the activity of DPPH radical catcher, FTC measurement (Ferry thiocianide), salt reduction method of Fremy, TEAC measurement (Trolox Equivalent Antioxidant capacity), etc. About the Scavenging mechanism of DPPH by the antioxidant, both xylo-oligosaccharide hydrolysis products from Hemicellulose of corn cobs have antioxidant activity because they have $IC_{50}$ values <100 ppm,[8] but these results suggest that xylo-oligosaccharide from Hemi B hydrolysis product have a $IC_{50}$ value greater than the that of Hemi A (Figure 2) that xylo-oligosaccharides from Hemi B is more active as an antioxidant than the Hemi A.

The antioxidant activity of oligosaccharides compounds was affected by Degree of Polymerization (DP) of the compound. Previous studies have succeeded in proving that the existence of the antioxidant activity from galacto-oligosaccharides obtained marine algae by acid hydrolysis, and are influenced by the degree of polymerization of is xylo-oligosaccharides.[12] The higher the degree of the polymerization of xylo-oligosaccharides compound, is the higher the antioxidant activity will be. In this study, xylo-oligosaccharide from Hemi B hydrolysis product based on the TLC has shown that they have a higher degree of polymerization than that from the Hemi A (Figure 1).

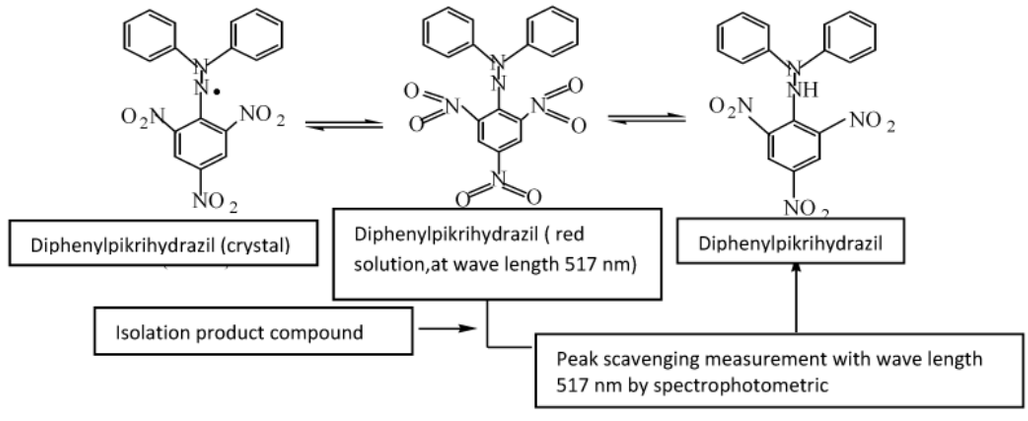


**Figure 3.** DPPH Scavenging mechanism with antioxidant.

But the spots of both of the xylo-oligosaccharide were tailing although we could distinguish the Rf value of xylo-oligosaccharide. This is because the tailing spots of xylo-oligosaccharides produced a mixture of xylo-oligosaccharides that have degrees of polymerization which are adjacent. We still have not been able to prove the influence of the degree of polymerization toward the antioxidant activity. In this study, the antioxidant activity in xylo-oligosaccharide from Hemi B hydrolysis product with variations hydrolysis time (6 hours, 12 hours, and 24 hours) (Table 2) was also tested. This is due to the very influential hemicellulose hydrolysis products and degree of polymerization of the product of hydrolysis. Hydrolysis time can produce hydrolysis products with low Degree of Polymerization (DP) longer such as sugar monomers. Hemi B was also used without hydrolysis as the polysaccharide production controller with a high molecular weight polysaccharide which was insoluble in water. From the calculation with SPSS, $LC_{50}$ value was obtained, with the average $LC_{50}$ for xylo-oligosaccharide of 400 ppm (Table 3). It shows that xylo-oligosaccharide has antitumor activity since it has $LC_{50}$ less than 1000 ppm.

## CONCLUSION

It can be concluded that hemicellulose of corn cobs hydrolysis product has higher antioxidant activity. Hemi B without hydrolysis (Polysaccharides) had no antioxidant activity, and had $IC_{50}$ values > 100 ppm because of its very large molecular weight that the antioxidant activity is influenced by the steric effect of the polysaccharide in reducing free radicals. To figure out the toxicity of xylo-oligosaccharide as an antioxidant compound, BSLT method was performed by counting the number of dead larvae in each test solution.

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