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Syntheses via phenolic oxidative coupling using crude peroxidase from *Brassica juncea* (L) Czern leaves and antioxidant evaluation of dimeric thymol

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Abstract: The ability of a crude *Brassica juncea (L)* Czern peroxidase as green and edible catalyst for phenolic oxidative dimerization of thymol is presented. Crude peroxidase was isolated and partially purified using precipitation method with gradient concentration of ammonium sulphate and dry ice-acetone combination as temperature regulator. The crude peroxidase was then analyzed qualitatively and quantitatively using guaiacol as a model substrate and amino antypirin activity test, respectively, and the positive result was confirmed. From the phenolic oxidative reaction, dimerization of thymol was carried out under extremely mild reaction condition in aqueous medium. The product was checked by using Fourier Transform Infrared (FTIR) and Gas Chromatography-Mass Spectrometry (GC-MS). Furthermore, the biological activity test using DPPH radical scavenging method was confirmed that the dimeric thymol showed 15 times greater than monomeric thymol in term of antioxidant capacity.

Keywords: *Brassica juncea;* peroxidase; oxidative coupling; dimeric thymol; antioxidant.

Introduction

Peroxidases (PODs), that widely distributed in plants species [1], are oxidoreductases that catalyzes a reaction in which hydrogen peroxide (H_2O_2) act as the acceptor and another compound acts as the donor of hydrogen atoms [2-4]. Most of them are heme proteins that contain iron (III) protoporphyrin IX (ferriprotoporphyrin IX) as the prostetic group [1,3]. It plays a significant role in the plant defense system by converting phenolic compounds to quinones, which are toxic to pathogenic organisms and pests. Plant differentiation and development are also regulated by this type of enzymes[5] together with other class of enzymes. Peroxidases have been reported to present in both soluble and membrane-bound forms [6,7]. PODs from several plants have been purified and studied, for example, oil palm leaf [8], sweet potato tubers [9], turnip [10-11], melon [12], Brussels sprouts [13-14], cabbage [15], barley [16], okra [17], oranges [18], tea leaves [19], pepper fruits [20], carrot roots [21], tobacco [22], wheat germ [23], mango [24], green pea [25-26], papaya fruit [27], spinach [28], Cox's apple pulp [29], rice [30], cotton [31], peanut [32], tomato [33-34], green asparagus [35], strawberry [36].

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In the presence of peroxide, for example hydrogen peroxide, PODs from plant tissues are able to oxidize a wide range of phenolic compounds, such as guaiacol, pyrogallol, chlorogenic acid, catechin and catechol [37]. One type of chemical reaction that catalyzed by peroxidases is coupling oxidative of phenolic. Phenolic compounds such as eugenol and guaiacol act as hydrogen donor and then forms a species, called phenoxy radical. This species is relatively unstable and get stabilisation by making dimeric compound via coupling. The previous research published by Anita et al. studied dimerisation of guaiacol to O-para guaiacol by crude of *Brassica oleracea* var *alboglabra* peroxidase [38].

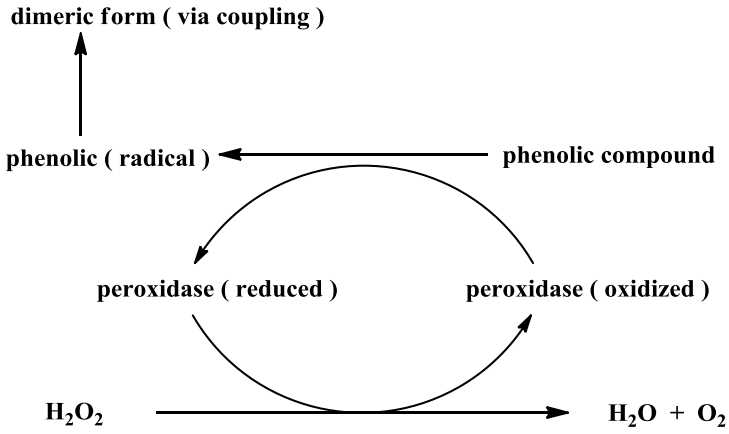


Figure 1. Scheme of phenolic oxidative coupling catalyzed by peroxidase

In this paper, we reported the isolation and partial purification of peroxidase from *Brassica juncea*. The crude enzyme was then applied in catalyzing oxidative coupling dimerisation of thymol in aqueous medium. Formation of dimeric thymol via carbon-carbon coupling was confirmed by LC-MS spectrometry, and the potential bioactivity of dimeric thymol as antioxidant has also been studied.

Experimental Section

General and Instrumentation

Fresh *Brassica juncea* was collected from local market. All chemicals were purchased from commercial suppliers, analytical grade, and used without any further purification. Analytical Thin Layer Chromatography (TLC) was performed on Merck silica gel 60 F_{254} plates. Fragmentation of dimeric thymol was recorded using Gas Chromatography – Mass Spectrometry (LC-MS) and infrared spectra was recorded on Shimadzu Prestige-21 Fourier Transform Infrared (FTIR) spectrophotometer. The condition of GC-MS analysis : DB column (30 m x 0.25 mm), carrier gas He, 1.2 mL/min constant flow, heating 40°C for 2 min, 40-100 $^{\circ}$C at 40 $^{\circ}$C/ min, 100-140 $^{\circ}$C at 2 $^{\circ}$C/ min, 140-340 $^{\circ}$C at 30 $^{\circ}$C/ min, system injector split 200$^{\circ C}$, system detector MSD 320$^{\circ C}$.

Isolation and Partial Purification of Peroxidase

Brassica juncea (250 g) was washed using distilled water to remove the physical impurities, and the rest was carefully cut into smaller size with an ordinary kitchen peeler, the phosphate buffer was added and the mixture was blended for 5 minutes at -4°C.

The homogenate was then filtered, and the filtrate was collected using a 250 mL beaker glass. The filtrate was purified using fractional precipitation method. The gradient concentration of ammonium sulfate (30, 50, and 70%) was used as precipitator, and temperature of environment was adjusted approximately to -30°C using dry ice-acetone.

Determination of Total Protein Concentration

Total protein content in the enzymatic system was performed using Lowry method with albumin from bovin (BSA) as protein standard. A crude peroxidase extract (1 mL) was added into 5 mL of Lowry reagent (mixture of 2 g of Na_2CO_3 in 100 mL of NaOH 0.1 N and 0.25 g of $CuSO_4.5H_2O$ in 50 mL of 1% K-Na tartrate solution). The mixture was stirred and let stand for 10 minutes. A 0.5 mL of 1N Folin solution was added carefully, let stand for 30 minutes, and the absorption was measured using UV-Vis spectrophotometer at 750 nm.

Qualitative Analysis of Crude Peroxidase Activity Using Guaiacol

Crude peroxidase activity was semiquantitatively determined using guaiacol as a model substrate. Various of stock enzyme solution (1, 2 and 4 mL) and guaiacol (0.2 mL) were added to a test tube containing 0.4 mL of 30% hydrogen peroxide. The mixture was shaken vigorously about 2 minutes, and the red colour was appeared after 30 s reaction time. The reaction were monitored by reading the increase in absorbance at 460 nm using Shimadzu 2450 double beam ultraviolet-visible (UV-Vis) spectrophotometer.

Determination of Specific Activity of Crude Peroxidase

2.5 mL of 2.5 μM 4-aminoantipyrin in phenol and 2.0 mL of 30% hydrogen peroxide were added into 0.2 mL of crude peroxidase. The mixture was incubated in boiling water for 2 min. The mixture of 4-aminoantipyrin, hydrogen peroxide and phosphate buffer pH 7.0 was used as control solution. The absorbance was recorded at 510 nm. Specific activity of crude peroxidase was calculated using the following equation :

$$\textbf{Specific activity} = \frac{\textbf{absorbance at 510 nm}}{\textbf{6.58 x total protein content (in mg/mL)}}$$

Dimerisation of Thymol

Two test tubes were prepared in this reaction procedure. The first tube as negative control which contains 5 mL of phosphate buffer pH 7.0 and the second test tube contains 5 mL of crude peroxidase. A 1.0 mL of thymol solution (1 M) was added into each test tube, followed by the addition of 0.1 mL of 30% hydrogen peroxide. The reactions were monitored using TLC analysis. After completion, crude product was extracted using ethyl acetate (2x50 mL). The combined fraction was dried over anhydrous Na_2SO_4. The product was concentrated, and the rest was purified using flash colum chromatography to get the pure dimeric thymol. Structure elucidation of product was performed using GC-MS and FTIR.

Antioxidant Assay

Antioxidant activity of thymol and dimeric thymol was determined using DPPH radical scavenging method. The DPPH radical-scavenging activities were assessed as described previously [39] with major modification. Briefly, 1.0 mL DPPH radical (0.5 mM) was mixed with 1.0 mL of thymol or dimeric thymol solution (in methanol). The volume of the mixture

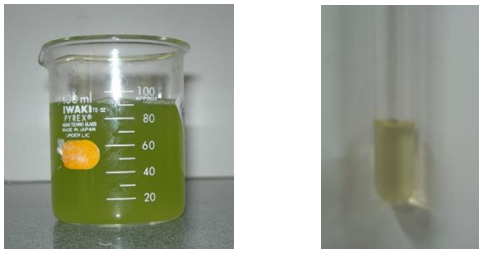
was adjusted to 5 mL by adding methanol. The concentration of thymol were varied from 1, 5 and 15 ppm, and dimeric thymol from 0.25, 0.5 and 1 ppm. The reaction was carried out at room temperature for 25 minutes. The decrease of DPPH radical concentration was monitored every 5 minutes by measuring the absorbance at 517 nm with a UV-Vis spectrophotometer (Shimadzu 2450 double beam). Antioxidant activity was measured as the decrease of DPPH radical concentration caused by the addition of sample (thymol or dimeric thymol), and was expressed as percent inhibition. The value of percent inhibition was calculated by following equation :

$$\% \text{ inhibition} = \frac{A_{\text{control solution}} - A_{\text{sample}}}{A_{\text{control solution}}} \times 100\%$$

Results and Discussion

Isolation and Determination of Specific Activity of Crude Peroxidase

Crude extract of *Brassica juncea* peroxidase has green colour. It signify the presence of impurities such as debris cells from it leaves. Partial purification was performed using gradient concentration of ammonium sulphate at approximately -30°C of environmetal temperature. Result of purification using 30% (by mass) ammonium sulphate solution, called fraction I. Fraction I was then followed by second and third purification stages using 50% and 70% (by mass) of ammonium sulphate, and called enzyme fraction II and III, respectively. Fraction III was used in this experiment in catalyzing oxidative dimerisation of thymol. Before used, determination of total protein content, qualitative analysis and specific activity of fraction III was perfomed and give the following result as described in the next paragraph.



a b

Figure 2. Crude extract of *B. juncea* peroxidase (a) and peroxidase fraction III (b)

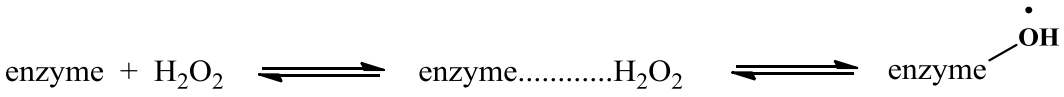
Qualitative analysis using guaiacol shows the positive result. The increase of absorbance at 460 nm (dimeric guaiacol) was recorded. The absorbance increase in the addition of more volume of fraction III peroxidase, as shown in the Table 1

Table 1 : the increase of absorbance at 460 nm in qualitative analysis of enzyme fraction III

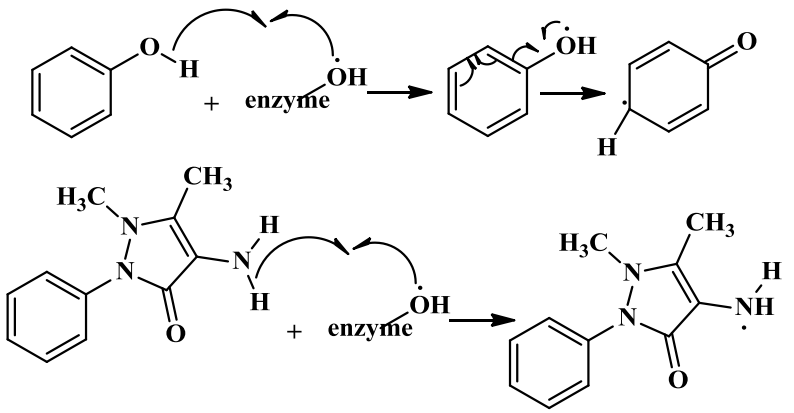
No.	Volume of fraction III (mL)	Volume of guaiacol (mL)	Absorbance at 460 nm
1	1	0.2	1.50
2	2	0.2	1.60
3	4	0.2	3.80

Total protein content of fraction III was determined using Lowry method and was found to be 0.59 mg/mL. As a part in determination of specific activity, peroxidase fraction III reacts with 4-aminoantipyrin (in phenol) which is act as hydrogen donor. This reaction can be observed because there is a change of colour solution to red colour, and monitored at 510 nm using UV-Vis spectrophotometer. the red colour formed is related to the presence of quinoneimine compound. From the quiantification using UV-Vis, absorbance at 510 nm was found to be 2.29. Therefore, by using formula given in experimental section, the specific activity of peroxidase fraction III was found to be 0.59 (unit/mg). The reaction mechanism of enzyme and 4-aminoantipyrin can be illustrated below.

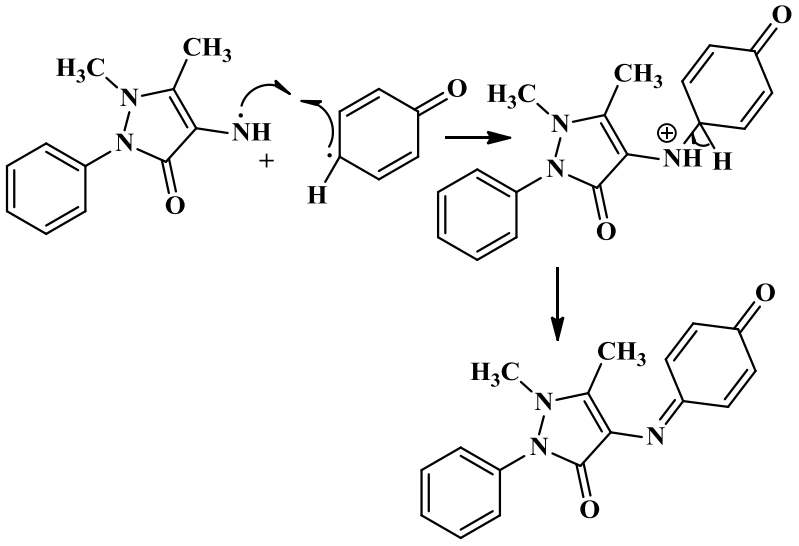
Stage 1 : initiation



Stage 2 : propagation



Stage 3 : termination



Quinoneimine (red)

Figure 3. proposed mechanism in determination of specific activity using 4-aminoantipyrin

Dimerisation of Thymol and Structure Elucidation

We presented a simple and green organic synthesis of dimeric form of thymol via radical C-C oxidative coupling using peroxidase isolated from *Brassica juncea* leaves. The reaction mixture of thymol, hydrogen peroxide and *B. juncea* peroxidase in aqueous medium containing phosphate buffer pH 7.0 was magnetically stirred, and the colour of solution change to orange after 30 minutes reaction time. The completion of the reaction reach after 1.5 hour reaction time. TLC analysis suggested the formation of new compound with higher polarity compared to starting material (thymol) and identified at Rf value of 0.47 (using n-hexane : ethyl acetate 6 : 1 as eluent). The crude product was extracted using ethyl acetate, the solid was purified by column chromatography using silica gel to get the pure desirable product.

Figure 4. Colour change in dimerisation of thymol using *B. juncea* peroxidase

The product was then analyzed using FTIR and GC-MS to confirm the structure of the product. The result of FTIR analysis as summarized in the Table 2. From FTIR analysis, there is no the significant change of IR absorption between thymol and desirable product (dimeric thymol) because both of them has no difference in functional group. We need more chemical instumentation to elucidate the structure of product.

Table 2 : Some of Important IR absorption of thymol and dimeric thymol

No.	**Wavenumber (cm^{-1})**		**Functional group**
	thymol	**dimeric thymol**	
1	3201.0	3418.4	-OH stretch
2	2962.2 ; 2929.7	2958.4 ; 2925.8	C-H sp^3 stretch
3	1619.4	1620.9	Aromatic ring
4	1428.5	1422.4	C-H sp^3 bend
5	1358.5	1379.9	Isopropyl, $-C(CH_3)_2$
6	1159.1	1155.0	C-O single bond stretch
7	733.8	739.2	2 or 3 substituted benzene

From the GC-MS analysis, there are four main peaks with the retention time 15.03 min (m/z 150), 16.64 min (m/z 278), 26.08 (m/z 198), and 28.54 min (m/z 298). The molecular mass analysis, signify that compound which has retention time 15.03 related to thymol structure with Mr 150 g/mol. The fragment with m/z 298 is suggested as dimeric structure of thymol after two thymol radical coupled together. Fragment m/z 298 = 2 x Mr thymol (150) minus two hydrogen atom. Fragment m/z 298 was then followed by some more fragmentation and has several main peaks at m/z 283, 241, 149, 123, 91, and 77 as shown on the picture below. The molecular cation m/z 298 fragmented to m/z 283 by losing one methyl group (-CH_3), molecular cation m/z 298 also fragmented in different pathway to form m/z 241 by losing isopropyl and –CH_2-. The molecular cation fragment m/z 298 was broken in C-C biphenyl bond to give fragment of m/z 149, corresponding to the cationic radical of thymol.

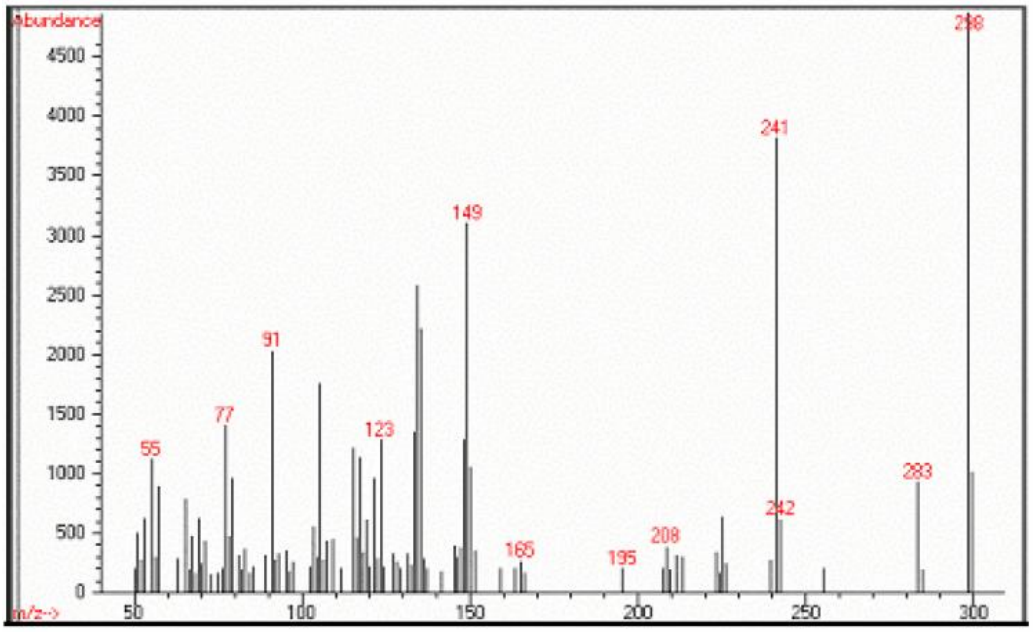


Figure 5. Mass spectra of dimeric thymol

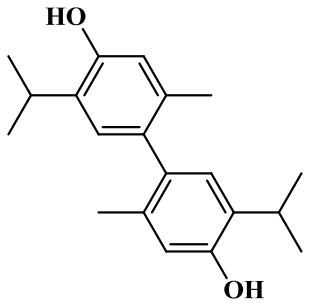


Figure 6. Structure of dimeric thymol

The mechanism of dimeric thymol formation can be described by the following processes. Peroxidase reacts with hydrogen peroxide as acceptor to form oxidized enzyme. Thymol as hydrogen donor give one hydrogen to the enzyme. Therefore, the enzyme is reduced and the thymol is oxidized to thymol radical (phenoxy, in general). Thymol radical stabilize it self by coupling with other thymol radical to form dimeric thymol.

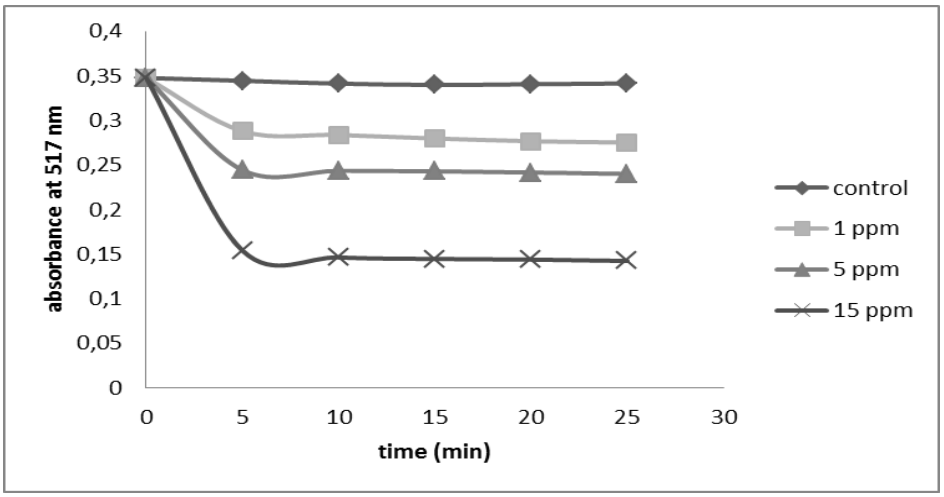
Antioxidant Activity of Thymol and Dimeric Thymol

Phenolics share the same general structure composed of an aromatic hydroxyl nucleus and exist in an approximated number of 8000 in nature. So far, plant phenolics constitute one of major groups of compounds acting as primary antioxidants or free radical terminators [40]. Antioxidant activity is defined as the ability of a compound to inhibit oxidative degradation like lipid peroxidation [40-41]. There are several method in determining of antioxidant activity of certain compound, such as ABTS scavenging activity [42], DMPD assay [43], cupric ion (Cu^{2+}) reducing assay [44-45], chelating activity of ferrous ion (Fe^{2+}) [46], and DPPH assay. The antioxidant activity of dimeric thymol and thymol were evaluated by the neutralizing of DPPH, a model of radical compound [47-48]. In DPPH assay, the antioxidants were able to reduce the stable radical DPPH (violet-coloured) to the yellow-coloured diphenyl-picrylhydrazine. This method is based on the reduction of DPPH in methanolic solution in the presence of a hydrogen-donating compound due to the formation of non-radical form of DPPH. The decrease of radical DPPH absorbance can be monitored quantified at 517 nm. The decrease of DPPH absorbance can be tabulated below.

Table 3. The decrease of DPPH absorbance at 517 nm as a function of time

time (min)	**absorbance of DPPH caused by addition of sample**						
	control	**thymol (ppm)**			**dimeric thymol (ppm)**		
		1	**5**	**15**	**0.25**	**0.5**	**1**
0	0.34755	0.34755	0.34755	0.34755	0.34755	0.34755	0.34755
5	0.34440	0.28788	0.24425	0.15400	0.29596	0.25244	0.14162
10	0.34119	0.28363	0.24355	0.14637	0.29370	0.25047	0.13037
15	0.33975	0.27969	0.24291	0.14465	0.29197	0.24641	0.12433
20	0.34051	0.27665	0.24157	0.14388	0.29079	0.24530	0.12100
25	0.34144	0.27511	0.23998	0.14239	0.28154	0.24406	0.11838

Table 3 illustrates a significant decrease in the concentration of DPPH radical due to scavenging ability of dimeric thymol. A 0.5 ppm of dimeric thymol has same ability of 5 ppm of thymol in decreasing the absorbance of DPPH from 0.34755. this indicates the effectiveness of dimeric thymol compared to monomeric of thymol structure, in term of ability in donating hydrogen atom to the radical. IC_{50} values for monomeric and dimeric structure of thymol were found as 12.08 and 0.77 ppm. A lower IC_{50} value indicates a higher DPPH free radical scavenging activity. DPPH scavenging capacity of dimeric thymol 15 times higher monomeric thymol.



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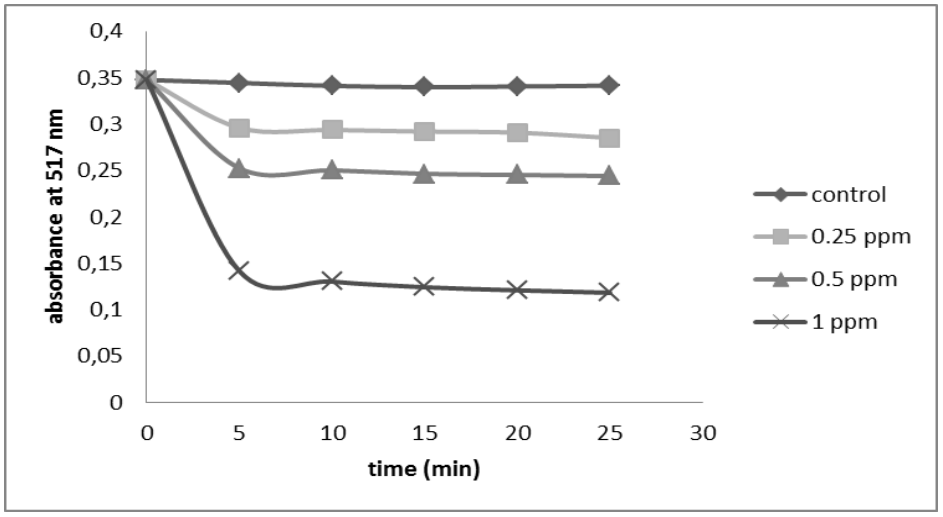

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Figure 7. The decrease of DPPH radical absorbance caused by the addition of a sample (a) thymol and (b) dimeric thymol

Table 4. IC_{50} values for monomeric and dimeric thymol

Tested compound	**Antioxidant activity using DPPH method (IC_{50}, ppm)**
Thymol	12.08
Dimeric thymol	0.77

Conclusion

The oxidative coupling dimerisation of thymol was succesfully achieved by using crude extract of *Brassica juncea* peroxidase as edible catalyst in the presence of hydrogen peroxide. Dimeric thymol was identified using GC-MS spectrometry at retention time 28.54 minutes with m/z value of 298. From antioxidant assay, dimeric structure of thymol has greater antioxidant capacity compared to thymol about 15 times. Biocatalytic dimerisation using crude peroxidase from plants provides a new way to synthesis organic molecules with interesting biological performances.

Acknowledgements

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