

## SYNTHESIZING DERIVATIVES FROM CYCLOPENTANONE ANALOGUE CURCUMIN AND THEIR TOXIC, ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES

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### Abstract

Three types of cyclopentanone derivatives have been synthesized from aromatic aldehyde and ketone derivatives under a base condition through aldol condensation. These cyclopentanone products were 2,5-dibenzylidene-cyclopentanone (a), 2,5-bis-(4-hydroxy-benzylidene)-cyclopentanone (b), and 2,5-bis-(4-hydroxy-benzylidene)-cyclopentanone (c) which has a yield of 63-99%. The chemical structure of these compounds were determined using UV, IR and NMR spectroscopy. In order to clarify the role of hydroxyl and amine moieties, toxic, antioxidant and anti-inflammatory activities were carried out. The toxic test indicated that the compounds showed strong toxicity. In addition, the presence of hydroxyl and amine groups on both rings of curcumin increased the antioxidant and anti-inflammatory activities.

*Keywords: antioxidant, anti-inflammatory, curcumin, toxicity*

### 1. Introduction

Curcumin compounds can be found in many types of *Curcuma* Genus and is the main pigment in turmeric (*Curcuma longa*). Some of the curcuminoid compounds found in turmeric are curcumin **1**, 4-dimethoxy-curcumin **2** and bisdimethoxycurcumin **3**, which are the derivative compounds of diarylheptanoid. In addition, an asymmetric derivative of curcuminoid, dihydrocurcumin **4** [1] can also be found (Figure 1). Synthetic and natural curcumin compounds are known to have varied biological activities, such as anti-inflammatory [2], antioxidant [3], antiviral, anti-infection, anti-allergy [4], and anti-HIV [5]. Their biological activities and simple structure have made curcumin the compound structure model for the target

that is going to be synthesized. Curcumin isolated from natural sources was found in a small amount of around 3-5% from the dry weight and has a limited structure variation; it becomes a problem in optimizing the function of curcumin [6]. Therefore, efforts are required to synthesize in the laboratory in order to obtain the analogue of curcumin derivative in the desired number and with a wide range of structure variation.

Biological activities from curcumin derivatives are related to unsaturated  $\alpha$ ,  $\beta$  ketones. The strength is affected by the substituent type and pattern that can be found in the aromatic ring. This study tests the effect of the difference in substituent, hydroxyl, and amines in the aromatic ring of curcumin towards toxic, antioxidant, and anti-inflammatory activities.

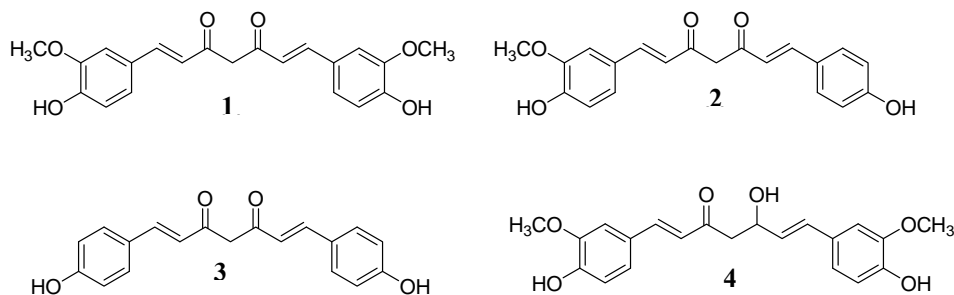


Figure 1. Structure of Several Curcumin Derivatives Found in Turmeric

The study uses the brine shrimp lethality test (BSLT), which is a method using brine shrimp as the test subjects. The preliminary test can support findings from anticancer and pesticide compounds. A lot of scientific findings showed that the antioxidant compound reduces risk of chronic diseases such as cancer and coronary heart disease. The main characteristic of antioxidant is its ability to capture free radicals [7]. The inflammation process is the body's defense mechanism to destroy harmful compounds on the wounded places, and prepare the condition for a tissue repair [8].

## 2. Methods

The tools used in this study include a round flask, a magnetic stirring bar, a reflux condenser, a mortar, a vacuum pump, a Buchner funnel, a thermometer, a Fisher-Johns melting point apparatus, an infrared spectrophotometer (FTIR Shimadzu, IR Prestige-21), a proton and carbon NMR spectrophotometer (Jeol Type ECA 500), and a UV-visible spectrometer (Hitachi U-2001).

The materials used in this study include a cyclopentanone reactor, a benzaldehyde, an ethyl acetate, a hexane, an ethanol, a methanol, a barium hydroxide octahydrate, a KLT GF254 plate, 96% ethanol, sea water, diphenylpicrylhydrazyl (DPPH), HCl, DMSO, murine monocytic macrophage cell line RAW 264.7, Dulbecco's Modified Eagle Media (DMEM) (2mM L-glutamine, 45 g/L glucose, 1 mM sodium pyruvate, 50 U/mL penicillin; 50 µg/mL streptomycin) with 10% foetal bovine serum (FBS), Griess reagent (2.5% sulfanilamide, 0.2% naphthylenediamine dihydrochloride, 2% sulphanilamide in 5% phosphoric acid).

**Synthesis of the cyclopentanone analogue of curcumin.** As much as (0.01 mol) cyclopentanone was mixed with barium hydroxide octahydrate (0.0055 mol) and 10 mL absolute ethanol in the round flask equipped with the magnetic stirring bar and reflux condenser. The reaction mixture was stirred and refluxed for 2.5 hours, while gradually adding aldehyde derivative (0.02 mol in 2 mL ethanol). The resulting sediment was then cooled and added with 50 mL HCl 1N, followed by filtration using the Buchner funnel. It was then washed with 50

mL aquadest and 50 mL hexanal respectively, and dried at a temperature of 40 °C for 24 hours. Hypothetical Combinatorial Molecule can be seen in Figure 2. All the compounds were tested for their biological activities (toxicity, antioxidant, anti-inflammatory).

**Toxic activity.** In order to determine LC<sub>50</sub>, data was derived from calculating the death of *Artemia salina* in different concentrations of curcumin derivatives within 24 hours. The data was analyzed on a confidence interval of 95% using LdP program.

**Antioxidant activity.** The sample was dissolved using DMSO 100% to achieve a concentration of 200 µg/mL. The sample was diluted with a different concentration of 100 µL in a 96-well microtiter plate from 6.25-200 µg/mL. Each well received an additional of 5 µL diphenylpicrylhydrazyl (DPPH) (1 mg/mL in methanol). The microtiter plate was then vortexed and incubated for 30 minutes in the dark room. The absorbance was measured on 517 nm wavelength, and the total percentage of radical scavenging activity was calculated based on the following formula (1):

$$[(A \text{ control} - A \text{ sample})/A \text{ control}] \times 100\% \quad (1)$$

**Anti-inflammatory test.** *In vitro* anti-inflammatory from the sample was done by using murine monocytic macrophage cell line RAW 264.7 inside Dulbecco's Modified Eagle Media (DMEM) (2mM L-glutamine, 45 g/L glucose, 1 mM sodium pyruvate, 50 U/mL penicillin; 50 µg/mL streptomycin) with 10% foetal bovine serum (FBS). The cell was incubated at 37 °C with 5% CO<sub>2</sub>. Activities of the sample that inhibit the production of NO were analyzed based on Griess method [9]. 1 x 10<sup>6</sup> cell/mL RAW 264.7 cell lines was applied in 96-well tissue culture plate and incubated for 4 hours at 37 °C with 5% CO<sub>2</sub>. The cell was then incubated using DMEM which consisted of 100 µL sample within DMSO and results in a concentration of 200 µg/mL in 0.1% DMSO. The cell was stimulated with 200 U/mL IFN-γ and 10 µg/mL LPS for 17 hours. The NO production in the media cell was analyzed using Griess reagent, and absorbance was read on a 550 nm wavelength using a microplate reader (Spectra Max Plus 384, Molecular Devices Inc., USA). The nitrate concentrate in the supernatant was calculated through

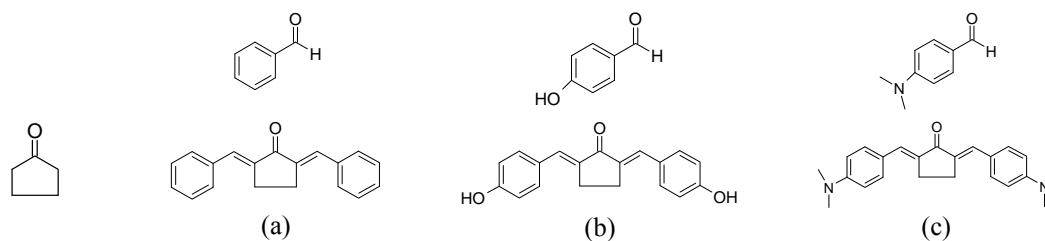


Figure 2. Hypothetical Combinatorial Molecule Libraries

comparison with the standard curve of sodium nitrate. The percentage of cell viability was analyzed using MTT method. L-NAME is used as a positive control.

### 3. Results and Discussion

The synthesis mechanism of the curcumin derivative compounds was conducted through a condensation reaction between ketone and aromatic aldehyde in an acidic or alkaline environment. In this report, the synthesizing of the curcumin derivative was performed in the alkaline condition using barium hydroxide. This enables the making of curcumin derivative with sufficient yield between 90-96%. The principle method was quite simple as it did not require complicated work, only the filtering and washing of the sediment gathered. One of the problems that occurred was sedimentation on reflux which lessened the product's yield. However, this could be solved a slight modification of the method that is changing the way aldehyde is added.

In the previous procedure, the three materials were mixed at the same time and refluxed together so that there was a build up of product and raw material. In this study, aldehyde was gradually added into the alkaline ketone mixture while being refluxed. Thus, the reaction could be controlled and it would happen gradually until all of the aldehydes had reacted. This could be seen from KLT which uses a solvent that matches the end product. The mixture was left to cool and washed so that the solids were made. The compound that had yet to become pure was separated with a silica gel chromatography column with a gradient polarity. The end result gives a good yield between 63-99%.

**Compound (2E, 5E)-2,5-dibenzylidene-cyclopentanone 5.** The compound was obtained in the form of bright yellow powder with a melting point of 76-77 °C and a yield of 89.55%. From the TLC results, yellow spots were observed with Rf value of 0.3 (dichlorometane hexane,3:2), Rf 0.68 (dichlorometane 100%), and Rf 0.72 (ethyl acetate hexane, 4:1). In the UV-Vis spectroscopy, this compound gives a maximum absorbance at 351.0 and 263.5 wavelength. In IR Spectroscopy, it showed absorbance on 3052.3; 3018.6; 3088.0; 2954.9; 2910.6 (C-H); 1689.6 (C=O); 1625.9; 1600.9; 1570.0 (C=C), and in <sup>1</sup>H-NMR spectroscopy (CDCl<sub>3</sub>, 500 MHz) gave chemical shift of 3.1252 (s, 4H, H2C-CH2); 7.59 (s, 2H, -CH=); 7.46 (m, 4H, H3, H5); 7.43 (m, 2H, H4); 7.61 (m, 4H, H2, H6).

**Compound (2E, 5E)-2,5-bis(4-hydroxy-benzylidene)-cyclopentanone 6.** The compound was obtained in the form of bright green powder with a 63.7% yield. The TLC result was bright green spot with Rf value of 0.52 (benzene ethyl acetate, 2:3), Rf 0.54 (ethyl acetate dichloromethane, 2:3), and Rf 0.64 (dichloromethane methanol, 9:1). On the UV-Vis spectroscopy, maximum

absorbance on wavelengths 240.5 and 216.0 were revealed. Furthermore, IR spectroscopy showed absorbance on 3371.5; 3394.7 (O-H); 3026.3; 3057.2; 2920.2; 2954.9 (C-H); 2850.7 (-CH<sub>2</sub>-), and on <sup>1</sup>H-NMR spectroscopy (DMSO, 500 MHz) showed chemical shift of 3.01 (s, 4H, H2C-CH2); 6.87 (d, J=11.6 Hz, 4H, H3, H5); 7.33 (s, 2H, -CH=); 7.54 (d, J=8.55 Hz, 4H, H2, H6); 10.07 (br, 2H, -OH).

**Compound (2E, 5E)-2,5-bis(4-dimethylamino-benzylidene)-cyclopentanone 7.** The compound was obtained in the form of greenish yellow powder with a melting point of 194-195 °C and a yield of 63.7%. The resulting TLC result was a yellow spot with Rf value of 0.53 (benzene ethyl acetate, 9:1), and Rf 0.76 (ethyl acetate dichloromethane). On the UV-Vis spectroscopy, it showed maximum absorbance on wavelengths 461.5; 381.0; 338.0; 275.0; and 235.0. On IR spectroscopy, it showed absorbance on 3084.2 (C-H); 2895.2; 2806.4 (-CH<sub>2</sub>-); 1635; 1597.1; 1525.6 (C=C); and 1438.9, and on <sup>1</sup>H-NMR spectroscopy (CDCl<sub>3</sub>, 500 MHz) showed chemical shift of δ3,07 (s, 4H, H2C=CH2); 6.70 (d, j=9.15 Hz, 4H, H3, H5); 7.57 (d, J=8,55 4H, H2, H6); 7,73 (d, J=9.15, 2H, -CH=). All structures can be seen in Figure 3.

**Toxic activity.** A program that sets out to search anti-cancer compounds has been developed by the National Cancer Institute. Toxicity can be described as the effects of having an element or a compound enter the body of an organism in an amount exceeding the amount that can be tolerated by the organism. Toxicity is also defined as the ability of a substance that has destructive cell characteristics especially related to the process of a cell in the body immunity system or anti-neoplastic drugs which can selectively kill cells [8]. Toxicity is the

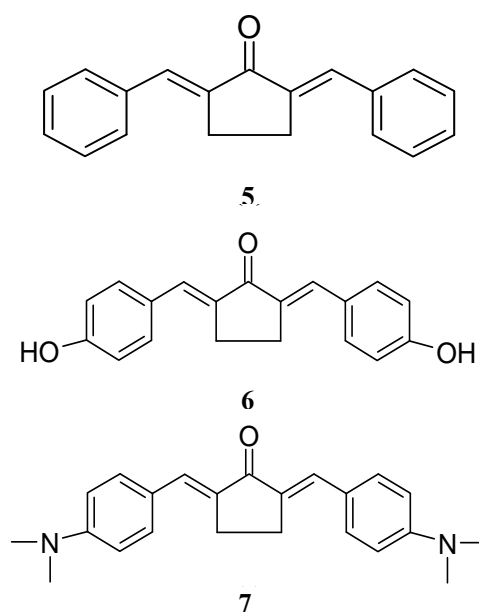


Figure 3. Structure of 5, 6, and 7 Compounds

characteristics of a compound which disturbs cell development, whether normal or cancerous [10]. Toxicity test is used to discover the influence of a poison produced by a single dosage of a chemical mixture on the test animal as the pre-screening test on the anti-cancer bioactive compound [11-12]. The toxicity test is correlated to the activities of the anti-cancer drugs.

According to Mayer *et al.* [13], an extract is considered very toxic if it has a value of  $LC_{50}$  below 30 ppm, toxic if the  $LC_{50}$  is between 30-1,000 ppm, and not toxic if  $LC_{50}$  is above 1,000 ppm. Regarding the toxicity test towards the curcumin compound resulting from the synthesis in Table 1, all of them had  $LC_{50} < 1,000$  ppm, which means the test compounds were toxic (Table 1). Compound 5 and 7 were very toxic compounds, while 6 was a toxic compound. The presence of active clusters such as phenolic and carbonyl could have toxic characteristics towards tested animal and the toxic compound could continue to anti-cancer activities test. Thus, the three curcumin synthesis compounds could proceed to the anti-cancer test.

**Antioxidant activity.** The antioxidant activity of the compounds was done by using free radical scavenging activity with DPPH (2,2-diphenyl-1-picrylhydrazyl) as a radical. DPPH is a synthetic radical that will dissolve in polar solvent, such as methanol [11]. Antioxidant compound will react with radical compound DPPH through the mechanism of hydrogen atom donation and cause the shedding of DPPH color from purple to yellow at 517 nm wavelength [12]. A linear regression to determine the value of  $IC_{50}$  can be made from the value of concentration and percentage (%) of the sample inhibition.  $IC_{50}$  is the compound concentrate of the synthesis result that can give a 50% free radical inhibition. The smaller the  $IC_{50}$ , the stronger its antioxidant power.

The results of the measurement of antioxidant power of the compound resulting from synthesis using DPPH method can be seen in Table 2. The table shows that

using DPPH method test compounds have the antioxidant power with  $IC_{50}$  value in the range of 49 to bigger than 200  $\mu\text{g/mL}$ . This  $IC_{50}$  value also showed that compounds 6 and 7 have powerful antioxidant activities because they have  $IC_{50}$  value of less than 200  $\mu\text{g/mL}$  [13]. From the  $IC_{50}$  value of the compound resulting from synthesis, it can be seen that compound 6 has 4.67% higher antioxidant activities than the ascorbic acid as the standardized, while compound 7 has 25.44% lower antioxidant activities than the ascorbic acid.

From Figure 4, it shows that the percentage of the inhibited sample correlates with the  $IC_{50}$ . The weakest compound inhibited by DPPH was 43.817%, while b and c, inhibited by a relatively similar value, were of 76.823 and 74.955%. These values were lower compared to ascorbic acid with a percentage of inhibition at 87.5% and at a concentration 200  $\mu\text{g/mL}$ . This activity might be due to the presence of OH and amine moiety in compound 6 and 7 respectively.

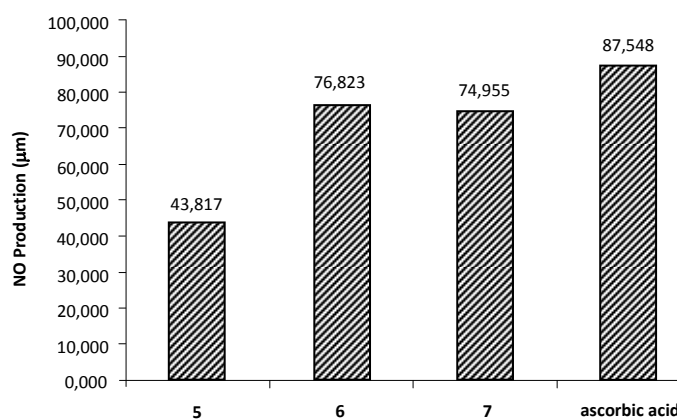
According to Ohkatsu and Yatoh [14], the presence of hydroxyl and methoxy moiety may increase antioxidant activities. In addition, it is known that compounds with more than one hydroxyl moiety in the ring will increase the antioxidant activity [15]. From the structure, the

**Table 1.  $LC_{50}$  from Synthesis Curcumin Compound**

Compounds	$LC_{50}$ (ppm)
5	10,000
6	146,893
7	11,103

**Table 2.  $IC_{50}$  Test Sample against DPPH Free Radicals**

Sample	$IC_{50}$ ( $\mu\text{g/mL}$ )
5	>200
6	49.1
7	64.6
Ascorbic acid	51.5



**Figure 4. The Percentage of Sample's Inhibitory Power against Free Radicals (DPPH) at a Concentration of 200  $\mu\text{g/mL}$**

presence of the simplest compound a that does not contain moiety to be donated is the reason structure 5 does not show antioxidant activities. Compounds 6 and 7 had two hydroxyl moieties and amine moieties respectively in each ring because those moieties could neutralize DPPH radicals by donating a proton. Further explanation can be seen through the reaction mechanism in Figure 5 and 6.

Furthermore, protons that are formed will bind DPPH radicals just as methyl radicals formed will also bind other DPPH radical molecules. Thus, this further enhances the ability of compound 6 and 7 in the DPPH radicals through the reaction mechanism in Figure 5.

**Anti-inflammatory activity.** Nitric oxide (NO) is a free radical generated by nitric oxide synthase (iNOS) and

serves as an important mediator in the regulation of physiological and pathophysiological mechanisms in cardiovascular and immune systems [16]. Abnormal expression of iNOS induction and overproduction of NO can lead to chronic inflammation and is associated with numerous diseases such as diabetes, hypertension, and others.

Anti-inflammatory activities using murine monocytic macrophage cell line RAW 264.7 in Dulbecco's Modified Eagle Media (DMEM) in all compounds synthesis could decrease levels of NO production in the cell line used, but the value of the decrease of NO production was lower than the positive control of L-NAME (N $\omega$ -nitro-L-arginine methyl ester hydrochloride). According to Kim *et al.* [2], inhibition activities of iNOS can be classified as very active if the

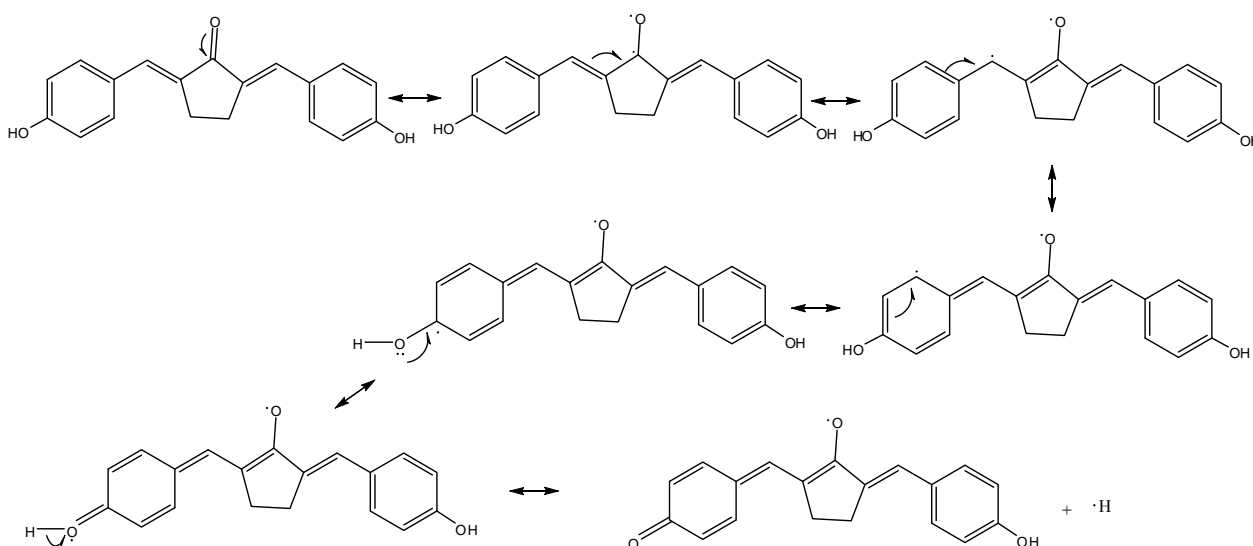


Figure 5. The Formation Mechanism of Protons which Act as Free Radicals Antidote on Compound 6

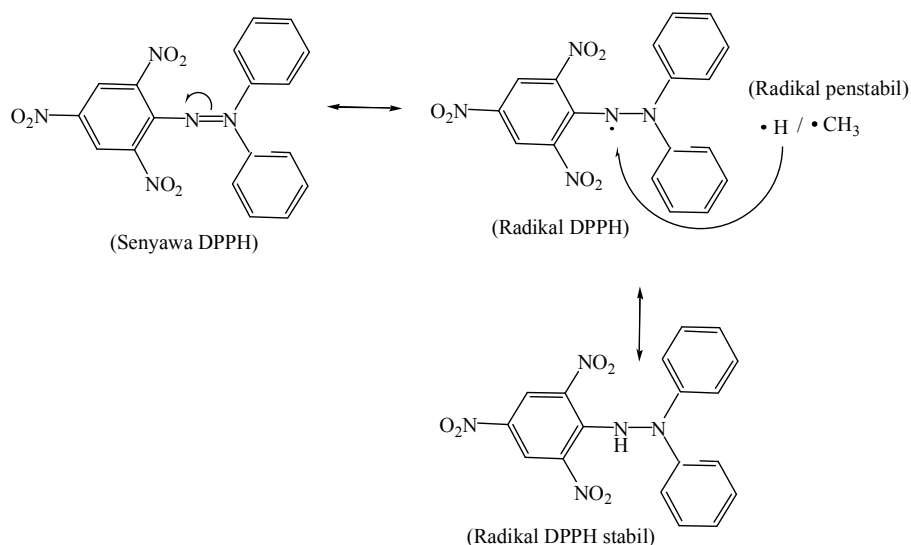
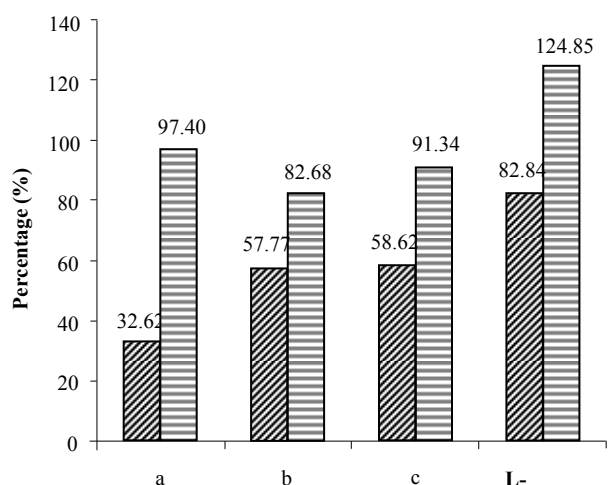


Figure 6. Mechanism of Reaction of Hydrogen Atom Donation/Methyl Radical to DPPH Radical



**Figure 7. Percentage of the Inhibition of NO Production and Cell Viability of RAW 264.7 Cell Line with Samples at a Concentration of 200 µg/mL. Inhibition (▨), Cell Viability (▨)**

percentage of inhibition reaches >70%. It is classified as moderate if the percentage inhibition activity is 50-69% and weak when the percentage inhibition of activity is 30-49%, and is not active if the percentage of inhibition is only <29%. Figure 7 shows that compound a showed a weak activity, while compound b and c showed a moderate activity compared to the control of L-NAME.

Compound a with no additional substituent on both aromatic rings has a high cell survival rate of 97.4%, but low inhibition activity. This suggests that although the inhibition of NO formation was low, the compound was safe and not toxic. Additional OH moiety and amine in b and c show an increase in the level of inhibition of NO production to 71.83 and 74.36% compared to compound a without these moieties. The third influence of the synthesis of compounds in cell survival is quite good and did not differ significantly as the cell may live between a range of 82.68 to 97.4%. Control L-NAME had the highest inhibition percentage of NO and cell survival rate with a value of 82.84 and 124.85%.

The existence of hydroxyl moiety and amines in cyclopentanone compounds derived from curcumin showed an increase in the ability of anti-inflammatory activities. Pan *et al.*, [17] mentions that the saturation of alkenes and carbonyl reduction on C7 curcumin compound showed a reduction of anti-inflammatory activities by the repression of NF-κB activities through the inhibition of IκB kinase activities. Anti-inflammatory activities of compound synthesis are correlated with antioxidant activities because of the NO produced in inflammatory processes are free radicals that can likely be neutralized by the curcumin synthetic compound.

## 4. Conclusion

Curcumin derivatives can be synthesized using Claisen-Schmidt condensation from ketone and aromatic aldehyde using the base catalyst barium hydroxide. Through this method, the result came out quite pure and the product yield was quite high. Thus, it can be made as a model to create other curcumin derivatives. Toxic, antioxidant, and anti-inflammatory activities gave different results for the three compounds. The toxicity test showed how the three compounds have high levels of toxicity, so that they can continue to receive further anti-cancer study. Additional OH and amines moieties on both curcumin rings show the existence of antioxidant and anti-inflammatory activities that were higher compared to those without moieties.

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