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Research Article

SYNTHESIS AND EVALUATION OF ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF 2-(4'-DIMETHYLAMINO BENZYLIDENE)-6-BENZYLIDENE CYCLOHEXANONE

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ABSTRACT

The study was designed to investigate whether 2-(4'-dimethylamino benzylidene)-6-benzylidene cyclohexanone (named code "B") can be synthesized by reaction of Claissen–Schmidt condensation with 4'-dimethylamino benzal-dehyde, benzaldehyde, and cyclohexanone as starting materials and to evaluate its antioxidant and anti-inflammatory activities. The study showed that compound B at the concentration of 5, 10, 20, 40, and 80ppm did not exhibit antioxidant activity. In order to access the anti-inflammatory activity, this compound was administered orally an hour before intra-plantar injection of carraagenan 1% on rat paw. Anti-inflammatory effect was evaluated by measuring the volume of edema every hour for 5h. Statistical analysis was performed by one way anova at 95% confidence level and followed by LSD test. "B" was potential as an anti-inflammatory agent at the dose of 132.40mg/200g BW by decreasing the volume of paw edema.

Key words: synthesis; 2-(4'-dimethylaminobenzylidene)-6-benzylidene cyclohexanone; antioxidant; anti-inflammatory

INTRODUCTION

In 2000 Sardjiman had successfully synthesized several new analogue compounds of curcumin which showed antioxidant, anti-inflammatory, and antibacterial activities (Sardjiman, 2000). These activities were correlated with its chemical structures. In the recent publication, the anti-allergenic activity of the PGV-0, and their correlation to the chemical structure have also been reported (Nugroho *et al.*, 2009). However, the chemical structure of bis-compound changes into other asymmetric compounds such as 2-(4'-dimethylamino benzylidene)-6-benzylidene cyclohexanone and the evaluation of the derivates biological activities have not been able to be carried out.

Sardjiman (2000) has synthesized the derivates of benzylidene-cyclohexanone such as 2,6-bis (4'-dimethylamino benzylidene) cyclohexanone and 2,5-bis (4'-dimethylamino benzylidene) cyclopentanone. Nevertheless, 2-(benzylidene 4'-dimethylamino)-6-benzylidene cyclohexanone has not yet been obtained. Thus, this study was aimed to synthesize the targeted compound by coupling 4'-dimethyl-amino benzaldehyde and cyclohexanone and reacting the coupled intermediate with benzaldehyde.

This study was propossed to determine whether 2-(4'-dimethylamino benzylidene)-6-benzylidene cyclohexanone ("B") was able to be synthesized through Claissen-Schmidt conden-sation which involved two steps of reaction: the reaction of starting material (4'-dimethylamino benzaldehyde) and cyclohexanone; and the further reaction of the produced intermediate with benzaldehyde. The purity of synthesized compound was further analyzed using thin layer chromatography (TLC) and its chemical structure was identified using infra red spectrophotometry, mass spectrometry and nuclear magnetic resonance (NMR) spectro-scopy. The chemical structure of targeted compound can be seen on figure 1.

METHODOLOGY

The materials used were benzaldehyde, cyclohexanone, 4'-dimethylamino benzaldehyde, KOH (Merck p.a), carrageenan, and Carboxy-methyl Cellulose (CMC). Instruments used in this research was plethysmograph. The animals used for testing were white male rats of Wistar strain.

Synthesis of 2-(4'-dimethylamino benzylidene)-6-benzylidenecyclo-hexanone ("B")

An approximate of 1.8625g (0.0125 mol) of 4'-dimethylamino benzaldehyde, 1.3266 g (0.0125mol) benzaldehyde, and 1.3mL (0.0125 mol) cyclohexanone were mixed sequentially in a three-neck boiling flask and stirred at 700rpm for 10min at 15°C. Tetrahydrofuran (THF) was then added while the mixture was being stirred. KOH as the catalyst was added dropwise for 2.5h until the mixture became opaque and there was no precipitate obtained. The stirring was continued for 1.5h after the last drop and the mixture was then allowed to stand for an hour.

Isolation of "B"

The synthesis product was transferred into glass beaker and neutralized with HCl 0.5N. The mixture was then added with cold distilled water to form the crystal of the product which was later filtered through a Bűchner funnel, dried, weighed, and calculated as rendement .

To purify the product, recrystallization was carried out using ethanol. The purity of "B" was evaluated by measuring its melting point using termopan and identification by thin layer chromatography. The 4'-dimethylamino benzaldehyde (DAB) standard was dissolved in ethanol. Sample and DAB standard were developed with the chloroform: *n*-hexane (6:4) as mobile phases. Visualization of the spots was performed with UV 254nm lamps.

Structure elucidation of "B"

Infra red and mass spectra were obtained using FTIR merck Shimadzu type Prestige-21 and LCMS Mariner Biospectrometry Workstation, respectively. H^1-NMR spectra were recorded using NMR Spectrometer merck Jeol type JNM-ECA 500.

Determination of antioxidant activity

Antioxidant activity was estimated using DPPH free radical scavenging method (Anonymous, 2006). The inhibition percentage which was correlated to radical scavenging activity of the compound was calculated using the following formula:

Inhibition (%):

$$\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

Determination of anti-inflammatory activity

Anti-inflammatory activity of synthesized compound was determined using method described by Chattopadhyay *et al.* (2012). The qualified 30 male white rats were randomly grouped into 6 groups (group I – VI) which each consisted of the same number of animals. All test animals were housed in a controlled condition and adapted to research environment before the experiment was performed. They were fasted for 18-24h with water was only given.

CMC 0.5% at a dose of 1mL/200g BW was administered orally an hour before intra-plantar injection of carraagenan 1% to the first group of rats, namely negative control group. Suspension of compound "B" was administered orally at doses of 33.10mg/200g BW, 66.20mg/200g BW, 99.30 mg/200g BW, and 132.80mg/200g BW to group II-V, respectively, an hour before intra-plantar injection of carraagenan 1% was given. The positive control group (fifth group of rats) received diclofenac sodium suspension at a dose of 25mg/200g BW before intra-plantar injection of carraagenan with the same dose and period as given to the other groups of rats. For scoring purpose, the paw edema volume was observed every hour for 5h for each group with plethysmograph.

Statistical analysis

Percentage of the paw edema inhibition among the groups were assessed and compared statistically by one-way analysis of variance (ANOVA) and LSD's post hoc tests between two groups were performed using SPSS Statistics (SPSS Inc.) with level of significance 0.05.

RESULTS AND DISCUSSION

About 85,50% yield of products was succesfully obtained through synthesis process. The product's purity was assessed using TLC the spot were detected by application of applying phosphomolybdic acid, potassium

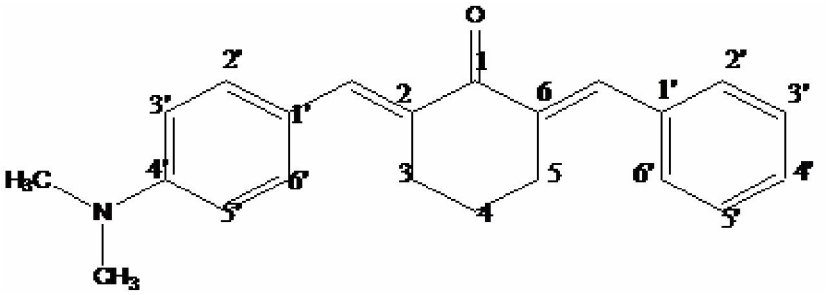

Figure 1. The chemical structure of 2-(4'-dimethylaminobenzylidene)-6-benzylidenecyclohexanone

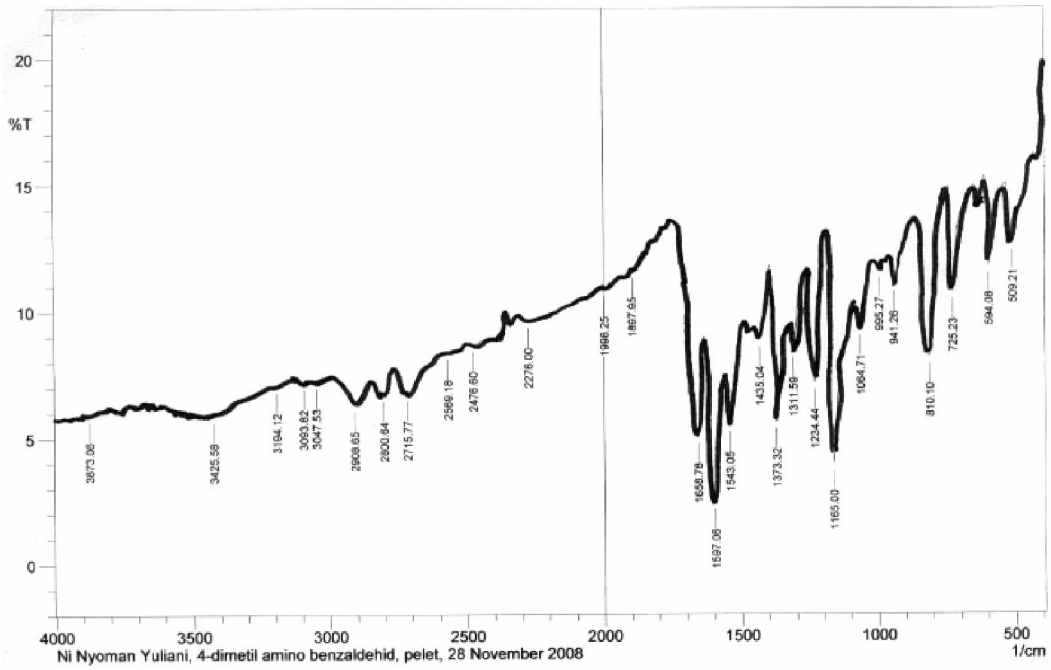

Figure 2. Infra Red spectrum of starting material (4'-dimethylamino benzaldehyde)

ferrocyanide, and DPPH as spot visualizing agents. Each agent gave the spot colors of brownish yellow, prussian blue and yellow, respectively, for dimethyl amino benzaldehyde (DAB) and no color for synthesized product. This result showed that there was no aldehyde group in the synthesized product "B" structure, thus the color was not formed.

The TLC assay using silica G as stationary phase and *n*-hexane:chloroform (4:6) as mobile phase with UV 254 nm visualization resulted a purple spot of DAB at R_f value of 3,9 and a dark yellow spot of compound B at R_f value of 2.7 which is shorter than DAB's.

The IR, NMR, and MS spectra of the starting material and "B"

IR spectra showed the presence of the absorption band for C-H alkyl due to dimethyl group attached to the N atom of either DAB or "B" which was identified at 2908.65 cm^{-1} and 2931.80 cm^{-1} respectively. This was consistent with the theoretical wave number for the alkyl C-H stretching vibration which are shown at 3000-2850cm^{-1} (Sastrohamidjojo, 2001). Aldehyde group of DAB was observed due to the C=O peak at 1597.06 cm^{-1} and C-H peak at 2715.77cm^{-1}, which was spesific for this unit. Furthermore, it can be seen that there was

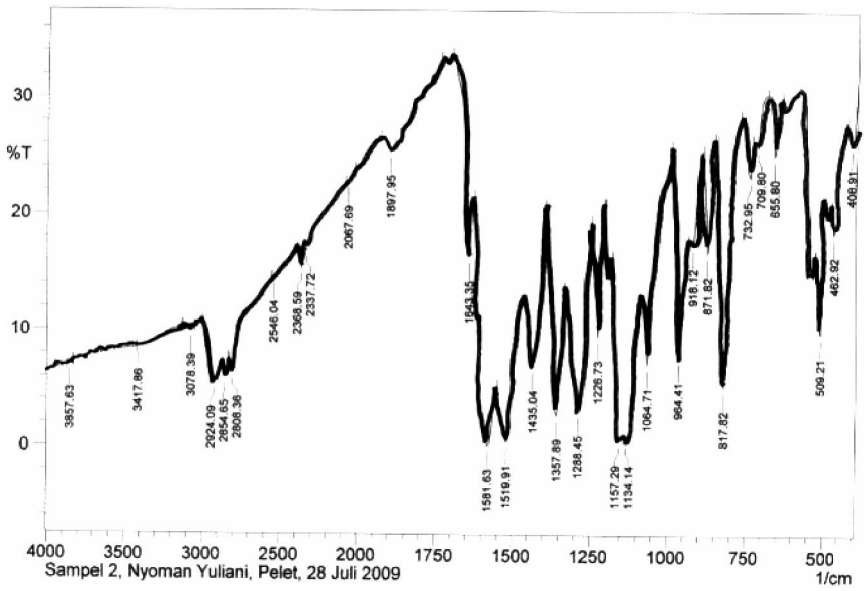

Figure 3.IR spectrum of the compound B

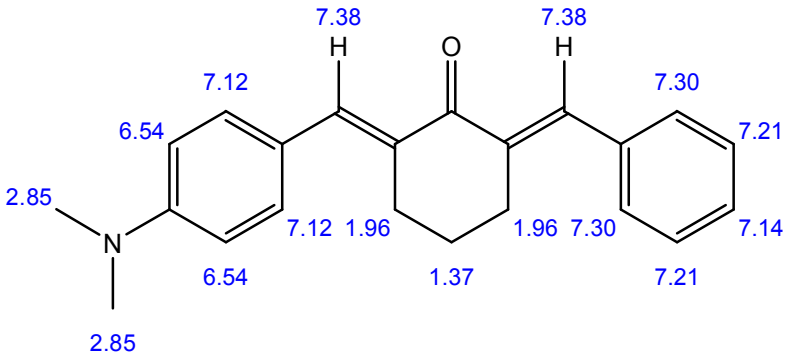

Figure 4. ^{1}H-NMR spectrum prediction of compound B according to Chem Draw

a strong band for C-N due to dimethylamino group at 1373cm^{-1} and a stretching vibration band for C-C aromatic at 1543cm^{-1}.

C-H aldehyde peak was not appeared in the IR spectra obtained from "B" (figure 3) as in DAB's. Nevertheless, the carbonyl vibration peak was shifted from 1597 cm^{-1} to the 1581 cm^{-1} due to conjugate extension of the -ene and ketone group of cyclohexanone. This indicated that the coupling reaction had been occured between the alpha carbon atom of cyclohexanone and dimethyl aminobenzal-dehyde.

Theoretically, compound "B" has 23 H atoms and several major peaks including A, B, C, D, E, F, G, H and I as shown on its ^{1}H-NMR spectrum (figure 3). Peak A was found to be a singlet at the down field area with the chemical shift (δ) as 7.7939ppm. Peak B have two benzylidene groups. The first group which attached to the N atom, unprotonated group, and alkene, appeared at δ 7.12 ppm and 6.700 ppm for peaks B, C, D, and F. While the second one was observed at deshielded down field area with δ 7.4480ppm; 7.4541-7,.4712ppm; and 7.3954ppm. The peak G which was found at δ 3.0063-3.0148ppm, was supposed to be protons of the dimethyl group as confirmed by the calculation of proton number based on integration comparison. It appeared as a singlet due to its proximity to unprotonated N atom. Whereas, the protons

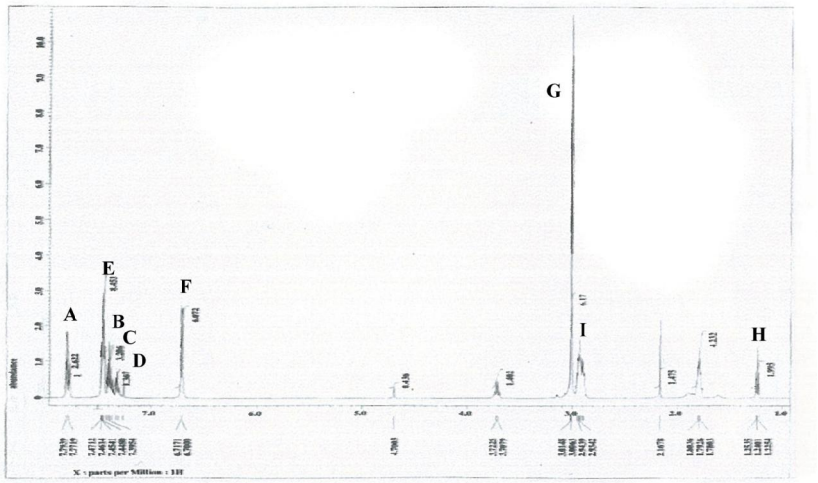

Figure 5. ^{1}H-NMR spectrum of the compound B

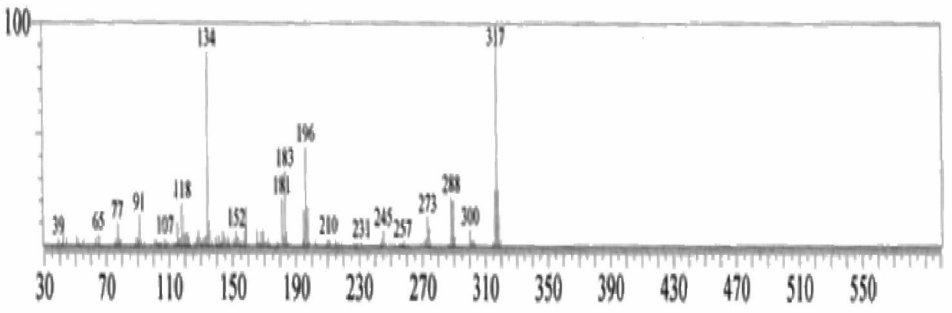

Figure 6. GCMS spectrum of the compound BGC: Retention time of compound B (MR=317) was 27,130 – 27,292 minutes and its AUC was 85,54 %.

which were close to the carbonyl of cyclohexanone, were shown as peak H and I. Peak H and I were found as an upfield triplet at δ 1.2535-1.2254ppm and a multiplet at δ 2.8865-2.9562ppm, respectively.

The chemical shifts pattern of the NMR spectrum of "B"could be predicted theoretically using Chem Draw. The involved steps included creating the chemical structure of the compound, activating Lasso tool, choosing the "Analyzed" menu and selecting "H-NMR" option.

MS spectrum showed that the elucidated compound had the M^+ peak at m/z 317 which was exactly same with the molecular weight (MW) of "B". The other ion fragments were found at m/z 288, 134, and 77 (figure 6). The possible fragmentation pattern was illustrated in figure 7.

The biological activity evaluation of "B"

The evaluation of antioxidant activity was summarized at table I. It can be seen that compound be at the concentration of 5-80ppm showed negative antioxidant activity percentage. Thus, the "B" (2-(4'-(dimethylamino benzylidene)-6-benzylidene cyclohexanone) exhibited no antioxidant activity at those levels. The negative values which was obtained, proposed that "B" is more likely to be an oxidant as it had no capacity for DPPH radical scavenging. This is due to steric hindrance provided by two methyl groups which bind to amine group. In adition, the previous study of dibenzylidene cyclohexanone using lipid peroxidase (LPO) inhibition assay (Sardjiman, 2000) reported that its scavenging activity was found to be 1.5% only at 4μM concentration. This activity was much lower

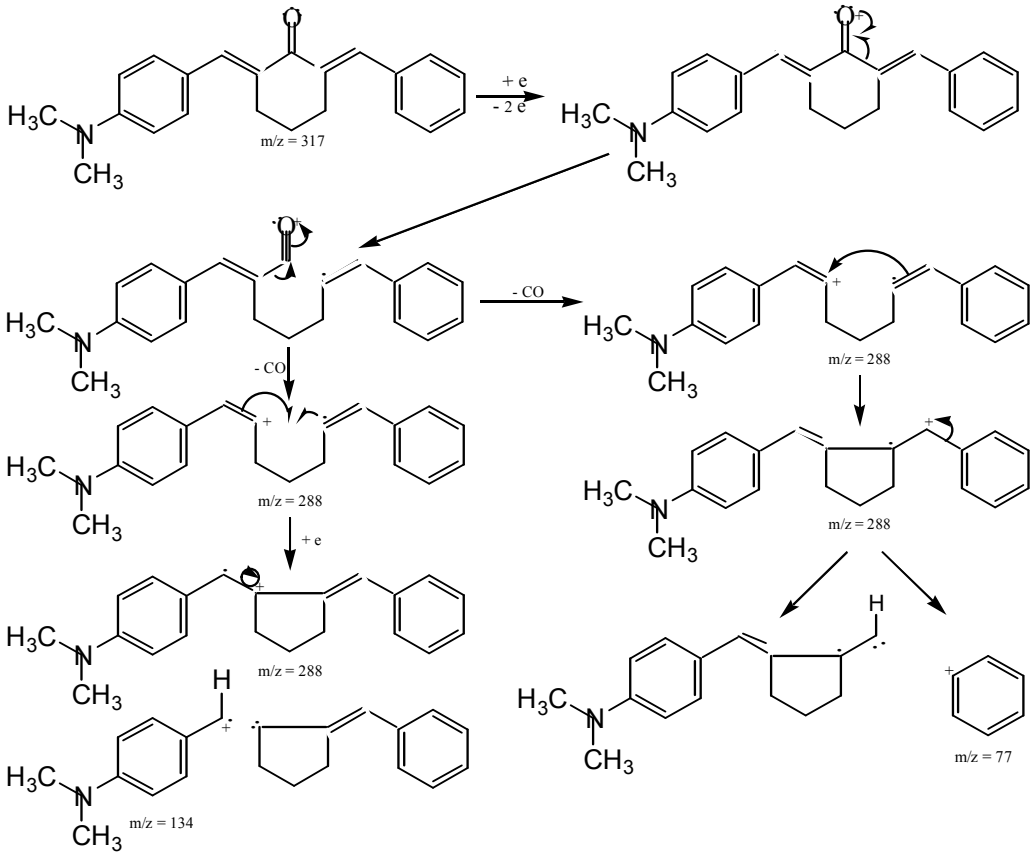

Figure 7. Possible Fragmentation Pattern for compound B

Table I. DPPH radical scavenging activity of "B at different concentrations

Concentration (ppm)	Activity (%)				
	Replication I	Replication II	Replication III	Replication IV	Replication V
5	-10.4762	-11.0012	-9.1021	-0.9920	-1.4907
10	-6.9409	-3.0000	-6.9100	-3.3635	-1.6435
20	-0.9975	-2.6250	-3.1726	-1.2438	-4.0404
40	-0.7643	-8.5784	-1.1990	-3.2594	-2.6515
80	-5.0505	-2.9691	-1.3580	-8.5158	-10.1966

than synthetic curcumin's (29%) at the same concentration.

Carrageenan-induced paw edema experiment was conducted to determine anti-inflammatory activity of "B". Optimum edema existed from the third to the fifth hour. The maximum swelling of rat paw was obtained at the fifth hour after an intra-plantar injection of carrageenan 1% was given to the rats hindpaw as depicted at figure 7. The measurement of edema volume and the calculation of edema inhibition percentage were shown at table II.

Compound "B" at dose 132mg/200g body weightand 99,60mg/200g BW showed 68,75% inhibition of the edema volume at fifth hour, compared to the positive control. "B" showed a dose-dependent anti-inflammatory activity in the carrageenan-induced rat paw edema test. The activities declined as its dose reduced, in which dose 66,20 mg /200 g BW

Table II. Anti-inflammatory activity of the compound B

Groups	Inhibiton percentage of edema				
	1 h	2 h	3 h	4 h	5 h
I	-	-	-	-	-
II	16.67 ±0.00	20.83 ± 8.32	36.36 ± 9.09	36.36 ± 9.09	42.61 ±13.25
III	29.17 ± 10.20	33.33 ± 8.33	49.96 ± 9.09	54.54 ± 0.00	65.91 ± 5.68
IV	33.33 ± 8.33	37.50 ± 0.00	54.50 ± 0.00	54.54 ± 0.00	68.75 ± 0.00
V	33.33 ± 8.33	37.50 ± 0.00	54.50 ± 0.00	54.54 ± 0.00	68.75 ± 0.00
VI	29.17 ± 10.20	33.34 ± 8.33	49.96 ± 9.092	54.54 ± 0.00	65.91 ± 5.68

I : Negative control group (CMC solution)
II : Compound B at dose 33.10 mg/200 gbody weight
III : Compound B at dose 66.20 mg/200 g body weight
IV : Compound B at dose 99.30 mg/200 g body weight
V : Compound B at dose 132.40 mg/200 g body weight
VI : Positive control group (Diclofenac Sodium solution)

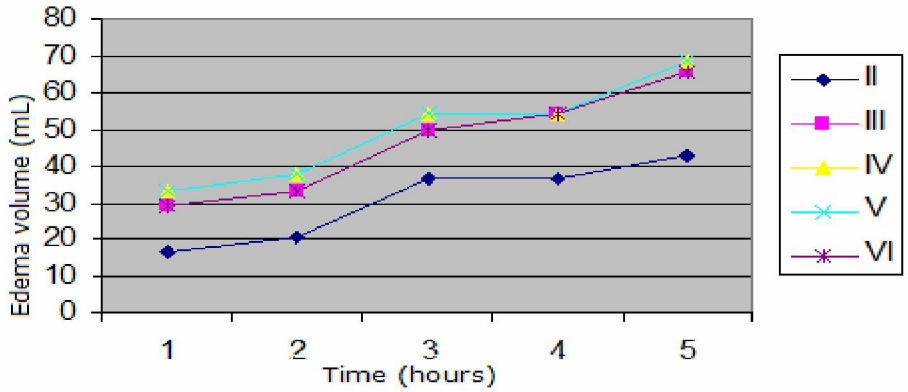

Figure 7. The graph of in vivo anti-inflammatory activity study of compound B when given at doses of 33.10mg/200g BW. 66.20mg/200g BW, 99.60mg/200g BW, and132 mg/200 g BW, compared to diclofenac sodium

and 33,10mg/200g BW gave 65,91% and 42,61% edema inhibition, respectively.

Moreover, the effects of "B" were not significantly different in all studied points ($P < 0.05$) compared to the effect produced by the positive control. Thus, it can be said that anti-inflammatory effect of "B" was equivalent to the effect of the diclofenac sodium.

According to the data of this study, one derivate of curcumin, 2-(4'-dimethylamino benzylidene)-6-benzylidene was successfully synthesized by two steps reaction of Claissen-Schmidt condensation. The starting material used, 4'-dimethylamino benzaldehyde, was reacted with cyclohexanone to produce the targetted compound, namely "B". Unfortunately, "B"showed no antioxidant activity according to the result obtained from DPPH free radical scavenging assay. Majeed *et al.* (1995) reported that based on the analysis of the chemical structure of curcumin and its analogues, the *para*-hydroxy groups of the two aromatic rings played important roles in contributing to the antioxidant activity. Previous publication said that the antioxidant activity of curcumin in capturing free radicals was greatly influenced by the two hydroxy linked to the aromatic rings called phenolic while the active methylene group affected slightly (Sun *et al.,* 2002). Curcumin is well known for its antioxidant activity because of those important groups exist in its structure.

Furthermore, curcumin and its derivates, demetoxy curcumin and bis-demetoxy curcumin, are also responsible for the antioxidant effect of turmeric (Tonnesen and Greenhill, 1992). However, in this study "B" as curcumin analogue was not found to be an antioxidant due to lack of *para*-hydroxyl group (-OH) directly attached to the two aromatic rings.

Nevertheless, "B" was observed as a potent anti-inflammatory agent. It was believed that α,β-unsaturated carbonyl group and double bond in the curcumin like structure were responsible for the anti-inflammatory activity of "B" (Majeed *et al.*, 1995).

CONCLUSION

Briefly, we can conclude that compound B could be synthetized by reaction of Claissen–Schmidt condensation using KOH as catalyst at cold temperature 15^0C. On the further antioxidant activity. However investigation, compound B did not exhibit, at the dose of 132 mg/200 g BW, it was potential as an anti-inflammatory agent with 68.75% inhibition of paw edema. IC_{50} value was found to be 32.9761mg/200 BW

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