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Synthesis and Antioxidant Activity of Some Dibenzylidene-Cyclohexanones

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ABSTRACT

Dibenzylidene-cyclohexanone is a curcumin analog, whose activity indicates antioxidative properties. This research aims to synthesize dibenzylidene-cyclohexanone compounds and to study their antioxidant activity. The synthesis was carried out according to the carbonyl condensation reaction, and the antioxidant activity was tested using the DPPH radical scavenging activity and FRAP assay. Compound entry 1 (2,6-bis-(3'-bromo-4'-methoxybenzylidene)-cyclohexanone) and entry 3 (2,6-bis-(2'-chloro-6'-fluorobenzylidene)- cyclohexanone) were obtained in 70% yield. Following the DPPH method, compound entries 1 and 2 have the IC_{50} values of 1565 μM and 1560 μM respectively. The values are not good enough if they are compared with vitamin E. Among the series, compound entry 1 has the best antioxidant activity via FRAP method with the IC_{50} value of 1486 μM.

Keywords: Synthesis, Dibenzylidene-cyclohexanone, Antioxidant, DPPH, FRAP

INTRODUCTION

Modern people's bad lifestyle nowadays, has changed the trend of diseases from communicable ones, like infectious diseases to non-communicable ones, such as degenerative diseases. In 2020, World Health Organization reported that non-communicable diseases are the cause of 70% human death in all ages (WHO, 2020). It is widely believed that degenerative disease is related to human's responses to stress, a condition that leads to exececssive accumulations of free radicals in the body (Ray et al., 2012). Acute stress is responded by human body through oxidative stress, a condition free radicals endogenous antioxidants. This unfavorable state, triggers the occurance of various disseases (Ray et al., 2012) (Nimse and Pal, 2015).

Free radicals are formed in the body as a response to the presence of stressors such as UV light radiation, drugs, pollutants, pesticides, and chemicals in food (Ray et al., 2012). Meanwhile, antioxidants are silent bodily compounds that can protect biological systems by scavenging excessive free radicals. There are two types of antioxidants. As endogenous antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GSH), and catalase (Murray, 2003) are produced by our body, exogenous antioxidants come from intakes such as fruits, vegetables, food supplement, herbs, spices, and drugs. The nature provides wide arrays of good external antioxidants with important substances such as vitamin E, vitamin C, and flavonoids are some of the examples (Nimse and Pal, 2015).

Dibenzylidene-cyclohexanone has two aromatic rings, connected by a cyclohexanone in the middle (Sardjiman et al., 1997; Ritmaleni et al., 2021). This structure is categorized as Curcumin analog (Ritmaleni, 2016). Curcumin is a natural compound isolated from turmeric (*Curcuma longa L.*) (Nelson et al., 2017) (Marchiani et al., 2013). Both curcumin and turmeric have been long reported to have many biological activities, some of which are anti-inflammatory (Panahi et al., 2016) (Biswas, 2016), antioxidant (Agnihotri & Mishra, 2011; Ak & Gülçin, 2008; Parihar et al., 2007) anticancer (Teixeira Lima et al., 2018; Wang et al., 2019; De et al., 2019), and hepatoprotective.

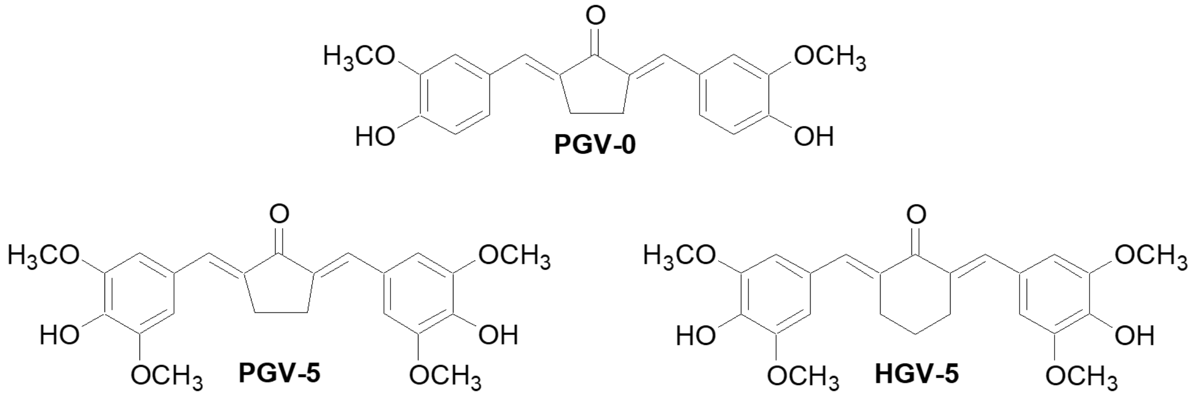


Figure 1. Curcumin analogs with dibenzylidene-cyclopentanone structures

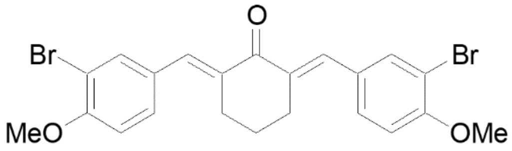
Curcumin analogs are the result of curcumin structure modification. Many of them have been studied for their biological activities (Tomeh et al., 2019; Allegra et al., 2016; Murwanti et al., 2020), including as antioxidant (Simon et al., 2018). Some studies have reported the discovery of compounds in this type, such as Pentagamavunon-0 (PGV-0, Pentagamavunon-5 (PGV-5), Heksagamavunon-5 (HGV-5) (Figure 1) (Reksohadiprodjo et al., 2004) (Yuwono & Oetari, 2004), Tetrahydropentagamavunon-0 (THPGV-0) (Ritmaleni & Simbara, 2010), Tetrahydropentagamavunon-1 (THPGV-1) (Ikawati et al., 2018), and Tetrahydropentagamavunon-5 (THPGV-5).

Several studies have predicted the antioxidant powers of curcumin analogs *in-silico*. The data resulting from past research examining the activity of those compounds, which have been successfully synthesized and tested, can be used as data sets. Although the *in-silico* test was conducted accordingly as Lipid Peroxidase Inhibition, the findings can be used as a foundation to conduct *in vitro* studies about the antioxidative properties of dibenzylidene-cyclohexanone derivatives. The prediction was carried out using a Quantitative Structure-Activity Relationship (QSAR) application named Build QSAR. Resulting in that 2,6-bis-(3’-bromo-4’-methoxybenzylidene)-cyclohexanone has 0.16 µM of IC_{50}. In this research, dibenzylidene-cyclohexanone derivatives have not been synthesized, and their antioxidant activity is still unknown. Therefore, this research aims to synthesize some dibenzylidene-cyclohexanone compounds and to study their antioxidant activity *in vitro* incorporating DPPH free radical scavenging and of ferric ion reduction. These methods of determining the antioxidant activity compounds are easy, cheap, fast, and very common.

MATERIAL AND METHOD

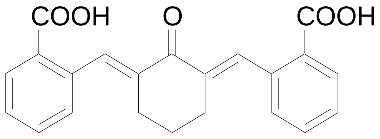
The materilas used in this research are 3-Bromo-4’-methoxybenzaldehyde, 2-Carboxy-benzaldehyde, 2-Choro-6-fluorobenzaldehyde, 2,4-Dichlorobenzaldehyde (Sigma-Aldrich), Cyclo-hexanone, TLC Plate (E-Merck), organic solvents (Ethanol, hexane, dichloromethane, acetone, ethyl acetate), and HCl

Synthesis of 2,6-bis-(3’-bromo-4’-methoxy-benzylidene)-cyclohexanone (Fatmayanti, 2019)



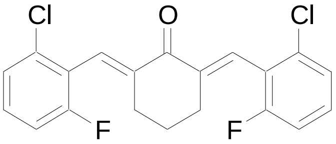
3-Bromo-4’-methoxybenzaldehyde (1g; 2.33 mmol), cyclohexanone (0.24 mL; 1.16 mmol), and HCl (0.2 mL) were added into a round bottom flask, stirred for 2 hours. The reaction was monitored using TLC. The crude product was washed with water:EtOH (2:3 v/v) until neutral. The product was then recrystallized, yielding $C_{22}O_3H_{20}Br_2$ in 70% as crystalline yellow product, m.p. = 179.7-180.6 ^{0}C (DCM/Hex); Rf = 0.45 (EtOAc : Hex, 1 : 5); IR (cm^{-1}, KBr) : 2939, 1589, 1458; ^{1}H-NMR (500 MHz, ppm, $CDCl_3$) δ 1.81 (2H, *q*, *J*=5.5 Hz), 2.87 (4H, *t*, *J*=5.5 Hz), 3.92 (6H, *s*); 6.92 (2H, *d*, *J*=8.5 Hz), 7.39 (2H, *d*, *J*=8.5 Hz), 7.65 (2H, *s*), 7,667 (2H, *s*); ^{13}C-NMR (500 MHz, ppm, $CDCl_3$) δ 22.75, 28.32, 56.23, 111.47, 111.55, 129.79, 131.36, 134.99, 135.08, 135.17, 155.94, 189.47; MS (m/z, %) 492 [M+] (100%)

Synthesis of 2,6-bis-(2'-carboxybenzylidene)-cyclohexanone (Tranggono, 2019)



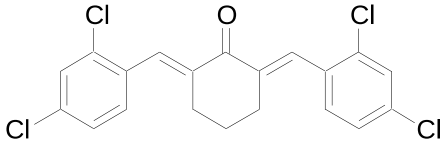
2-Carboxybenzaldehyde (1 g; 6.66 mmol), cyclohexanone (0.35 mL; 3.33 mmol), and NaOH (126 mg, 3.15 mmol) were added into a round bottom flask, stirred for 2 h. The reaction was monitored using TLC. The crude product was washed with water until neutral. The product that was then recrystallized, yielding $C_{22}H_{18}O_5$ in 45% as green crystalline product, m.p. = 204.6-205.9°C (Acetone : Hex); Rf = 0.60 (EtOAc:$CHCl_3$:Toluene, 2:3:2); IR (cm^{-1}, KBr) 3055, 2939, 1705, 1604, 748,38; MS (m/z, %) 362 [M+] (4%), 334 (9%), 316 (6%), 199 (6%), 188 (13%), 170 (12%), 133 (100%, base peak), 105 (20%), 77 (13%), 51 (7%), 39 (2%)

Synthesis of 2,6-bis-(2'-chloro-6'-fluorobenzylidene)-cyclohexanone (Ekananda, 2019)



2-Choro-6-fluorobenzaldehyde (1 g; 6.31 mmol), cyclohexanone (0.33 mL; 3.15 mmol), and NaOH (126 mg; 3.15 mmol) were added into a round bottom flask, stirred for 2 hours. The reaction was monitored using TLC. The crude product was washed with EtOAc:water (2:3 v/v) until neutral. The product was then recrystallized, yielding $C_{20}H_{14}OF_2Cl_2$ in 70% as yellow crystalline compound, m.p. = 124.6-125.0°C (EtOH); Rf = 0.2 (DCM : Hex , 1 : 1); IR (cm^{-1}, KBr) 3086, 2939, 1566, 786; ^{1}H-NMR (500 MHz, ppm, $CDCl_3$) δ 1.71 (2H, *q*, *J*=6,25 Hz), 2.51 (4H, *t*, *J*=6 Hz), 7.01 (2H, *dt*, J_1=8.5 Hz; J_2=1.83 Hz), 7.22 (2H, *dd*, J_1=8 Hz; J_2=1,5 Hz), 7.24 (2H, *ddd*, J_1=8 Hz; J_2=8 Hz; J_3=3 Hz), 7.56 (2H, *s*); ^{13}C-NMR (500 MHz, ppm, $CDCl_3$) δ 22.54, 28.87, 28.83, 114.49, 114.31, 123.65, 123.51, 125.45, 125.44, 128.55, 130.23, 130.16, 135.47, 135.43, 140.82, 160.89, 158.90, 188.41; MS (m/z, rel) 343 [M^+ with lose one atom Cl] (100%)

Synthesis of 2,6-bis-(2',4'-dichlorobenzylidene)-cyclohexanone (Arsani, 2019)



2,4-Dichlorobenzaldehyde (0.5 g; 2.86 mmol), cyclohexanone (0.15 mL; 1,42 mmol), and HCl (0.2 mL) were added into a round bottom flask, stirred for 2 hours. The reaction was monitored using TLC. The crude product was washed with water:EtOH (2:3 v/v) until neutral. The product was then recrystallized, yielding $C_{20}H_{14}Cl_4O$ in 26% as yellow crystalline product, m.p. 165.9 – 166.2°C; Rf = 0.10 (DCM : Hex, 1 : 2); IR (cm^{-1}, KBr) 2916, 1597; ^{1}H-NMR (500 MHz, ppm, $CDCl_3$) δ 1.77 (2H, *q*, *J*=5,5 Hz), 2.75 (4H, *t*, *J*=5.5 Hz), 7.26 (2H, *d*, *J*=8.5 Hz), 7.26 (2H, *d*, *J*=8.5 Hz), 7.46 (2H, *s*), 7.82 (2H, *s*); ^{13}C-NMR (500 MHz, ppm, $CDCl_3$) 22.75, 28.32, 56.27, 111.47, 111.55, 129.80, 131.36, 134.99, 135.08, 135.17, 155.94, 189.47; MS (m/z, %) 377 [M+] (100%)

Antioxidant Activity test, DPPH Free Radical-Scavenging Activity (Eryanti et al., 2011)

Dibenzylidene-cyclohexanone stock samples, vitamin-E as control, and 0.4 mM of DPPH solution were prepared before assaying antioxidant activity test. Five hundred microliter of a series with different concentrations (500, 600, 700, 800, and 1000 µM) of curcumin analogs was mixed with 1.0 mL of DPPH 0.4 mM and 3.50 mL of methanol as solvent. The control solution was 1 mL of DPPH 0.4 mM and 4 mL of methanol. Vitamin E with the concentrations of 100, 150, 200, 250, and 300 µM was used as the positive control. Following the operating time (20 min), the absorbance was measured using spectrophotometer at 517 nm. According to the published method, DPPH solution gives the maximum absorbance at wavenumber 517 nm. Hence, the measurement of the sample solution and control was conducted at λ_{max} value of DPPH 517 nm.

The percentage (%) of antioxidant activity was calculated using the equation below.

$$\text{\% Antioxidant activity} = \frac{\mathbf{A - B}}{\mathbf{A}} \text{ x } 100\%$$

A= Absorbance of control; B= Absorbance of sample

Antioxidant activity test, Ferric Reducing Antioxidant Power (FRAP) Assay. (Tanvir et al., 2017)

Solutions of 0.05% 1.10 phenanthroline (A) and $FeCl_3.6H_2O$ 1200 µM (B) were prepared. One milliliter curcumin analogs (with concentrations of 500, 600, 700, 800, and 1000 µM) were mixed with 0.5 mL of solution A and 1.0 mL of solution B. The blank was 1.0 mL of solution A and 1.0 mL of solution B. The absorbance of the reaction mixture was measured at 593 nm. According to the published method, ferric ion gives the maximum absorbance at 593 nm. Hence, the measurement of the sample solution and control was conducted at λ_{max} value of ferric ion 593 nm.

The % FRAP value was calculated using the equation below, and the value of $FeSO_4.7H_2O$ 1200 µM is considered to be 100% FRAP.

$$\% \text{ FRAP} = \frac{\text{FRAP sample value}}{\text{FRAP } FeSO_4.7H_2O} \times 100\%$$

Data Analysis

The entire data of the antioxidant activity test was analyzed in triplicate and was expressed as mean ± standard deviations. The statistical analysis was performed in SPSS using *one-way*-ANOVA with the confidence level of 95% ($P<0.05$). Then a linear regression was incorporated to identify the DPPH and FRAP IC_{50} values for each compound.

RESULT AND DISCUSSION

The new curcumin analogs are dibenzylidene-cyclohexanone derivatives, and they have been synthesized successfully in this study. The proposed reaction mechanism to obtain dibenzylidene-cyclohexanone derivatives is the aldol condensation that uses HCl as the acid catalyst. This mechanism is one of the carbonyl condensation mechanisms, involves a carbonyl compound that may come from aldehyde and ketone compounds. Here, aldehyde becomes the electron acceptor (electrophilic species), while ketone becomes the electron donor (nucleophilic species) (Pudjono et al., 2008). The proposed reaction mechanism of carbonyl condensation is shown in (Figure 2).

The aldol condensation for the synthesis of dibenzylidene-cyclohexanone derivatives occurred at room temperature and was followed by water dehydration during the reaction. The carbonyl condensation reaction under acidic conditions involves the formation of conjugated enones and α,β-unsaturated bonds (Murry, 2004). The reaction started with the protonation of a lone pair of electrons of oxygen at the carbonyl side, which in this research occured in both aldehyde and ketone compounds. Then, the release of two moles of H_2O occurred during the reaction. The specific product was obtained since there have been one source of donor electron from ketone. Hα was only found at cyclohexanone (ketone), and there was no Hα at aldehyde, hence aldehyde acted as electron acceptor, and cyclohexanone acted as electron donor. The presence of Hα is important because at least one of the involved carbonyl compounds must have Hα, in order aldol condensation to occur. In addition, aldol condensation under acidic condition gives a specific compound without creating any by-product, while aldol condensation under basic conditions can compete with Cannizzaro reaction and produced white precipitate of benzoate salt (Ismiyarto et al., 2001).

The synthesis gave moderate yields. The first compound, which has 70% of yield, was obtained when the benzyl ring has *meta*-bromo and *para*-methoxy substituents, while the second one which also has 70% yield, was obtained when the benzyl ring has both *ortho*-chloro and fluoro substituents, while *meta*-carboxy only gave produced 45%. The lowest yiled was obtained when the benzyl ring has *ortho* and *para*-chloro substituents (Table I). *Ortho* and *para*-chloro substituents has the lowest yield all of the series because the substituents of chloride atom (Cl) also other halogen atoms (Br, F, and I) especially at the *ortho* and *para* position to the alkyl have weak benzene ring deactivating properties. Therefore the ring became less reactive in yielding products through aldol condensation reaction than other compounds. These phenomena will have different results if the substituents have benzene ring activating properties for instance methoxy group (OCH_3). Compound entry 1 has a methoxy substituent with greater effects as a benzene ring activator than the weak benzene ring-dactivating halogen atom substituent (Br). The methoxy substituents became benzene ring activators through electron induction. Hence, the ring became more reactive and gave the highest percent of yield (Table I).

All of the compounds that have been tested have no better antioxidant activity than vitamin E. The DPPH method produced compounds with *meta*-bromo and *para*-methoxy substituents (entry 1) and *ortho*-carboxy substituents with better activity than other substituents in the series, while the FRAP method produced, a compound entry 1 which has the best result of all in the series.

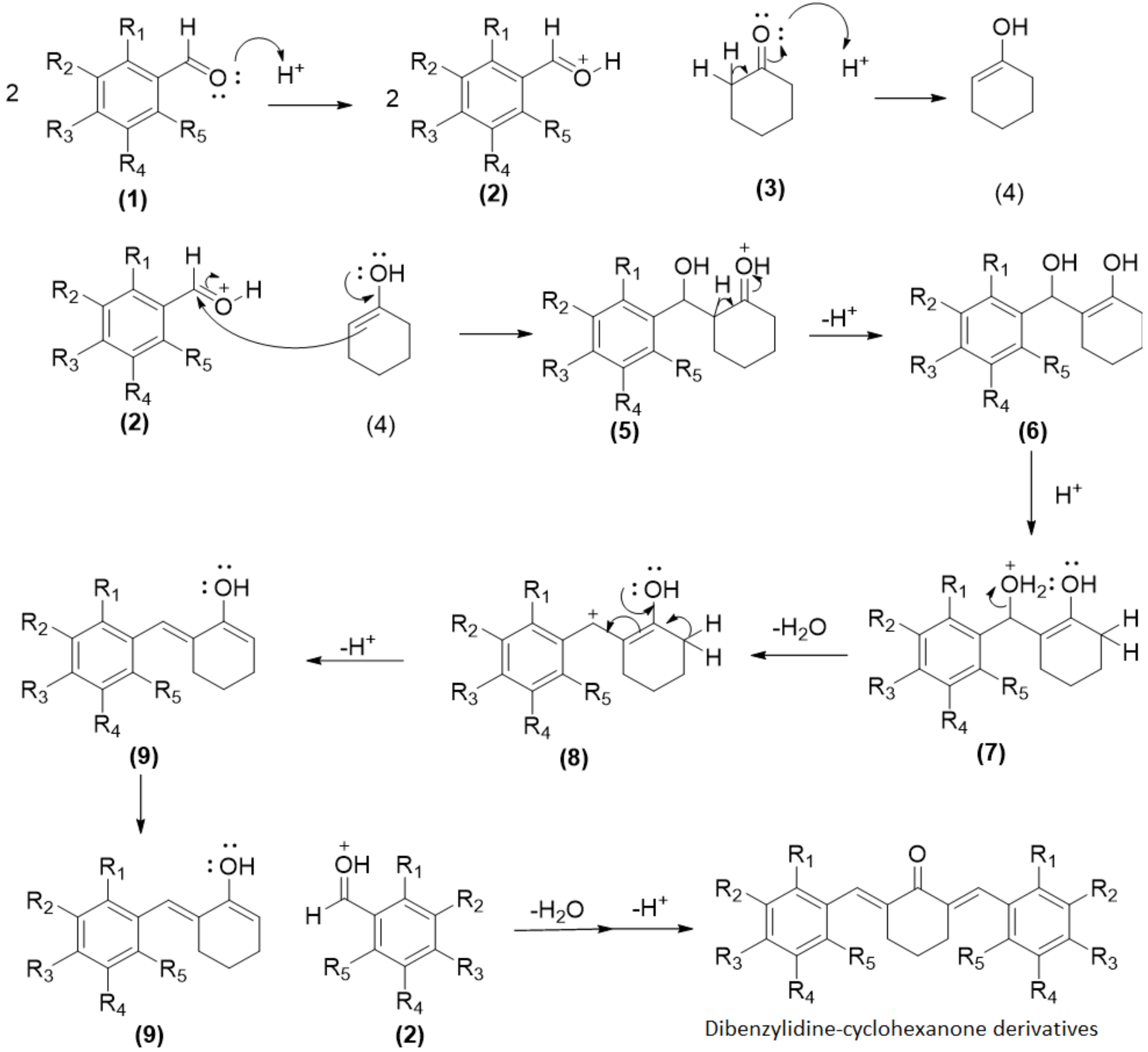
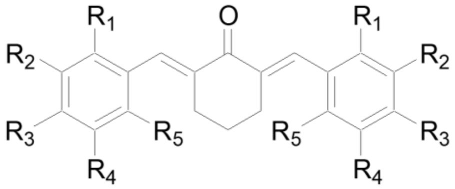


Figure 2. Proposed mechanism for carbonyl condensation

The DPPH method relies on the fact that compounds with antioxidative properties are able to capture free radicals from DPPH by releasing hydrogen which carries a single electron towards that DPPH, forming a stable DPPH-H compound (Molyneux, 2004). The antioxidative properties of the dibenzylidene-cyclohexanone derivatives are very weak due to the possibility of H on =C-H vinyl breaking heterolytically to produce hydrogen ions (H^+) without bearing the free electrons which are used to bond with the free electrons of the DPPH radicals. Thus, the free electrons on the DPPH radical will remain and cannot be stabilized. Meanwhile, the effect of methoxy substituent ($-OCH_3$) and carboxylic acid substituent (-COOH) is insignificant in increasing electron density because of the presence of C=C benzene and the steric factor of halogen substituent. The steric hindrance was also experienced by compound number 3, which possesses both Cl and F atoms, preventing potential H atom to be released as DPPH reductant agent and thus resulting in low IC_{50} compared to other compounds. Weak Electron Withdrawing Group (EWG) substitution such as F, Cl, and Br has a large electronegativity that would likely deactivate benzene ring by negative induction mechanism. The electron was carried out from benzene to EWG substituent, thus the compound's ability as donor electron to radical electron and stabilization of radical electron decreased (Sardjiman, 2000). This causes the low percentage (%) of antioxidant activity.

Table I. The structure, the yield and the IC$_{50}$ of tested compound



No.	R1	R2	R3	R4	R5	Yield (%)	IC$_{50}$ (DPPH)	IC$_{50}$ (FRAP)
1	-H	-Br	$-OCH_3$	-H	-H	70	1565 μM	148 μM
2	-COOH	-H	-H	-H	-H	45	1560 μM	1608 μM
3	-Cl	-H	-H	-H	-F	70	In Active	48230 μM
4	-Cl	-H	-Cl	-H	-H	26	2138 μM	3104 μM

IC$_{50}$ Vit. E (DPPH) = 224 μM; IC$_{50}$ Vit. E (Ferric Ion) = 240 μM

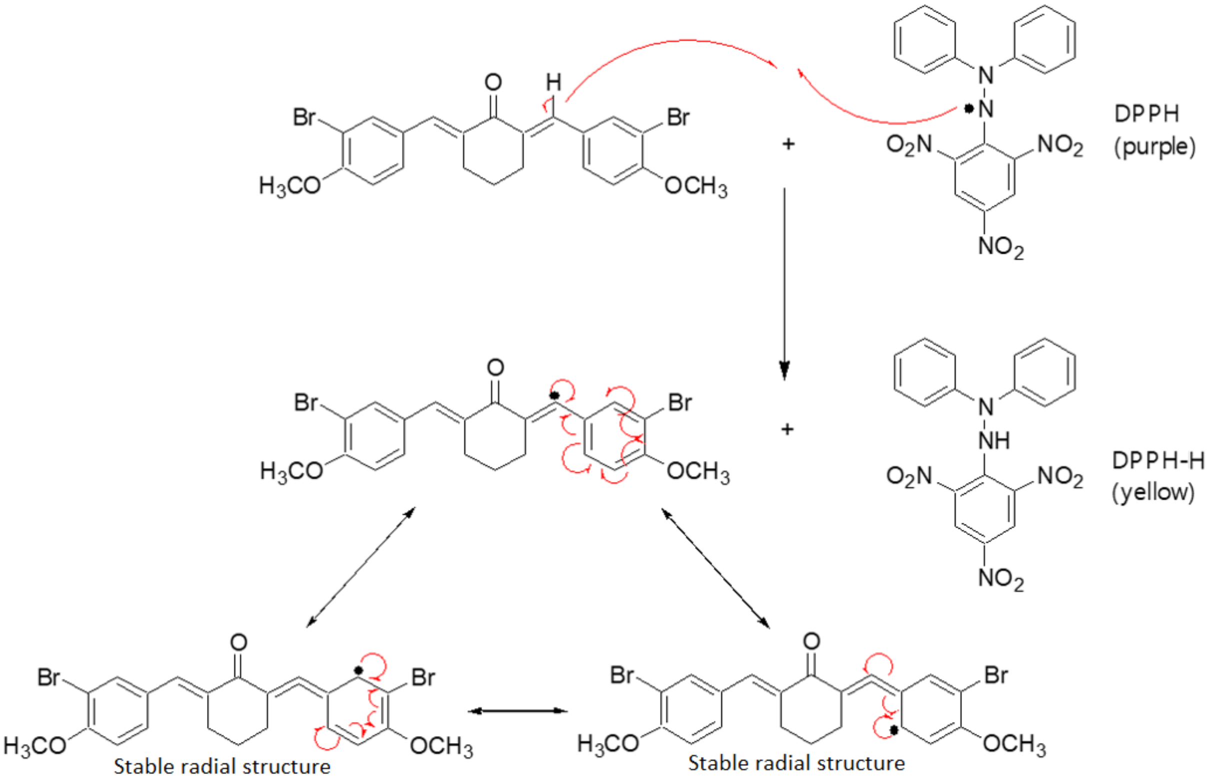


Figure 3. Proposed mechanism of radical scavenging DPPH by compound entry 1

Some literature mention that a compound with hydroxyl group on its structure has a better potency as an antioxidant. However, in curcumin analogs (dibenzylidene-cyclopentanone), random results were obtained. Here, the proposed mechanism of compound entry 1 is given, including why it has antioxidant activities when the DPPH method was applied. The radical from DPPH was scavenged by the electron that forms a bond between hydrogen and carbon alkene. The radical was then stabilized by the benzylic ring on its structure (Figure 3).

The mechanism of independent regulation by 2,6-bis-(3'-bromo-4'-methoxybenzylidene)-cyclohexanone (Figure 3) applies the effect of H atom binding on =C-H (vinyl) due to conjugation with carbonyl and aromatics, resulting in an anisotropic effect (electron effect), which causes the H atom bond to the =C-H vinyl easily separated while binding one free electron to bind to the free-electron radical DPPH (Nimse and Pal, 2015). The formed 2,6-bis-(3'-bromo-4'-methoxybenzyliden)-cyclohexanone radical can be easily stabilized by intramolecular stabilization in the presence of conjugation system in the structure of the compound. The methoxy substituent groups in these compounds also increase electron density in the benzene ring. Hence, the compound radicals can be stabilized properly. The mechanism of DPPH free radical scavenging by 2,6-bis-(3'-bromo-4'-methoxybenzyliden)-cyclohexanone is in accordance with the opinion of Nimse and Pal (2015), where curcumin and curcumin analogs can form radicals in the presence of a strong resonance effect on bond length and under the influence of the double in the middle of the structure.

The principle of the FRAP method is the reduction-oxidation (redox) reaction where a compound with antioxidative properties acts as a reductant agent, reducing ferric ion (Fe^{3+}) into ferrous ion (Fe^{2+}) by donating one electron (Kedare and Singh, 2011). The FRAP value, compound entry 1 (a compound with *meta*-bromo and *para*-methoxy substituents) indicates the best antioxidant activity of all compounds in the series, although its antioxidant activity is lower than vitamin E. The ability of compound entry 1 to reduce ferric ion is assumed because it has carbonyl groups on its structure. One electron from the carbonyl groups can fill the empty orbital of iron atom (Fe) to form a positive charge on its oxygen. By gaining this one electron, the ferric ion turns into the ferro ion (Figure 4).

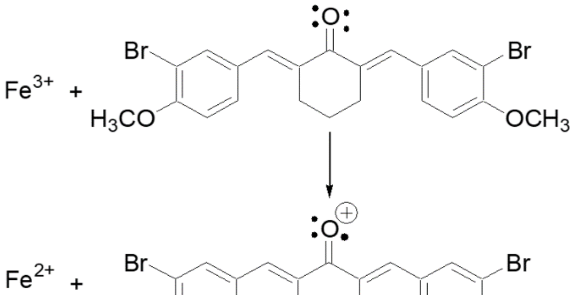


Figure 4. Proposed mechanism of reduction reaction of compound entry 1

The methoxy group helped donating an electron to the carbonyl group through the resonance and positive inductive effect in the structure. The possibility of becoming a reductant agent was increased by this methoxy group. The structure of the four synthesized dibenzyliden-cyclohexanone derivatives does not possess a hydroxyl group (-OH) which acts as the main reducing agent and plays an important role in determining antioxidative properties. In addition, the presence of an α,β-unsaturated carbonyl bond in all compounds causes the structure to give polar properties because of the effect of carbonyl resonance. Thus, the electron density is reduced and the reduction properties are also decreased (Sari, 2015). The presence of halogen substituent diminishing the electron density also decreases the reduction abilities of the compound (Sardjiman, 2000). Although methoxy ($-OCH_3$) and carboxylic acid (-COOH) substituents are present, their effects are not strong enough to increase electron density because of the presence of C=C benzene and the steric factor of the halogen atom. The effect of the steric factor and electronegativity of the substituent groups are depicted to significantly decrease antioxidative properties through the results of compound number 3 which showed inactivity in DPPH method and very low activity in FRAP method.

Overall, antioxidative properties of the compounds should be able to be enhanced by the presence of strong Electron Donating Group (EDG) such as hydroxyl group (-OH) and elimination of other hindrances such as steric factor and Electron Withdrawing Group (EWG). In the opposite to reductant agent properties, Electron-Withdrawing Group substituents are potential of becoming oxidizing agents. Generally, a compound with a good oxidizing agent tends to be a good antibacterial agent (Sardjiman, 2000) (Widyaningtyas, 2015). Thus, the dibenzylidene-cyclohexanone derivatives that have been successfully synthesized in this research may have other biological properties for, antibacterial and anticancer agent, etc. Further studies and activity screenings are still needed to know the best biological activities of dibenzylidene-cyclohexanone derivatives.

CONCLUSION

The synthesis of dibenzylidene-cyclohexanone derivatives occurred through carbonyl condensation reaction under the acidic condition at room temperature. Dibenzylidene-cyclohexanone of entries 1 and 2 have higher antioxidant activities than other entries in the series according to DPPH radical scavenging activity, but they have lower activites than vitamin E. Compound entry 1 is the best antioxidant agent of all entries in the series, even higher than vitamin E. Compound entry 1 is the best antioxidant agent of all in the series. The presence of the electron-donating group especially the hydroxyl group, is very important for maintaining the antioxidative properties of curcumin analogs. Studies exploring other potential bioactivity properties of dibenzylidene-cyclohexanone analogs, for instance as antibacterial agents and anticancer agents, are still needed.

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