Synthesis and *in vitro* Antioxidant Activity of chloro-substituted Hydrazone

N Afriana, Y Nurulita, A Zamri, J Jasril*

Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Riau, Kampus Bina Widya, Jl. HR. Soebrantas, Km. 12.5, Pekanbaru, 28293, Indonesia

*jasril.k@lecturer.unri.ac.id

**Abstract.** Hydrazones have been reported for various biological activities, including their scavenging radical activity as antioxidants. The presence of azomethine group (-NHN=CH-) makes this compound one of the important classes in many synthetic products. In this study, *ortho* and *meta*-positions of chloro-substituted hydrazones have been synthesized through condensation reaction between substituted benaldehydes and phenylhydrazine under microwave irradiation. The structure of the synthesized compounds was confirmed by UV, FTIR, $^1$H-NMR, and GC-MS spectroscopic data while antioxidant activity was tested by the DPPH method. Hydrazone compounds were successfully synthesized in good yield and short-time reaction. The results of *in vitro* antioxidant activity assay showed that chlorohydrazone have very good antioxidant activity, whereas *meta* has better activity than *ortho*-position with IC$_{50}$ value of 25.8 and 57 µg/mL respectively. The results demonstrated that these active compounds may use to support as a decent stand for further investigation in a way to ascertain innovative antioxidant medicines.

**1. Introduction**

Free radical generation and propagation have been identified as a major factor in oxidative damage which can cause various diseases such as atherosclerosis, cancer, diabetics, rheumatoid arthritis, cardiovascular diseases, aging, and other degenerative diseases in humans [1]. To overcome this, an antidote of free radicals or antioxidants are needed that serve to inhibit or stop these negative effects. The essential role of antioxidants is to prevent, neutralize, and reduce the effects of reactive oxygen species (ROS) reactions, thus preventing them from damaging biomolecules like protein, DNA, and lipids [2].

Hydrazones have been reported for various biological activities, including their scavenging radical activity as antioxidants. Moreover, the combination of the hydrazone group with other functional groups leads to compounds with unique physical and chemical properties. Rahmani *et al.* have synthesized series of substituted hydrazone bearing benzotriazole moiety and were screened for *in vitro* antioxidant activity by DPPH and FRAP assay. However, compounds substituted with electron-donating groups showed higher antioxidant activity [3]. Yilmaz *et al.* have synthesized 5-chloroindole hydrazone derivatives and almost all the tested compounds possessed strong scavenging activity against the DPPH radical with IC$_{50}$ values of 2 to 60 µM [4].
Formation of hydrazone can be catalyzed either with acids or bases, but generally with acids such as acetic acid \cite{5}. Some synthesis methods have been used to synthesize hydrazone such as reflux and stirring but this conventional method required more time and low yield compared to microwave-assisted \cite{6}. The ease of preparation, a tendency toward crystallinity, and increased hydrolytic stability relative to imines are all desirable characteristics of hydrazones. Due to the capability to react with nucleophilic and electrophilic reagents, hydrazones are widely used in organic synthesis, especially for the preparation of heterocyclic compounds \cite{7}.

One of methods for the antioxidant assay is DPPH (1,1-diphenyl-2-picyrylhydrazyl) method. This method is a popular, simple, substrate-free, and rapid analysis for antioxidant assay \cite{8}. The method can be characterized by the formation of stable radicals with electrons that can be delocalized and give a deep purple color, and the density of this color can be reduced into yellow if reacted with a compound that can donate hydrogen atoms \cite{9}. In this study, we synthesized hydrazone with chloro-substituent to increase its antioxidant activity which is evaluated by DPPH assay.

2. Methodology

2.1. Materials

The materials were used in this study are 2-chlorobenzaldehyde (Merck), 3-chlorobenzaldehyde (Merck), phenylhydrazine (Merck), glacial acetic acid (Merck), sodium hydroxide (NaOH) (Merck), 1,1-diphenyl-2-picyrylhydrazyl (DPPH) solution (100 µg/mL), distilled water, and some organic solvents such as absolute ethanol (Merck), ethyl acetate (EtOAc), n-hexane, chloroform (CHCl₃), and dichloromethane (DCM).

2.2. Instrumentation

The synthesis of compounds 3 and 4 was performed in a domestic microwave, Samsung (ME109F). The progress of reaction was observed by TLC analysis using a silica gel plate, GF₂₅₄ (Merck). The spots on the TLC plate were observed under UV lamp 254 nm (Camag). The HPLC analyzes were performed in a Shim-pack VP-ODS column (250 x 4.6 mm), with methanol and water as mobile phases in the gradient system for 20 min and a flow rate of 0.75 mL/min (UFLC Prominance-Shimadzu LC solution, UV detector SPD 20AD). The melting point of the synthesized compounds was measured in a Fisher John melting point apparatus (Uncorr.). The molecular mass was determined in a Gas Chromatography-Mass Spectrometer (GC-MS), Agilent 6890 GC). The ultraviolet (UV) spectra were measured by UV-Vis spectrophotometer (Genesys 10S UV-Vis v4.002 2L9N175013). IR spectra were recorded on the FTIR spectrophotometer (FTIR Shimadzu, IR Prestige-21). The ¹H-NMR spectra were measured by the NMR spectrometer (Agilent). The antioxidant activity assay was performed by 96-wells microplate reader (Berthold).

2.3. Procedure

2.3.1. Synthesis of 1-(substituted-chlorobenzylidene)-2-phenylhydrazine (3 and 4). Chloro-substituted benzaldehyde (1) (0.1405 g; 1 mmol) in 5 mL of absolute ethanol was put into Erlenmeyer flask. Then, 2 mL of glacial acetic acid and phenylhydrazine (2) (0.1081 g; 1 mmol) was added into the solution, then the mixture was homogenized. The compound mixture was irradiated by microwave at 180 Watt for 3 minutes. Progress of the reaction was observed every 60 seconds using TLC. If the reaction was finished, the mixture was then neutralized by NaOH 3N and then added 3 mL of cold aqua DM. The mixture was cooled in the refrigerator for 24 hours. The obtained solid compounds were then filtered by Buchner funnel and washed with cold aqua DM and n-hexane, and then dried at room temperature. The synthetic pathway of compound 3 and 4 are depicted in figure 1.
1-(2-chlorobenzylidene)-2-phenylhydrazine (3). The product was obtained as yellow solid. Yield: 96.23%, m.p. 70°C, UV (EtOH): \( \lambda_{\text{max}} = 209, 249, 302 \) and 355 nm. FTIR (KBr, cm\(^{-1}\)): 3316 (N-H str.), 3053 (C-H str.), 1604 (C=N str.), 1519 and 1445 (Ar C=C str.), 1250 (C-N str.), 754 (C-Cl str.).

\[ ^1H-\text{NMR} \ (500 \text{ MHz, CDCl}_3 \) \delta (ppm): 8.12 (s, 1H, azomethine H); 8.09 (dd, 1H, Ar-H); 7.86 (br-s, 1H, NH); 7.36 (d, 1H, J = 8 Hz, Ar-H); 7.29 (m, 3H, Ar-H); 7.23 (td, 1H, Ar-H); 7.14 (d, 2H, J = 9.5 Hz, Ar-H); 6.91 (t, 1H, J = 7 Hz, Ar-H). GC-MS: \( t_R = 12.330 \text{ minutes, m/z} [M]^- = 230.1 \text{ (100%)}, \ 232.1 \text{ (32%)}. \]

1-(3-chlorobenzylidene)-2-phenylhydrazine (4). The product was obtained as yellowish-white solid. Yield: 88.57%, m.p. 120°C, UV (EtOH): \( \lambda_{\text{max}} = 208, 249, 301 \) and 354 nm. FTIR (KBr, cm\(^{-1}\)): 3323 (N-H str.), 3053 (Ar C-H str.), 1585 (C=N str.), 1516 and 1441 (Ar C=C str.), 1250 (C-N str.), 748 (C-Cl str.).

\[ ^1H-\text{NMR} \ (500 \text{ MHz, CDCl}_3 \) \delta (ppm): 7.68 (br-s, 1H, NH); 7.61 (s, 1H, azomethine H); 7.49 (d, 1H, J = 7.5 Hz, Ar-H); 7.29 (m, 5H, Ar-H); 7.13 (d, 2H, J = 7.5 Hz, Ar-H); 6.91 (t, 1H, J = 7 Hz, Ar-H). GC-MS: \( t_R = 12.492 \text{ minutes, m/z} [M]^- = 230.1 \text{ (100%)}, \ 232.1 \text{ (31.02%)}. \]

2.3.2. In vitro Antioxidant Assay. Compounds 3 and 4 were prepared with a certain concentration in methanol. Approximately 100 \( \mu \text{L} \) of the samples were put into row A of microplate (plate consist of A-H rows, each row consists of 12 wells). Two-fold dilutions of the compounds were added to the next row so that the concentration of each A-F line were 1000 \( \mu \text{g/mL} \), 500 \( \mu \text{g/mL} \), 250 \( \mu \text{g/mL} \), 125 \( \mu \text{g/mL} \), 62.5 \( \mu \text{g/mL} \), and 31.25 \( \mu \text{g/mL} \) respectively. A-G rows were added with 80 \( \mu \text{L} \) of DPPH with a concentration of 100 \( \mu \text{g/mL} \). After that, it was incubated for 30 minutes in the dark room [10]. Then, the absorbance was measured in triplicate and the total percentage of radical scavenging activity was calculated based on the equation (1).

\[
\text{Inhibition (\%)} = \frac{(\text{Absorbance Control} - \text{Absorbance Sample})}{\text{Absorbance Control}} \times 100\%
\]

Then, the inhibition concentration 50 (IC\(_{50}\)) values were calculated based on the linear regression equation \( y = ax + b \) from the curve by plotting the Ln concentration on the x-axis and percentage of inhibition on the y-axis.

3. Results and Discussion

3.1. Synthesis chloro-substituted hydrazone

Compound 3 and 4 were synthesized through a condensation reaction between chloro-substituted benzaldehyde (1) in ortho and meta position with phenylhydrazine (2) which was catalyzed by acetic acid glacial as depicted in figure 1. MAOS (Microwave-Assisted Organic Synthesis) is the best method to synthesize hydrazone because of shorter time reaction and higher yields of products than the conventional method [6]. Reaction time for compound 3 was 1.5 min with yields 96.23% and compound 4 was 3 min with yields 88.57% (figure 2). The purity of the compounds was shown by a single spot in TLC with different eluents, sharp melting point, and a single sharp peak in HPLC.
Figure 2. Product of synthesized compounds: yellow solid of compound 3 (a) yellowish-white solid of compound 4 (b).

The structural identifications of compounds 3 and 4 were not much different in the UV, FTIR, and GC-MS spectra. In UV spectra, maximum absorption at wavelengths ($\lambda_{\text{max}}$) around 210, 250, 300, and 350 nm indicated the presence of conjugated double bonds in title compounds. In FTIR spectra, stretching and bending vibration indicated the certain presence of functional groups in the title compounds. The FTIR spectra of synthesized compounds showed the formation of azomethine groups in hydrazone with absorption bands around 3300 cm$^{-1}$ that indicated the presence of N-H bond of the azomethine group. In addition, the absorption bands from C=N in azomethine groups appeared around 1600 cm$^{-1}$. The GC chromatogram showed that the synthesized compounds possess high purity and the MS spectra showed the molecular ion peak [M]+ at m/z 230.1 with a relative abundance of 100% which is the molecular weight of the title compounds according to the molecular formula C$_{13}$H$_{11}$N$_2$Cl. Furthermore, there is an isotope peak with a relative abundance ratio of 1:3 in [M+2] that shows typical chloro-substituent fragment that is caused by the abundance of the isotope $^{37}$Cl and $^{35}$Cl in nature, which is 25% and 75%.

Figure 3. $^1$H-NMR spectra of compounds 3 and 4.
$^1$H-NMR spectra were performed to observe total proton, positions, and the environment of protons which results in different signals of compounds 3 and 4. $^1$H-NMR spectra of the title compounds showed the characteristic of azomethine group by the appearance of a broad singlet signal (N-H proton) at $\delta$ 7.86 and $\delta$ 7.68 ppm and singlet signal (H-1) at $\delta$ 8.12 and $\delta$ 7.61 ppm for compound 3 and 4 respectively. Methine proton (H-1) of compound 3 is more deshielding (downfield) than compound 4 because of the effect of the reduced electron density of methine proton due to electronegative atom, chloro (-Cl) in ortho position (see figure 3). All the spectroscopic data showed that the obtained compounds corresponded to be expected molecule targets.

3.2. *In vitro* Antioxidant Assay

The antioxidant activity of the title compounds was analyzed by using the free radicals scavenging activity (DPPH) method. This method can be characterized by the formation of stable radicals with electrons that can be delocalized and give a deep purple color, and the density of this color can be reduced into yellow if reacted with a compound that can donate hydrogen atoms $^9$. The results of antioxidant assay of the hydrazone compounds are presented in table 1. A higher percentage of inhibition indicated a better ability to scavenge the free radical. Compounds 3 and 4 were able to scavenge the DPPH radical with >90% by concentration 1000 µg/mL and could change the color of DPPH radical from purple to yellow. Based on the IC$_{50}$ value from the linear regression equation (figure 4), compound 3 and 4 are strong antioxidant with IC$_{50}$ 57 and 25.8 µg/mL respectively $^{11}$.

| Concentration (µg/mL) | % inhibition |  |  |
|------------------------|--------------|--------------|--------------|
|                        | Compound 3   | Compound 4   |              |
| 1000                   | 92.297       | 95.261       |              |
| 500                    | 84.208       | 87.027       |              |
| 250                    | 72.938       | 80.656       |              |
| 125                    | 60.395       | 70.868       |              |
| 62.5                   | 51.307       | 62.711       |              |
| 31.25                  | 41.309       | 50.126       |              |
| **IC$_{50}$ (µg/mL)**  | **57**       | **25.8**     |              |

**Table 1.** The percentage of inhibition of compound 3 and 4 in various concentration.

![Figure 4](image.png)

*Figure 4.* The plot of Ln concentration and percentage of inhibition to calculate IC$_{50}$: (a) compound 3 and (b) compound 4.
Based on their structure, the hydrazone compounds can act as electron or proton donating group to the DPPH radical further because the conjugation system will direct the resonance into the aromatic ring so that free radicals are stabilized. Jasril et al. suggested an antioxidant mechanism for hydrazone compounds, the group that donates their proton are NH proton from the azomethine group\textsuperscript{[12]}. Furthermore, the chloro-substituent may contribute to making higher antioxidant activity in hydrazone compounds as an electron-donating group\textsuperscript{[3]}. Chloro substituent \textit{in meta} has better activity than \textit{ortho}-position, this may be due to the steric effect of \textit{ortho}-position so it is more difficult to donate proton NH to DPPH radicals.

4. Conclusion

Chloro-substituted hydrazone compounds have been successfully synthesized in \textit{ortho} and \textit{meta} positions under microwave irradiation with high yields. Then, the synthesized compound was evaluated for its \textit{in vitro} antioxidant using the DPPH assay. The synthesized compounds possessed strong antioxidant activity with IC\textsubscript{50} values of 25.8 and 57 \textmu g/mL. The results demonstrated that these active compounds may use to support as a decent stand for further investigation in a way to ascertain innovative antioxidant medicines.

Acknowledgement

The final manuscript of this publication was presented at Webinar SEMIRATA 2020 on The 4th International Conference on Mathematics, Science, Education and Technology (ICOMSET) in conjunction with The 2nd International Conference on Biology, Science and Education (ICoBioSE) virtually at Universitas Negeri Padang, Padang City, Indonesia on September 19th, 2020.

References

[1] Uttara B, Singh A V, Zamboni P and Mahajan R T 2009 \textit{Curr. Neuropharmacol.} \textbf{7} 65–74
[2] Aruoma O I, Spencer J P E and Mahmood N 1999 \textit{Food Chem. Toxicol.} \textbf{37} 1043–53
[3] Rahmani S E and Mokhtar L 2018 \textit{Research J. Pharm. and Tech.} \textbf{11} 4104–7
[4] Yilmaz A D, Tulay C and Suzen S 2012 \textit{J. Enzyme Inhib. Med. Chem}. \textbf{27} 428–36
[5] Kodisundaram P, DuraiKannu A, Balasankar T, Ambure P S and Roy K 2015 \textit{Int. J. Mol. Cell. Med.} \textbf{4} 128–137
[6] Bekdemir Y and Efıl K G 2014 Microwave assisted solvent-free synthesis of some imine derivatives \textit{Org. Chem. Int.} \textbf{2014} 1–6
[7] Belskaya N P, Dehaen W and Bakulev V A 2010 \textit{Arkivoc} \textbf{1}: 275–332.
[8] Antolovich M, Prenzler P D, Patsalides E, McDonald S and Robards K 2002 \textit{Analyst} \textbf{127} 183–98
[9] Hendra R, Masdeatresa L, Abdullah R and Haryani Y 2020 \textit{AIP Conf. Proc.} \textbf{2243} 030007
[10] Lu Y, Hendra R, Oakley A J and Keller P A 2014 \textit{Tetrahedron Lett.} \textbf{55} 6212–15
[11] Marjoni M R and Zulfisa A 2017 \textit{Pharm. Anal. Acta} \textbf{8} 1–8
[12] Jasril, Ikhtiarudin I, Nurulita Y and Nurisma \textit{AIP Conf. Proc.} \textbf{2242} 040041